CHARLES UNIVERSITY IN PRAGUE

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DOCTORAL DISSERTATION

SICKNESS BEHAVIOUR IN THE EARLY ADJUVANT ARTHRITIS

(ROLE OF NEUROINFLAMMATION AND OXIDATIVE STRESS?)

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CONTENT

DECLARATION

I honestly declare that I elaborated the doctoral dissertation entitled *"SICKNESS BEHAVIOUR IN THE EARLY ADJUVANT ARTHRITIS (ROLE OF NEUROINFLAMMATION AND OXIDATIVE STRESS?)"* with results and literature sources mentioned. I also declare that this doctoral dissertation was not submitted for evaluation before. I agree to provide this doctoral dissertation for study purposes.

I declare that the printed and electronic versions are identical.

PharmDr. Martina Škurlová

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LIST OF ABBREVIATIONS

AA - adjuvant arthritis

ACTH - adrenocorticotrophin

nArc - nucleus arcuatus

anti- CCP - antibodies to cyclic citrulinated peptide

ANOVA - analysis of variance

AVP - argininevasopressin

BDNF - brain derived neurotrophic factor

CAE - closed arm entries

CAT - closed arm time

CC - central circle

CE - central entry

cFA - complete Freund adjuvant

CT - central time

CNS - central nervous system

CORT - corticosterone

CRH - corticotrophin

CRH 1 - CRH type 1 receptor

CRP - C-reactive protein

5- HT - serotonin

HPA-axis - hypothalamus- adenopituitary- adrenocortical axis

EPM - elevated plus maze

IgG - immunoglobulin G

IL-1β - interleukin 1beta

IL-1 RA - interleukin- 1 receptor antagonist

IL-1RI - interleukin- 1 receptor type I

IL- 2 - interleukin- 2

IL- 4 - interleukin- 4

INF-γ - interferon- gamma

Lp - Legionella pneumophilla

LTP - long term potentiation

MC4 R - melanocyty stimulating hormone receptor type 4

MMPs - metalloproteinases

α- MSH - alpha- melanocyty stimulating hormone

MWM - Morris water maze

NADPH - nicotinamide adenine dinucleotide phosphate-oxidase

NOX 1 - nicotinamide adenine dinucleotide phosphate-oxidase (type I)

NOX 2 - nicotinamide adenine dinucleotide phosphate-oxidase (type II)

L-NAME - N-nitro- L-arginine methyl ester

NGF - neurotrophic factor

NOS - nitric oxide synthase

NE - norpinephrine

NF-κB - nuclear factor κappa B

NO - nitric oxide

NPY - neuropeptide Y

NREM - non rapid eye movement sleep

OAE - open arm entries

OAT - open arm time

OF - open field

PAMPs - pathogen associated molecular patterns

PVN - paraventricular nucleus

PC - peripheral crossings

PGE_2 - prostaglandin E_2

PT - peripheral time

RA - rheumatoid arthritis

RF - rheumatoid factor

RIA - radioimmunoassay

ROS - reactive oxygen species

RNS - reactive nitrogen species

TE - total entries

TNF-α - tumor necrosis factor- alpha

TT - total time

SOUHRN

Úvod: Revmatoidní artritida je inflamační autoimunitní polyartritida. I když toto onemocnění primárně nepostihuje mozek, poruchy funkcí CNS patří mezi její projevy. Neuropsychiatrické poruchy v artritidě se liší svojí závažností od těžkých forem psychóz až k poruchám paměti. Etiologie behaviorálních změn v artritidě není objasněna.

Cíle: Předkládána práce zkoumá incidenci behaviorálních změn v rané fázi adjuvantní artritidy. Rovněž zkoumá, zdali změny chování v rané artritidě souvisí s neuroinflamací a oxidačním stresem v hippokampu.

Metodika: Experimentální artritida byla vyvolána jednorázovým podáním cFA potkanům kmene Lewis ve věku sedm týdnů. Byly zkoumány první čtyři dny rané artritidy. Parametry jako tělesná hmotnost a příjem potravy byly měřené denně. Testy bolesti, chování a biochemické analýzy byly provedeny ve dvou intervalech rané experimentální artritidy (den 2 a 4). Reakce na bolest byla hodnocena v plantar testu, a zvlášť zkoumána na předních a zvlášť na zadních končetinách a na ocase. Prostorové učení jako i navigace v prostoru byly testovány v Morrisově vodním bludišti. Úzkostné chování bylo hodnoceno za pomocí testů zvýšeného kříže a otevřeného pole. V hippokampu byly zkoumány genové exprese mRNA IL-1β, IL-6, iNOS, NADPH oxidáz, CRH a BDNF.

Výsledky: U artitických potkanů ve všech měřených intervalech (den 1 až den 4) rané fázi experimentálního zánětu klesá příjem potravy a rovněž tělesná hmotnost. Ve 2 dni po inokulaci artrogenem se u artritických potkanů přechodně objevuje hyperalgezie na končetinách i na chvostě. Kromě toho se od druhého testovacího dne artritičtí potkani hůř učí (prodloužená latence a dráha) v Morrisově vodním bludišti a mají horší schopnost orientace oproti kontrolám. Artritičtí potkani vykazují od druhého dne rané artritidy známky úzkostného chování v obou testech. V testu zvýšeného kříže u nich klesá poměr počtu vstupů a čas strávený v otevřeném rameni. V testu otevřeného pole se snižuje počtu vstupů a doba strávená v centrální oblasti. Na zvýšenou emocionalitu u artritických potkanů poukazuje i delší doba strávená v thigmotaktické zóně Morrisova vodního bludiště. Od 2 dne se u artritických potkanů objevují zánětlivé změny v periferii i v centrálních oblastech. V plasmě stoupají hladiny CRP a recipročně klesají hladiny albuminu. V hippokampu stoupá genová exprese mRNA IL-1β, IL-6 co indikuje na inflamační změny v této oblasti mozku. V hippokampu dále stoupá genová exprese mRNA pro iNOS, NOX1, a klesá genová exprese pro CRH. Tyto změny genových expresí jsou nepřímým důkazem oxidačního stresu v hippokampu. V této fázi artritidy se nemění genová exprese mRNA pro BDNF.

Závěr: Raná AA zhoršuje prostorové učení a vyvolává úzkostné chování. Kognitivní deficit a úzkost v rané experimentální artritidě souvisí s neuroinflamací a oxidačním stresem v hippokampu.

SUMMARY

Background: Rheumatoid arthritis is an inflammatory autoimmune polyarthritis. Although, it is not a CNS involvement disease, affective disorders and alterations of cognitive functions occur in rheumatic patients and may vary in their relevance from serious psychosis to memory disorders. Aetiology of sickness behaviour in arthritis is not known yet.

Aims: The aim of the present work was to study incidence of behavioural components of sickness in the early phase of experimental arthritis, and to confirm an association between behavioural components of sickness and neuro- inflammatory / chemical alterations in the hippocampus in this phase of the disease.

Methods: Experimental arthritis was induced to Lewis rats by a single injection of cFA. First four days of experimental arthritis were studied. The body weight and food intake were measured daily. Pain reactivity, behaviour and biochemical analysis in plasma and hippocampus were done on day 2 and on day 4. Pain reactivity was measured separately on limbs and on tail in plantar test. Spatial learning abilities and swim strategies were examined in MWM. Anxiety behaviour was tested in EPM and open field tests. In plasma, concentration of CRP, albumin, ACTH, corticosterone, leptin, ghrelin were estimated. In hippocampus, mRNA gene expression of IL-1β, IL-6, iNOS, NADPH oxidase enzymes, CRH, and BDNF was estimated.

Results: In arthritic rats, the body weight decline accompanied by decreases in food intake was found in all disease intervals measured (day 1 to day 4). Early AA also manifested with transient hyperalgesia measured on limbs and on tail on day 2. In this time interval, spatial learning deficit in arthritic rats was apparent by increased time, and longer distance in MWM. Since the 2nd day swim navigation worsened in arthritic rats opposite to controls. No difference in swim speed was observed between groups. Early arthritis evoked behavioural changes. Anxiety behaviour occurred in arthritic rats from day 2 onwards as shown by decreased open arm time and /or entries ratio. Moreover, arthritic rats entered less into the central area of the OF. In the area they spent less time and were less active compared to controls. Increased time spent in thigmotaxis area of MWM indicated for higher emotionality in arthritic rats too. Inflammatory changes were observed in plasma and in hippocampus since day 2 of AA onwards. Inflammation in plasma manifested by increased CRP and decreased albumin levels. In the hippocampus, up-regulation of IL-1β, and IL-6 mRNA indicated for inflammation. Moreover, increases of NOX1, iNOS mRNA together with decreases of CRH mRNA gene expression indicated indirectly for oxidative stress in that brain area. BDNF mRNA transcripts were not altered in early AA.

Conclusions: Early AA caused spatial learning impairments and anxiety behaviour. Incidence of behavioural components of sickness associates with neuro-inflammatory / chemical changes in the hippocampus in that phase of disease.

1. INTRODUCTION

Inflammation and / or infection often start as subjective feelings of malaise, lassitude, fatigue, numbness, coldness, and reduced appetite. All of these inflammatory features are part of sickness behaviour syndrome. Peripheral pro- inflammatory cytokines released from activated immune cells are believed to induce sickness behaviour syndrome. Increased production of peripheral cytokines associates with enhanced expression of pro-inflammatory cytokines in the brain. It is widely documented that locally produced brain cytokines interfere with behaviour under sickness (Dantzer, 2004).

Rheumatoid arthritis (RA) is common form of arthritis in a female/male ratio of 2.5:1, and incidence about 1% of the world population. Rheumatics are deprived of homeostatic regulation leading to long-term immune activation that has serious health consequences. The chronic progressive evolution of pain is often associated with deterioration in psychological well-being leading to an impaired quality of life of rheumatic people. Although RA is not a typical CNS involvement disease, approximately 30% of rheumatics are impaired in cognitive functioning. Incidence of affective disorders including depression and/ or anxiety is about 20%. Symptoms of depression in RA include feelings of sadness, loss of pleasure, and fatigue. The symptoms can range from mild to severe and from transient malaise to persistent debilitating episodes. Cognitive deficits in arthritis manifest as impaired attention, loss and/ or memory insufficiency (Lorton et al., 2008).

Thus, while behavioural components of sickness have been documented in RA patients, the biological mechanisms responsible for these alterations are not clear yet.

1.1 RHEUMATOID ARTHRITIS

1.1.1 AETIOLOGY AND PATHOGENESIS

RA is an autoimmune inflammatory joint disease. The aetiology of RA is a multistep process, and is not completely understood. Traditionally, an antibody reactive against antigenic determinants on the Fc fragment of the IgG molecule called rheumatoid factor (FR) is believed to trigger the RA process. The RF is currently the only serologic diagnostic criteria for presence of RA according the American College of Rheumatology (Kaneko et al., 2011). The severity and activity of RA correlates with RF levels, and "seropositive" RA is associated with more aggressive articular disease, a higher frequency of extra-articular manifestations and increased mortality and morbidity (Zhang and Bridges, 2001). Various autoantigens, infectious agens (viruses, bacteria) and environmental factors can initiate RA too (Kokkonen et al., 2010). An environmental factor (tobacco smoke) evokes specific citrullination of proteins, which initiate autoimmune reactions in genetically susceptible individuals (Pavelková, 2009). Different T and B cell- derived antigens induce, sustain, and modify course of RA (Bläss et al., 1999).

The pathogenesis of this migratory polyarthritis connects with proliferative inflammatory synovitis leading to structural and functional destructions of joints. Synovial inflammation and swelling primarily starts at small joints of hands then spreads to feet and wrists. Over time, uncontrolled inflammation affects all organs over the body (Goldbach-Mansky, 2009). Pro-inflammatory cytokines, proteases, reactive oxygen intermediates and other products of infiltrated leucocytes as well as of resident synovial macrophages and fibroblasts promote systemic inflammation (Ayer et al., 2000).

T lymphocytes play a pivotal role in orchestrating the inflammatory response (Gerli et al., 2002). Activated T cells release pro- inflammatory cytokines like tumor necrosis factor-α (TNF- α), and interleukin-1β (IL-1β). These cytokines facilitate leucocyte infiltration into synovium by inducing expression of adhesive molecules on vasculae endothelium and on synovial fibroblasts and by stimulating chemokine production. Together with monocytes, and macrophages T cells infiltrate the synovium. Functionally interconnected network of T cells, macrophages and synoviocytes sustain amount of the inflammatory synovium contributing to oedema development (Firestein, 2005). B cells help to maintain chronic inflammation in late phases of arthritis. B cells secrete especially TNF-α, a well known cytokine to interfere with inflammation throughout chronic phases (Panayi, 2005).

1.1.2 PHASES OF RHEUMATOID ARTHRITIS

1.1.2.1 EARLY RHEUMATOID ARTHRITIS

RA is a complex disease unique to humans, and there is currently no universal agreement upon definition and duration of early RA. It has been suggested that early RA is temporarily limited to less than a year (Morel and Combe, 2005). Patients who will develop RA have a distinct but transient synovitis in their clinical picture.

Immunopathology of synovium relates to humoral changes. T cell and stromal cell-derived cytokines were found in synovium of these patients (Raza et al., 2005).

Furthermore, pro-inflammatory cytokines are expressed and functionally active in the synovial tissue very shortly after the disease onset (McInnes and Schett, 2007). Moreover, synovial concentration of neuropeptide Y (NPY) has direct correlation to

duration of symptoms, the earlier phase of the disease, the higher concentration of NPY is found in the synovium (Appelgren, 1993). RF is detectable in plasma (Raza et al., 2005).

The cytokine presence in synovial fluid might predispose to arthritis development. Interleukin- 2, and interleukin- 4 (IL-2, and IL-4) were found in synovial fluid of screened individuals five years before the diagnosis had been confirmed (McInnes and Schett, 2007). Positive, plasma antibodies to cyclic citrulinated peptide (anti-CCP) have a predictive value to early RA development too (Raza et al., 2005).

Early RA synovitis is particularly apparent in the morning. The synovitis is followed by hyperplasia of synovial cells, expansion of synovial fluid, and development of pannus. Symmetric polyarthritis starts at small hand and feet phalangeal joints. No radiologic changes are present. Joints are tender on palpation. Morning stiffness lasts one hour before maximal improvement (Grassi et al., 1998).

The preclinical phase of arthritis last long. Defective hypothalamus-adenopituitary-adrenocortical axis (HPA) -axis function may be present in some patients during preclinical phase of arthritis (Chikanza, 1996). The lack of immune control due to disturbed neuro-endocrine activity may encourage disease progression.

1.1.2.2 CHRONIC RHEUMATOID ARTHRITIS

RA is a progressive disease. The clinical course of RA varies, depending on the number of involved joints. The classic morphological triad of chronic RA includes: sustained proliferative synovitis, cartilage and bone destruction (Sewll and Trentham, 1993). The destruction of cartilage and bone in chronic arthritis are consequences of synovitis. The process of synovitis and concomitant erosive changes of cartilage and bone are mastered by TNF-α, and IL-1β. These pro-inflammatory cytokines are found in

increased quantities in synovial tissue in the course of chronic arthritis (Kirkham et al., 1999). The synovial levels of TNF-α are in parallel with the extent of synovial inflammation and bone erosion (Neidel et al., 1995). Additionaly, the tissue destruction in RA is partly a result of extracellular matrix degradation by proteolytic enzymes including matrix metalloproteinases (MMPs) and the release of calcium by prostaglandin E_2 (PGE_2). IL-1β and TNF-α both produce MMPs and PGE_2. TNF-α stimulates activity of inducible nitric oxide synthase enzyme (iNOS). Anti TNF-α therapy significantly suppresses serum nitric oxide (NO) oxidation products in severe rheumatoid arthritis patients (Gonzales-Gay et al., 2009). Several clinical studies confirmed increased endogenous NO synthesis in patients with established RA (Onur et al., 2001; Ersoy et al., 2002). Serum nitrite/ nitrate are enhanced in RA patiens, but they are irrespective plasmatic markers of disease activity (Choi, 2003). Instead, NO has been implicated in the process of bone damage in arthritis (Escandell et al., 2007).

Insufficient neuro-endocrine and autonomic inhibitory feedback mechanisms participate in the chronicity of arthritis. Cortisol in RA patients is inappropriately low in relation to serum concentrations of cytokines and general inflammation suggesting blunted HPA- axis responses. Furthermore, serum levels of adrenal/gonadal androgens are low in RA patients with still normal levels of oestrogen (Straub and Cutolo, 2001; Bijlsma et al., 1999). Paradoxically, the number of glucocorticoid receptors in rheumatic patients, which have not received glucocorticoid therapy is normal or even elevated (Sanden et al., 2000). Quite normal adrenocorticotropin (ACTH) plasma secretion without accompanying rises in plasma cortisol suggests normal central response to inflammation in arthritis, opposite to diminished adrenal gland responsiveness. Furthermore, the anti-inflammatory neurotransmitters of the sympathetic nervous system are secreted in low amounts in

chronic arthritis. This may lead to continuous imbalance in synovial microenvironment and perpetuation of the disease (Straub and Cutolo, 2001; Miller et al., 2000).

The research opportunities in human rheumatoid arthritis are limited since the structures of interest are not directly accessible especially in the early phase of disease. Experimental models of arthritis serve to clarify mechanisms of the early inflammatory processes in the joints as well as the whole body.

1.2 ADJUVANT ARTHRITIS

1.2.1 AETIOLOGY, PATHOGENESIS, AND RAT MODEL

Adjuvant arthritis (AA) is an experimental model of arthritis which was first described in rats by Pearson and Wood (1959). The model resembles to human rheumatoid arthritis (Chowdrey et al., 1995). The aetiology of AA is not fully understood, although a cross-reactivity of mycobacterial wall antigens with cartilage proteoglycans, heat shock proteins or interactions with intestinal flora may be involved (Bendele, 2001).

The disease has a dynamic progression. Preclinical phase (until day 10) is followed by clinical arthritis (until day 20) (Pearson and Wood, 1959). AA is a T cell mediated autoimmune disease. B lymphocytes are also of pathological relevance in AA, because administration of proteins interfering with B lymphocyte function to rats suppressed inflammation in fully developed phase of AA (Veselsky et al., 2001).

Among other rat strains Lewis rats are often used as experimental arthritic model because of its high sensitivity to arthritis development (Holmdahl, 1995). Susceptibility to arthritis in Lewis rats is determined by various factors. At first, Lewis rats exert defective

adreno-cortical responses to immunological stressors (Sternberg et al., 1989). At second, T suppressor cell functions are defective in Lewis rats (Piatier- Tonneau et al., 1982). Lewis rats also express high levels of thymus glucocorticoid type receptor II (Dhabhar et al., 1993). At third, Lewis rats overproduce pro-inflammatory cytokines (Perretti et al., 1993).

1.2.2 PHASES OF ADJUVANT ARTHRITIS

1.2.2.1 EARLY ADJUVANT ARTHRITIS

Early AA manifests with humoral changes in plasma and in the joints. Plasma level of C-reactive protein (CRP) rises since day 1 (Philippe et al., 1997) followed by increases of plasma nitrates since day 3 (Ling and Jamali, 2005). Interleukin- 6 (IL-6) protein level increases in inflammed synovium since day 4 of AA (Szekanecz et al., 2000). Together with IL-6, IL-1β, TNF-α, and interferon-γ (IFN-γ) cytokines occur in the inflammed synovium (Waksman, 2002). Furthermore, leucocytes, mainly neutrophils accumulate into inflammed synovium from day 4 of AA onwards (Johnston et al., 1998). The expression of IL-6 mRNA increases in macrophages and endothelium from day 7 of AA. Increased expression of TNF-α and IFN-γ mRNA was also described in lymph nodes from that day (Ayer et al., 2000). Moreover, since day 7 of AA neuro-endocrine activity is enhanced (Sarlis et al., 1992). In addition, increased concentration of IL-1β was observed in spleen in that phase of the disease. (Stofkova et al., 2006). All above mentioned changes indicate on systemic inflammatory changes in the course of early experimental arthritis. The systemic inflammatory changes precede pain, which is not manifested before day 8 of AA (Nagakura et al., 2003).

1.2.2.2 ACUTE ADJUVANT ARTHRITIS

Oedema and joint swelling are cardinal features of acute arthritis. Synovial fluid of arthritic joints is infiltrated with pro-inflammatory cytokines of T cell origin (Bush et al., 2001). The cytokines inhibit synthesis of cartilage (Willbrink et al., 1992). IL-1β is a bone erosive cytokine promoting subchondral bone resorption by osteoclasts (Bolon et al., 2004). Furthermore, pro-inflammatory cytokines denervate joints expressing immunoreactive agens as substance P and calcitonin- regulatory peptide (Konttinen et al., 1992).

Plasma levels of ACTH and CORT are elevated in acute AA suggesting lack of inhibitory control of HPA- axis (Harbuz et al., 1992, Sarlis et al., 1992, Stephanou et al., 1992). Acute AA interferes with circadian rhythms of adenopituitary hormones and cytokines. The circadian profiles of growth hormone and prolactin are diminished. Gonadal hormones lose their daily rhythms in acute AA too (Roman et al., 2003). The daily rhythm of IL-6 mRNA is preserved in acute AA, while that of IL-1β mRNA is lost (Šereš et al., 2004).

1.3 SICKNESS BEHAVIOUR SYNDROME

1.3.1 DEFINITION AND SIGNIFICANCE

Episodes of viral and bacterial infections connect with subjective feelings of sickness in the form of malaise, lassitude, fatigue, humbness, coldness, muscle and joint aches and a reduced appetite. These features of sickness are often accompanied by alterations in behaviour, especially when inflammatory response is exaggerated (Lorton et al., 2008).

Sickness behaviour include: (1) Changes in cognition (lack of concentration learning and memory deficits) (2) Changes in affect (depression, anhedonia, letargy). Sickness behaviour together with other features of sickness are collectively termed "sickness behaviour syndrome" (Lorton et al., 2008).

Several theories clarify the significance of sickness behaviour syndrome may play under infection or inflammation. A "motivational theory" of sickness behaviour syndrome describes it as alteration of motivation. Under inflammatory conditions, sick individual continues in pleasure activities, but avoids the aversive and energy consuming. For example, motivation to rest is increased in sick individual while feeding and reproduction behaviour are reduced (Konsman and Dantzer, 2001).

The "adaptive theory of sickness" favours the idea about sickness behaviour syndrome as a highly organized adaptive response that facilitates organism’s recovery (Dantzer, 2001). Acording to adaptive theory, sick individual uses its typical behaviour to adapt to potential threat and to maintain homeostasis. This concept was elaborated by Hart (1988).

Sickness behaviour syndrome also respresents highly organized strategy to fight infection and / or inflammation. The higher body temperature that is achieved stimulates proliferation of immune cells and supresses growth of bacteria and viruses (Dantzer, 2001). Inflammatory hyperalgesia encourages recuperative behaviours like licking the injured body site (Watkins and Maier, 2000). Inflammatory anorexia spares energy. Sick individual preferres consuming carbohydrates to fat because fat is decayed during inflammation by cytokines what would lead to hyperlipidemia and excess heat loss (Johnson, 1998).

Sickness behaviour syndrome is a good example of periphery to brain communication mediated by pro-inflammatory cytokines (Dantzer, 2004). The physiologic and behavioural components of sickness behaviour syndrome are regulated by brain cytokines. Cytokine receptors are found in limbic brain areas. The highest expression of IL-1RI mRNA was detected in the hippocampus, in the basolateral amygdala, and in the basomedial nucleus of the hypothalamus (Ericsson et al., 1995). IL-1 receptors were found on neurons, glial cells, and endothelial cells in these brain areas. Moreover, it was shown that brain IL-1 receptors are similar to those found on peripheral immune and nonimmune cells. For this reason, several pathways of immune-to-brain communication are proposed for the action of proinflammatory cytokines on the CNS, from the induction of prostaglandins in brain areas devoid of a functional blood-brain barrier to the existence of specific saturable transporters. Additionaly, proinflammatory cytokines act indirectly on the CNS activating afferent nerves.

1.3.2 PATHOGENESIS

1.3.2.1 ACTIVATION OF PERIPHERAL IMMUNE SYSTEM

Pro-inflammatory cytokines are involved in regulation of sickness behaviour syndrome because peripheral administration of TNF-α, IL-1β, and IL-6 mimic all of its symptoms: including fever, activation of HPA- axis, anorexia, social withdrawals (Dantzer, 2008).

Sickness behaviour syndrome does not start by direct action of pathogens on the brain usually. Brain alone cannot detect pathogens instead immune system cells express surface receptors, which enable them to recognize invading viruses or bacteria. After recognition dendritic cells, macrophages and mast cells get activated and release pro-inflammatory cytokines that inform the brain about antigens. Cytokines are major signalling molecules of the immue system, and also potent neuromodulators (Elenkov et al., 2005).

Very early in inflammation, macrophages, and mast cells sectrete TNF-α. TNF-α triggers inflammatory response by: releasing chemokines, cytokines at the inflammatory sites, by promoting their adherence and migration at sites of microbial invasion, and by activation of leukocyctes. TNF-α stimulates release of other cytokines like IL-1 and IL-6. IL-1 induces expression of many immune cells, cytokines, chemokines. IL-6 is a major mediator of the acute-phase protein release (Tizard, 2008).

Peripheral cytokines as regulators of sickness behaviour syndrome were first noticed in cancer patients. The patients developed flu- like symptoms after cytokine therapy. Upon repetition of injections, some of them displayed acute psychotic episodes (Capuron and Dantzer, 2003). Mice or rats treated with cytokines show signs of sickness behaviour syndrome as social deprivation, anorexia and decreases in locomotor activity (Dantzer, 2001). Systemic administrations of IL-1 and TNF-α suppress feeding and drinking. This effect has been observed using various measurements of food and water intake in *ad libitum* as well as deprived conditions (Plata- Salaman, 1996). Somnolence as an important component of sickness behaviour syndrome is cytokine-mediated. Muramyl peptide infection – based sleepiness was attenuated by pretreated with IL-1 and /or TNF-α antagonists. Peripheral IL-1β mediates hyperalgesia under infection or inflammation (Fukuoka et al., 1994). Mice lacking IL-1 type I receptor (IL-1RI) will not develope social withdrawal after peripheral IL-1β (Bluthe et al., 2000).

1.3.2.2 NEUROINFLAMMATION

Systemic inflammation reflects in high circulating levels of proinflammatory cytokines, which may impact the CNS (Perry, 2004). The local and in action non- toxic up-regulation of brain pro-inflammatory cytokines due to increases in their circulating levels is called neuroinflammation. The mechanism of neuroinflammation is complex including activation of resident immune brain cells, the microglia (Lynch, 2009). Upon activation microglias secrete pro-inflammatory cytokines (Benveniste, 1997). Prolonged overexpression of IL-1β leads to recruitment of neutrophils into brain parenchyma (Ferrari et al., 2004).

Microglial IL-1β is a driving force in process termed “the cytokine cycle” where pro-inflammatory cytokines participate in a spectrum of events and continuously feedback and influence each other promoting more neuroinflammation (Griffin et al., 1998).

In vivo and *in vitro* studies have suggested neuroinflammation as a driving force of sickness behaviour syndrome under inflammation. Cognitive deficit in inflammatory brain diseases such as meningitis emerged due to neuroinflammation (Liraz-Zaltsman et al., 2010). The mnemonic deficits in early autoimmune encephalomyelitis were strongly associated with activation of microglia following release of pro-inflammatory cytokines (Mandolesi et al., 2010). LPS treated mice exerted signifiant gliosis in cortical and hippocampal regions together with memory deficits (Liraz-Zaltsman et al., 2010). Similarly, in IL-1β ^{XAT} transgenic mice, an experimental model of neuroinflammation, a delayed aquisition of Morris water maze (MWM) spatial learning task was ascribed to focal neuroinflammation in the hippocampus (Moore et al., 2009). TNF-α when overexpressed impaired cognitive capabilities in mice (Fiore et al., 2000). Chronic brain overexpression of IL-1β altered spontaneous behaviour in rats as evident on decreased burying and locomotor activity (Campbell et al., 2007).

1.3.2.3 OXIDATIVE STRESS

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are a by-product of oxidative phosphorylation process. When the pro-oxidant/ antioxidant balance is disturbed towards higher concentrations of ROS/ RNS a state termed “oxidative and nitrosative stress” develops. Oxidative stress results also from impaired oxidative defense mechanisms, such as depletion of enzymatic and non-enzymatic antioxidants (Hovatta et al., 2010).

The brain is highly vulnerable to oxidative stress due to its high oxygen consumption, its modest antioxidant defenses and its lipid- rich constitution (Bouayed et al., 2009). Oxidative stress can alter neurotransmission, neuronal function and overall brain activity. In the brain, ROS and RNS are produced by activated microglia and / or by endothelial cells via enzymes like are the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and the NOS (Emerit et al., 2004).

In a field of anxiety research, gamma amino- butyric acid -ergic and serotonergic nerve pathways were mostly studied, but recently a link between oxidative stress and affective disorders has been established. Concentration and activity of antioxidant enzymes is increased in anxiety, depression disorders. Increased concentration of superoxide dismuthase was found in homogenates prepared post-mortem from frontal regions of patients with depressive disorder (Michel et al., 2007). Activities of glutathione reductase, and glyoxylase 1 were the highest in hyperanxious mice (Hovatta et al., 2005). In these mice increased redox status was confirmed in neuronal as well as glial cells of cerebellum and hippocampus (Rammal et al., 2008; Bouayed et al., 2009). Oxidative stress – based anxiety is NADPH- mediated. Suppression of buthionine sulfoximine, an oxidative stress inducer, decreased expression of NADPH oxidase subunits (p47 phox and gp91 phox) in amygdala and hypothalamus of hyperanxious mice (Masood et al., 2008).

Oxidative stress and inflammation are highly interconnected, and both relate to sickness behaviour (Hovatta et al., 2010). Blood granulocyte oxidative status was interrelated with anxiety behaviour in mice (Bouayed et al., 2007). Antioxidant treatment normalized activity of SOD dismutase and catalase enzymes in the cerebral cortex and hippocampus, and also reversed spatial learning and memory deficits in autoimmune mice (Kuhad and Chopra, 2008). Increased plasma levels of TNF-α together with superoxide anion have been found in anxious women (Arranz et al., 2007).

1.3.2.4 NEUROTROPHINS

Neurotrophins are family of neurotrophic factors including the brain- derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin- 4/5. BDNF is a major neurotrophic factor in the CNS, and is critically involved in regulation of synaptic transmission, plays a role in formation of long-term potentiation (LTP) thus influencing learning and memory processes (Yamada et al., 2002). Learning enhances BDNF mRNA gene expression in hippocampus (Shaw et al., 2001; Hall et al., 2000; Kesslak et al., 1998). On the other hand, blockade of BDNF activity icv. impairs learning (Ma et al., 1998; Mu et al., 1999). BDNF knockout mice failed to learn in the MWM (Linnarson et al., 1997). BDNF also influences process of memory consolidation (Alonso et al., 2002). In the hippocampus, primarily in the CA1 region, BDNF enhances synaptic transmission (Schinder and Poo 2000). Up-regulation of BDNF mRNA was described after induction of LTP (Patterson et al., 1996; Bramham et al., 1996). On the other hand, pre-treatment with BDNF antibodies attenuated LTP in hippocampal slices (Figurov et al., 1996). Furthermore, BDNF restored impaired LTP at CA3-CA1 hippocampal regions in mutant BDNF mice (Korte et al., 1995; Korte et al., 1996).

Inflammatory- based cognitive deteriorations emerge due to deficits in BDNF signalling at hippocampal level (Tong et al., 2008). Administration of IL-1β into CA1 area of the hippocampus impaired BDNF synthesis in the area (Barrientos et al., 2004). An intrahippocampal injection of interleukin-1β receptor antagonist (IL-1RA) prevents BDNF-induced memory impairment (Barrientos et al., 2003). Cognitive deficit in LPS model of inflammation was ascribed to downregulation of BDNF mRNA mediated by IL-1β cytokine (Bilbo et al., 2008).

1.3.2.5 NEUROPEPTIDES

Neuropeptides are signalling molecules, produced by neurons, and capable of eliciting effects on neuronal functions. They are released into cerebrospinal fluid. Intracerebrally released neuropeptides are involved in regulation of sickness behaviour (Landgraf, 2001).

Arginine vasopressin (AVP), a stress- related neuropeptide, also regulates affective behaviours (Rotzinger et al., 2010). Beyond its hypothalamic location, vasopressin-containing neurons, and receptors are distributed in the neocortex, the brain septum, and the amygdala (Caldwell et al., 2008). AVP modulates anxiety and/ or depression -related behaviour via V1a receptor type localized in septal area of the brain. Blocking septal V1a mRNA reduces anxiety behaviour. Followed in the study, the authors have demonstrated preference for open arm of the EPM by higher number of entries and time spent in the area (Landgraf et al., 1995). On the other hand, over-expression of septal V1a gene in mice increased anxiety behaviour as the mice spent less time in the light part of the light/ dark box (Bielsky et al., 2005). AVP affects depression- related behaviour. Forced swim task, an animal model of depression, increases AVP release from the ventral septum and the amygdala. Followed in the study, application of V1a antagonist into septal and amygdalar brain area reduced floating time indicating on antidepressant effects in this paradigm (Caldwell et al., 2008).

Corticotrophin-releasing hormone (CRH) is another stress and/ or affect regulatory neuropeptide. Together with AVP it is colocalized in hypothalamic PVN, but its receptors are found in cortex and limbic areas such as hippocampus and amygdala (Rotzinger et al., 2010). Endogenous CRH exerts its anxiogenic effect throuhout type 1 receptor (CRH1).

Mice, that overexpress CRH1 are anxiogenic when exposed to novel environment of the elevated plus maze test (Liebsch et al., 1999). On the other hand, CRH1- knockout mice are more explorative when exposed to open field test indicating less anxiety behaviour in these mice (Timpl et al., 1998). Followed in the study hyperlocomotion was found in these mice. It is of speculate if deficits in locomotor activity could contribute to anxiety behavior in response to novelty. Smith et al., (1998) reported that locomotor activity in CRH1 knockout mice in novel open field is indistinguishable from wild-type mice. CRH1 antagonist administration increases time spent in light area of the light-dark testing box (Griebel et al., 1998). Moreover, direct administration of CRH into basolateral amygdala prolongs time to social contacts indicating for its anxiogenic activity (Sajdyk et al., 1999). On the other hand, icv. blockade of CRH activity increases preference for open arm of the EPM as well as time spent in the area (Skutella et al., 1998). Beyond regulation of anxiety behaviour, CRH modulates depression. CRH 1 antagonist suppressed depressive behaviour in different models of depression as the olfactory bulbectomy (Okuyama et al 1999), and the learned helplessness (Contarino et al., 1999).

1.4 FEATURES OF SICKNESS BEHAVIOUR SYNDROME UNDER ACUTE INFLAMMATORY CONDITIONS: FROM PERIPHERY TO BRAIN

1.4.1 ANOREXIA

Anorexia and body weight loss are common complications of inflammatory states, including infections. Anorexia is part of acute phase reaction, and is an early peripheral feature of sickness behaviour syndrome during inflammation / infection episodes (Rothwell, and Hopkins, 1995; Langhans, 2007). Anorexia is relatively discrete feature of sickness behaviour syndrome as it interferes only with motivation to eat, and not with decreases in locomotor activity or pain, which may occur during infection / inflammation (Aubert et al., 1997).

Microbial products like lipopolysaccharide (LPS) or muramyldipeptide (MDP) are strong anorectic factors as they both reduce food intake. The anorectic effect of peripherally administered LPS and / or MDP is characterized by reduction in meal number, and does not depend on concomitant inhibition of water intake or gastric emptying (Langhans, 2000). The magnitude of bacterial anorexia may involve interfering with brain pro/ anti- inflammatory cytokine ratio, LPS increasing IL-1β, and TNF-α levels (Plata-Salamán, 2001).

Pro-inflammatory cytokines (TNF- α, IL-1β) are also implicated in inflammation / infection anorexia (Swiergiel and Dunn, 1999; McCarthy, 2000). Peripheral administration of TNF- α, and / or IL-1β directly decreases food intake. Pro-inflammatory cytokines reduce meal number and meal size and do not inhibit water intake (Langhans, 2000).

Cytokines reduce protein intake (Aubert et al., 1995). Cytokine- based anorexia depends on the type and dose of cytokine used (Sonti et al., 1996; Plata-Salamán, 1995).

Humoral and neural pathways interfere with inflammatory/infection- based anorexia indicating that peripheral and central mediators are involved in its regulation. The center regulating food intake is the hypothalamic arcuate nucleus (nArc), where the orexigenic and anorexigenic pathways, integrate. The main orexigenic pathway involves neuropeptide-Y (NPY) promoting increase in food intake. Under physiological conditions leptin, a cytokine-like hormone from adipose tissue inhibits NPY neurons directly through its receptors to decrease food intake. On the other hand, the gastric hormone ghrelin has antipodal effects to leptin. Through its receptors (Ghsr) it stimulates NPY in nArc to induce feeding (Kalra and Kalra, 2003). LPS and/or pro-inflammatory cytokines increase expression and production of leptin in plasma and in adipose tissue. IL-1β and TNF-α reduce food intake by interfering with leptin levels in adipose tissue. Increases in serum leptin levels and fat leptin mRNA expression have been demonstrated after TNF-α and/or IL-1β admnistration (Sarraf et al., 1997). A positive correlation between increased leptin levels and food intake inhibitory effects of cytokines exist. Moreover, it was found that neutralization of circulating leptin reverse the feeding- inhibitory effect of LPS (Langhans, 2007). IL-1β exerts its anorectic effect also via the vagus nerve. The nucleus of solitary tract in the brainstem receives projections from the vagus nerve and plays an important role in meal termination. The nucleus of solitary tract in turn sends projections to hypothalamic PVN and to central amygdala. Neuropeptide- containing neurons of hypothalamic PVN stimulate catabolic pathways and promote anorexia. Central amygdala regulates appetitive aspects of food intake (Konsman and Dantzer, 2001).

Peripheral cytokines activate CNS structures involved in food intake mainly in the hypothalamus in different ways including transport from peripheral circulation via the circumventricular organs (Langhans, 2007). The part of the hypothalamus just adjacent to the median eminence, arcuate nucleus, contains capillaries rich in vesicles, suggesting transport of circulating cytokines this way. Moreover, arcuate nucleus of hypothalamus contains phagocytic cells. IL-1β immunoreactivity was found increased in the area after peripheral administration of LPS. Phagocytic cells may be a source of IL-1β in arcuate nucleus of the hypothalamus under infection. Central anorectic effects of circulating IL-1β include acting on specialized neurons, interference with food regulatory neurotransmitters and neuropeptides (Konsman and Dantzer, 2001). Pretreatment with a serotonin (5-HT) receptor antagonists blocked anorectic effect of central IL-1β (von Meyenburg et al., 2003). Administration of CRH antagonist into 3-rd ventricle attenuated IL-1β-induced anorexia (Uehara et al., 1989). Icv. injections of NPY prevented IL-1β- induced anorexia (Sonti et al., 1996).

1.4.2 HYPERALGESIA

Hyperalgesia, an increased sensitivity to pain, occurs early after onset of acute systemic infection / inflammation (Hori et al., 2000). Inflammatory hyperalgesia is characterized by increased sensitivity to mechanical or heat stimuli (Sommer and Kress, 2004). Cunha et al. (2000) in their study described incidence of inflammatory hyperalgesia 3 hours after administration of carrageenin / LPS. Almost complete recovery was observed 24 h (rats) and 48 hours (mice) after peripheral administration of LPS (Kanaan et al., 1996).

During infection / inflammation various inflammatory agents act synergistically to induce and maintain inflammatory hyperalgesia. TNF-α is the main mediator of carrageenin- induced mechanical hyperalgesia, because neutralization of TNF-α activity blocked the algesic response of the cytokine completely. The primary role of TNF-α on mediating mechanical hyperalgesia during acute infection / inflammation was also confirmed on LPS – pain model (Cunha et al., 1992). Moreover, IL-1β is a potent mechanical and thermal hyperalgesic agent when injected into different peripheral tissues (Cunha et al., 2000; Ren and Torres, 2008).

At least, in cutaneous tissue, a distinct algetic cytokine cascade unfolds during acute inflammation. TNF- α stimulates release of IL-1β and IL-6. Both cytokines then promote the release of prostaglandins. TNF-α also stimulates the release of neutrophil chemoattractant 1, which induces pain by stimulating release of sympathetic amines (Loram et al., 2007). The release of pro- inflammatory cytokines, known to mediate hyperalgesic response in the skin, is delayed in muscle under inflammation (Loram et al., 2007). In muscles, instead of cytokines, NO involvement on inflammatory hyperalgesia development was described. Pretreatment with NOS inhibitors attenuated muscle hypersensitivity after capsaicin treatment (Lee et al., 2009a). IL-1β additionally up-regulates other nociceptive mediators like neurotrophic growth factor (NGF), substance P, IL-6, and prostaglandins (Ren and Torres, 2009).

Inflammatory hyperalgesia occurs locally, at site of inflammation, or is diffused body-wide indicating that it results from peripheral and / or central sensitization. IL-1β directly excites nociceptive fibres as has been evidenced on nerve- skin preparations (Fukuoka et al., 1994). IL-1β injected intrathecally enhanced dorsal horn neuronal responses. Moreover observed changes correlated with development of mechanical allodynia and hyperalgesia (Reeve et al., 2000).

1.4.3 COGNITIVE IMPAIRMENTS

Inflammation and /or infection are often accompanied by decline of cognitive functioning. As was shown on different rodent animal models administration of LPS deteriorates learning and memory process in mice (Arai et al., 2001) and rats (Sparkmann et al., 2005).

Inflammatory cytokines are mediators of cognitive deficits under inflammation / infection. Neutralizing circulating IL-1β in Legionella pneumophilla (Lp) mice normalized learning in these mice (Gibertini et al., 1995). The IL-1RA injection restored consolidation of memory after peripheral LPS administration in fear conditioning task (Rachal Pugh et al., 2001).

Pro-inflammatory cytokines directly impair cognition. In this regard IL-1β was studied mostly, and was repeatedly shown to impair memory process. Oitzl and colleagues were the first to demonstrate suppressive effects of IL-1β on memory. Intracerebroventricular IL-1β administration caused a transient memory deficit in the first trail of the following training day, which normalized by second trial on that day. The authors suggested that IL-1β specifically affects memory retention (Oitzl et al., 1993). IL-1β negatively influences also consolidation of memories during learning process. In a fear-conditioning paradigm intrahippocampally administered IL-1β, immediately after the pre-exposure to the context, impaired the freezing response, which is usually indicator of learning in this task, to a much greater extent than a similar administration 24 h after the context presentation and at the time of context- shock association (Barrientos et al., 2002).

Cognitive deficits observed under acute inflammatory conditions accompanies to alterations at hippocampal level. Increased IL-1β mRNA gene expression and protein were found in the hippocampus after peripheral administration of LPS together with impaired fear- conditioning learning (Nguyen et al., 1998; Rachal Pugh et al., 2001). One of the mechanisms by which peripheral inflammation may affect cognitive functions could be by interfering with neurotrophins. Neurothrophins including BDNF are known neuromodulators they enhance LTP, and increase synaptic transmission (Lu and Chow, 1999). Up- regulation of BDNF expression was observed in the hippocampus after learning in a water maze (Yamada et al., 2002). Peripheral LPS significantly decreases BDNF in the hippocampus (Guan and Fang, 2006). Study by Shaw et al. (2001) decribed spatial learning deficit after one single LPS injection. No significant difference in BDNF mRNA expression was found under these conditions. The authors concluded that acute inflammation by a single LPS injection triggered no neuronal damage thus no activating BDNF synthesis (Shaw et al., 2001). In another study by Richwine et al. (2009) LPS-treated mice showed a working memory deficit in matching- to place paradigm, but without changes in dendrite length and spine density on CA1 hippocampal neurons.

1.4.4 AFFECTIVE DISORDERS

Acute inflammatory and/ or infectious episodes trigger behavioural alterations. Psychomotor retardation, fatigue, suppression of explorative activities, social deprivation, and disappereance of body-care activities described in animal studies all indicate on depressive behaviour (Yirmiya, 1996). LPS treatement was found to decrease exploratory behavior (Engeland et al, 2001), to cause social deprivations, and to increase anhedonia in different animal models (De La Garza, 2005). Furthermore, LPS treated mice were described as sleepy and less active towards the outside world. Also, decreases in grooming behaviour were found in LPS- treated mice (Fiore et al., 1996).

IL-1β has been demonstrated as the main mediator of affective disorders under acute inflammatory conditions. Administration of IL-1RA prior to LPS injection restores locomotion and social deprivation in LPS- treated mice. (Abraham and Johnson, 2009). In addition, IL-1β, alone induces affective disorders as administration of IL-1β, peripherally or into cerebral ventricles, mimics depressive symptoms. Systemic IL-1β decreases social and non social exploratory behaviour as presented by reductions in number of contacts with novel stimulus (Spadaro and Dunn, 1990), and less time spent with juvenile exploration (Bluthé et al., 1997). Mice treated with IL-1β are less involved in non ambulatory behaviours like digging, rearing, but more involved in passive responses as sitting immobile (Abraham and Johnson, 2009). Icv. injection of IL-1β causes social deprivation in mice (Johnson et al., 1998; Segreti et al., 1997). Central administration of IL-1RA reduces social deprivation after peripheral IL-1β administration (Bluthé et al., 1997). Intracerebroventricular administration of the IL-1RA improved escape learning in an animal model of depression the inescapable shock (Dantzer et al., 2009).

Anxiogenic effect of IL-1β was described in different tests of anxiety as the elevated plus maze (Swiergel and Dunn, 2007) or the open field (Song et al., 2004). The anxiogenic effect of IL-1β depends on the dose and the route of administration (Song et al., 2003; Cragnolini et al., 2006; Anisman et al., 2008; Swiergel and Dunn, 2007). While higher doses of IL-1β exert anxiogenic effect (Connor et al., 1998, Montkowski et al., 1997), low doses of IL-1β are anxiolytic (Montkowski et al., 1997). IL-1β had greater anxiogenic effect after icv than after ip administration, what points at central regulation of anxiety behaviour by the cytokine (Song et al., 2006). The anxiogenic mechanism of IL-1β is not clear yet. Cragnolini et al. (2006) in their study suggested that anxiogenic effect of IL-1β is mediated by alpha- melanocortin (α- MSH) as the hormone antagonist reversed IL-1β-induced anxiety. Further in the study, it was shown, that anxiogenic effect of α-MSH is mediated by type 4 receptor (MC4-R). Other anxiogenic mechanism of IL-1β involves changes in monoamine turnover. Anxiety behaviour observed after central administration of IL-1β correlated with increased dopamine turnover (Connor et al., 1998).

LPS- derived depressive symptoms correlated with up- regulation of IL-1β mRNA in hippocampus indicating on involvement of the brain area on regulation of affective disorders under acute inflammatory conditions (Abraham and Johnson, 2009).

1.5 SICKNESS BEHAVIOUR IN RHEUMATOID ARTHRITIS: KNOWN FACTS

Sickness behaviour in RA may vary in their relevance from serious neurological disorders and psychosis to light forms as headaches, mood changes and cognitive deficits (Appenzeller et al., 2004).

Rheumatic patients in fully developed state of arthritis are cognitively impaired. Patients with RA have a significantly worse outcome in verbal fluency and memory (Apenzeller et al., 2004). Generally they are less attentive. The attention score in RA patients moves below normal range (Bartolini et al., 2002). Several hypotheses were formulated about mechanisms determining cognitive deficits in RA. Firstly, RA may cause a slow, but progressive sublinical CNS dysfunction in a form of hypoperfusion of some cortical areas of the brain. It was suggested that hypoperfusion is caused by vasculitis located at small penetrating arterioles in this brain areas. Second, cognitive deficits in RA emerge due to defective propioceptive input to the brain from joints altered by inflammation. Inappropriate elaboration and planning motor activities at the brain level worsen cognitive functioning in chronic phase of arthritis. The study by Bartolini (Bartolini et al., 2002) confirmed the second hypothesis as functional imaging (SPECT) in chronic RA patients revealed cerebral hypoactivity in the parietal and frontal lobes together with memory deficits in these patients (Bartolini et al., 2002). On the other hand, Appezeller et al., (2004) asserted in their study that memory deficits in fully developed state of arthritis are independent from illness duration, therapy, and disability.

Patients with RA are depressed twice as likely as people from general population (Ang et al., 2005). The prevalence of depression in RA ranges from 13 to 20% (Dickens et al., 2002; Smedstad et al., 1997). Depression in RA contributes to higher mortality, low quality of life, and disability (Bruce, 2008; Löwe et al., 2004). Physical components of arthritis like pain, and comorbidities contribute to development of depression in arthritis (Hider et al., 2009). The

symptoms of depression in RA patients include feelings of sadness, loss of pleasure, fatigue and may vary from transient malaise to persistent debilitating episodes (Lorton, 2008). Central fatigue which is a result of neurotransmitter abnormalities in CNS often coexists with depression and anxiety in arthritis (Stebbings et al., 2010). Depression has been described as a predictor of fatigue in RA patients (Mancuso et al., 2006). In arthritis the degree of depression is proportional to the level of pain (Dickens et al., 2002). Arthritic pain is the strongest factor that positively correlates with depression in arthritis according to Beck depression inventory score (Melikoglu and Melikoglu, 2009). Depressive arthritics more often complain of pain (Pincus et al., 1996). Also, psychological distress may be a link between depression and pain in arthritis. Depressive arthritics women reported more frequently about stressful interpersonal events and pain when evaluated weekly (Zautra and Smith, 2001). The trait anxiety scores are significantly higher in rheumatic people.

The incidence of anxiety in arthritis is 20 % (Chandarana et al., 1987). Anxiety in arthritis is not related to disease duration, but is a predictor of pain (Smith and Zautra, 2008). Depression and anxiety in arthritis occur at early phases of arthritis (Schieir et al., 2009, Dobkin et al., 2009). Higher depression and anxiety scores were found in early arthritic women (Ramjeet et al., 2005). It was suggested that depression and/or anxiety shifts between periods of autoimmune outburst and remission in RA (van Dyke et al., 2004). Both depression and anxiety assess general distress in arthritis as they overlap each other. Depression in RA and plasma levels of CRP are closely inter-related what points at activation of immune system in the development of arthritic depression (Kojima et al., 2009). CD4 activation and higher IL- 6 plasma levels were found in depressed arthritic females (Zautra and Smith, 2001). On the other hand disease activity score 28 (DAS 28) in depressive anti TNF-α- treated rheumatics was only partially reduced after 3 month of treatment (Hider et al., 2009). Whether neuropsychiatric complications in arthritis are result of inflammatory outburst or associated comorbidities still remains to be solved.

2. HYPOTHESES AND AIMS OF THE STUDY

Sickness behaviours were described in fully developed state of arthritis, but their pathogenesis has not been clarified yet. The study tested two hypotheses: firstly, that early adjuvant arthritis manifests with sickness behaviour, and secondly that neuro-inflammation and oxidative stress in the hippocampus rather than hyperalgesia interferes with the development of sickness behaviour observed in the early adjuvant arthritis.

The main aims of the study to be answered were as followed:

1a. What are the clinical features of early adjuvant arthritis?

1b. Does the early adjuvant arthritis change pain sensitivity?

2. Does early adjuvant arthritis impair cognitive functions?

3. Does early adjuvant arthritis induce anxiety behaviour?

4. Does early adjuvant arthritis cause neuro-inflammatory/chemical alterations in the hippocampus?

3.1 ANIMALS

Male, Lewis rats at 6-week of age (Charles River, Germany) were used in the study. After arrival they were housed under standard conditions: a 12-h light/dark cycle (lights on at 6:00 am., and lights off at 6:00 pm.), controlled humidity and temperature in an animal room at the Department of Normal, Pathological and Clinical Physiology Third Faculty of Medicine, Charles University in Prague. The rats were housed four per cage with free access to water and standard pellet diet ST-1® (Velas, Czech Republic), and were daily handled by the experimenter to minimize impact of manipulation stress. The rats were tested after one week of acclimatization period. The rats were treated in accordance with the national law of the Czech Republic on the Use of Laboratory Animals no. 246/1992 (fully compatible with European Community Council directives 86/609/EEC) based on the project approved by the Committee for Protection of Experimental Animals at the Third Faculty of Medicine, Charles University in Prague.

3.2 ADJUVANT ARTHRITIS INDUCTION

Adjuvant arthritis was induced to Lewis rats at 7 week of age by a single subcutaneous injection of 100μL of complete Freund´s adjuvant (cFA) 2 cm from the base of the tail (Figure 1). The cFA composed of heat killed *Mycobacterium butyricum*, which was suspended in paraffin oil, saline and Tween 80 (1:1:0,16) to the concentration of 5 mg/ml. Control rats were injected by a single dose of 100 μL saline instead of vehicle mixture (paraffin oil, saline, and Tween 80) because of its´ immunological effect.

FIGURE 1: ADJUVANT ARTHRITIS:

AN EXPERIMENTAL MODEL OF RHEUMATOID ARTHRITIS

RAT MODEL (UPPER PART)

ADJUVANT ARTHRITIS INDUCTION (LOWER PART)

3.3 DESIGN OF THE STUDY

The study was divided into four series. The study started with clinical characterization of the early phase of AA (series 1) which was followed by tests of cognitive abilities (series 2), behaviour (series 3), and evaluations of neuro-inflammatory/ chemical changes in the hippocampus (series 4). The experimental groups used in the study were: control rats and rats on day 2 and on day 4 after cFA administration (early arthritic rats).

3.3.1 CLINICAL CHARACTERIZATION OF EARLY ADJUVANT ARTHRITIS

In series 1 the clinical characterization of the early phase of AA was done. Body weight, food intake, pain reactivity, and inflammatory changes were evaluated. Each group consisted of 8 rats. The body weight and food intake were measured daily. Pain reactivity was evaluated separately on limbs and on tail in plantar test on day 2, and day 4 after cFA administration. In parallel experiment plasma analyses of leptin, ghrelin, ACTH, corticosterone, albumin, and CRP were done in the same disease intervals. The rats were decapitated between 8 to 9 a.m.to aviod the effects of hormonal fluctuations. Trunk blood was collected into cooled tubes containing EDTA-Na_2 (Sigma-Aldrich, Czech Republic) and centrifuged. Immediately after decapitation thymus and spleen weight was measured. Plasma was stored at -70 ºC before use.

3.3.2 COGNITIVE TESTING

In series 2 spatial learning abilities and swim strategies were examined in Morris water maze throughout first four days of AA. The tests were done between 8 and 10 a.m. Each experimental group consisted of 11 rats.

3.3.3 BEHAVIORAL TESTING

In series 3 behavior was tested in elevated plus maze and open field tests. The tests were done in the phase of the day with the highest animals' activity, from 3 to 5 p.m. on the 2^{nd} and 4^{th} arthritis day. 14 control rats and 13 early arthritic rats were used for EPM tests, and 7 control rats and 7 early arthritic rats were used for open field tests.

3.3.4 NEURO-INFLAMMATORY/ NEUROCHEMICAL CHANGES IN THE HIPPOCAMPUS

In series 4 neuro-inflammatory/ chemical changes in hippocampus were done on the 2^{nd} and 4^{th} arthritis day. The mRNA expressions of IL-1β, IL-6, iNOS, NADPH oxidases (NOX1, NOX2), BDNF and CRH were measured in both hippocampi (left and right) separatelly. In plasma the levels of CRP were estimated. Each experimental group had 8 rats.

3.4 METHODS

3.4.1 BODY WEIGHT AND FOOD INTAKE MEASUREMENTS

Body weight and food intake were measured daily in the morning hours (8-9 a.m.). Food intake was calculated as the difference between the initial and remaining weight of pellets from the previous day. Day 0 was the cFA application day. The food intake value presented was a mean value of a cage (four rats per cage). The statistical evaluation was not appropriate in this case.

3.4.2 PLANTAR TEST

Pain reactivity was measured by plantar test (Ugo Basile Comercio, Italy). The value represents the mean withdrawal latency (s). Forelimbs, hind-limbs and tail were evaluated separately (Figure 2). The intensity of noxious thermal stimuli was set to 40% and maximum cut-off time was set to 22 s to prevent tissue damage. In one session, reaction of each body site was tested three times with 5 min. break intervals, and the mean value was used for further analysis. Before testing the rat was placed into clear plastic box (size 27 x 17 x 14 cm) with a clear glass floor. The rat could move freely in the box. The testing box was cleaned after each rat to avoid smell traces.

FIGURE 2: PAIN RACTIVITY MEASUREMENTS

FORELIMB AND HIND-LIMB (UPPER PART)

THE PLANTAR TEST (LOWER PART)

3.4.3 MORRIS WATER MAZE TEST

The Morris water maze (MMW) apparatus (Figure 3) was a circular pool, made of blue plastic (1.98 m in diameter and 0.47 m high). The maze was divided into four equal quadrants. A circular escape platform was positioned in north-east quadrant very close to start position labeled “east”. The platform was made of white plastic with a textured surface (13.0 cm in diameter). Four different start positions labeled “north” “south” “east” and “west” were equally spaced around the perimeter of the pool. Throughout testing the maze was filled with water (20°C) to a depth of 20.0 cm. The platform was submerged 1.0 cm below the water level. MWM tests were performed in the room illuminated with a 40 W light, and fitted with hanging pictures and posters which provided extra-maze landmarks. Swimming path was recorded with a video camera, suspended above the centre of the pool, connected to a video tracking system Etho Vision® 3.1 (Noldus, Netherlands). Each rat was put into the maze facing toward the maze wall. The rats received 8 trials per day, each trial started from a new position in following order: north, south, east, and west. Search time was limited to 60 s. After 60 s rats were manually guided to the hidden platform. The inter-trial interval was 30 s. The following parameters were measured: (1) latency (s) – time needed for rats to find the escape platform, (2) distance (cm) – the length of the trajectory (3) swim speed (cm/s) – the ratio of distance per time, and (4) time spent in the thigmotaxis zone of a diameter of 20.0 cm from the side wall of MWM. Tests were done in between 8 and 10 a.m.

3.4.3.1 SWIM STRATEGIES ANALYSIS

The swim strategies were recorded by the EthoVision® software (Noldus Neatherlands), and analyzed by two independent observers (Figure 3). The strategies were compared with those from the study of Janus (2004). The strategies studied were as followed: (1) thigmotaxis – swimming along the wall of the pool, (2) random search – swimming around the wall with excursions to the opposite side of the pool, (3) scanning – more systematic searching that involved circular swims over the central area, (4) chaining – circular swims at a fixed distance from the wall, (5) circling – swimming in tight circles around the pool towards the platform, (6) focal search in the incorrect quadrant – searching for the platform with loops and turns in the wrong quadrants, (7) focal search in the correct quadrant – searching for the platform with loops and turns in the correct quadrant, (8) direct swim – a direct movement to the platform from any starting position.

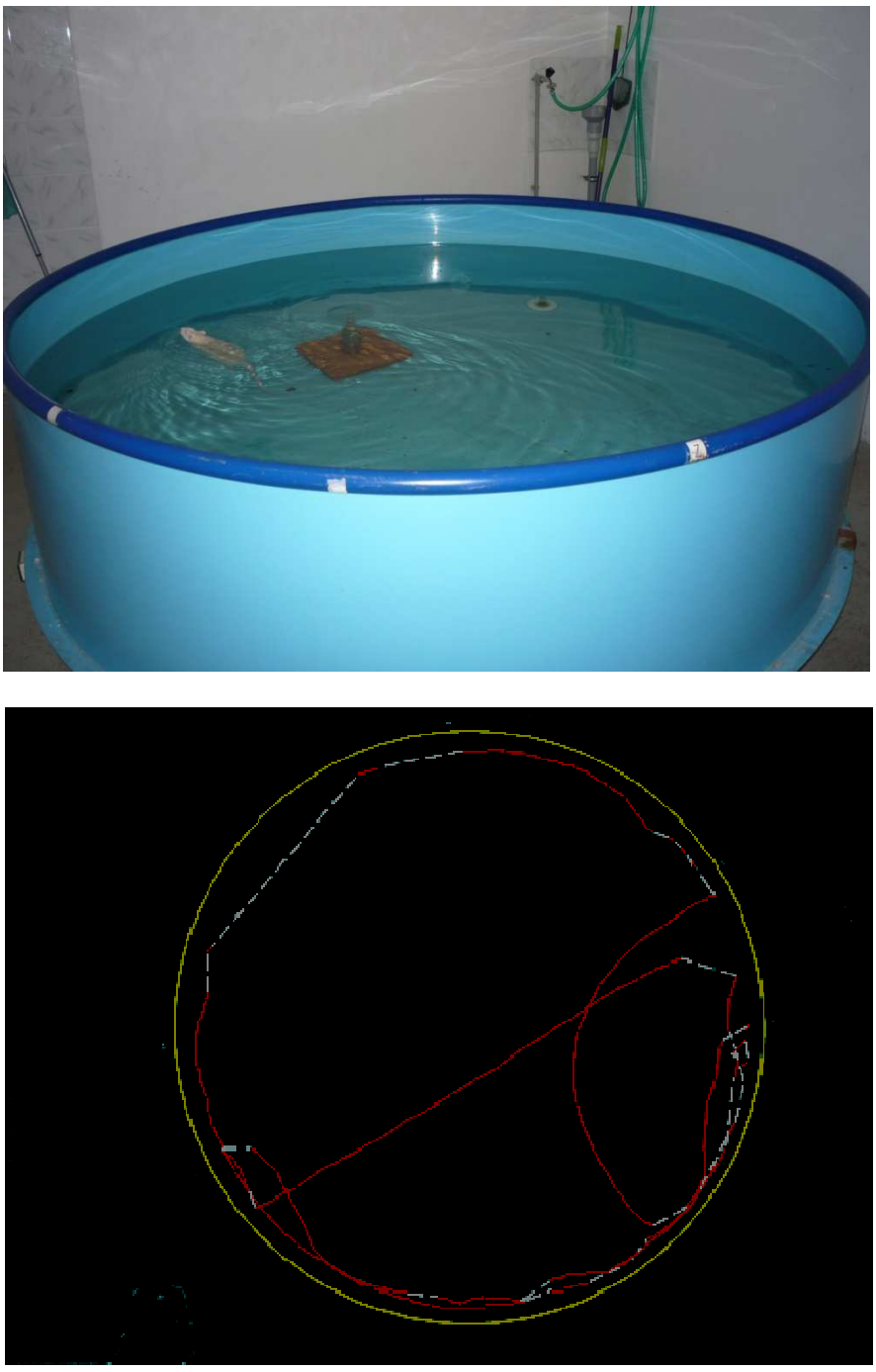

FIGURE 3: LEARNING ABILITIES MEASUREMENTS

THE MORRIS WATER MAZE (UPPER PART)

REPRESENTATIVE TRAJECTORY (LOWER PART)

3.4.4 ELEVATED PLUS MAZE TEST

The elevated plus maze apparatus (Figure 4) consisted of two open arms (10 x 30 cm) and two closed arms (10 x 30 cm). Both arms extended from a central platform (size 10 X 10 cm) were elevated by a single central support to a height 40 cm above the floor. The open arms were surrounded by a transparent plexiglass edge (0.5 cm) to support exploration in the arms. Each rat was individually positioned into central platform facing forward open arm, and was allowed to explore the maze freely for 5 minutes. On the testing day rats were moved to experimental room where they remained in their home cages for one hour acclimatization period. Behavioural testing was done during hours with the rats´ highest activity from 3 to 5 p.m. The manipulation room was provided with dim red light, and kept free from excessive noise during testing. Each rat was tested only once. After each rat the arena was cleaned, and dried thoroughly to avoid influence of smell traces on the behaviour. The rat's performance was videotaped, and evaluated by an independent observer using ODLog® Software (RegSoft.com).

3.4.4.1 BEHAVIOURAL DATA

In the elevated plus-maze following variables were measured: number of open (OAE), and close arm entries (CAE), time spent in open (OAT) and close (CAT) arms of the maze. Also total time spent in both arms of the maze (TT) and sum of entries into both arms (TE) were calculated. Anxiety index was expressed as ratio of open arm time to total time (OAT/TT (%)) as well as ratio of open arm entries to total entries (OAE/TE 100 (%)). An entry was characterized as movement into the arm with all 4 paws in the arm. The arm time was characterized as time spent in the arm with all 4 paws in the arm.

3.4.5 OPEN FIELD

The open-field (OF) was a transparent plastic box. The floor (total size: 45 X 45 cm) was divided into peripheral area (12 squares), and a central circle (on 4 squares). On the testing day (day 2, 4 of AA), rats were moved to experimental room where they remained in their home cages for one hour acclimatization period. The manipulation room was provided with dim red light, and kept free from excessive noise during behavioural recordings. Behavioural testing started after placing the rat into central circle of the OF. Each rat explored the arena for 5 minutes, no longer, to prevent habituation to the apparatus. Behavioural tests were performed during hours with the rats´ highest activity from 3 to 5 p.m. Each rat was tested only once. Between two rats the arena was cleaned, and dried thoroughly to avoid influence of smell traces on the behaviour. Rat´s performance was videotaped, and evaluated by an independent observer using ODLog® Software (RegSoft.com).

3.4.5.1 BEHAVIOURAL DATA

In the open field, following parameters were recorded: the number of squares crossed in the central circle (CC), and in peripheral area (PC), the time spent in the central circle (CT), and the number of the central visits (CE). A square crossing was a movement from one square to another when all four paws were inside the square. A central visit was an entry with all four paws into the central circle of the open field. The central time was the time spent in the central circle of the open field with all four paws in the central circle.

FIGURE 4: ANXIETY MEASUREMENTS

THE ELEVATED PLUS MAZE (UPPER PART)

THE OPEN FIELD (LOWER PART)

3.4.6 ISOLATION OF nARC AND HIPPOCAMPUS

Brain was quickly removed and ice-chilled at -30 °C in isopentane (Sigma Aldrich, Czech Republic) for one minute. Thereafter it was frozen in liquid nitrogen and stored at -70 °C. The frozen brains were placed into a Reichert® cryocut to adapt to - 20°C working temperature. Thereafter, the brains were cut horizontally until the beginning of the median eminence appeared. The nArc (including the median eminence) was isolated by 1 mm deep punch, using a needle with 0.6 mm of inner diameter. The isolated tissues were collected into 1.5 ml Eppendorf® tubes and kept under - 70°C until used for poly(A)RNA isolation. Right and left hippocampus was removed separately from fresh brain tissue, snap-frozen in liquid nitrogen until used for total RNA isolation.

3.5 PLASMA ANALYSES

Leptin and ghrelin concentrations in plasma were determined by RIA using commercial Linco® kits (Linco Research, USA) following the manufacturer´s instructions. The assay sensitivity for leptin was 0.5 ng/ ml, and 93 pg/ ml for ghrelin.

Plasma ACTH was determined by a double antibody RIA using a commercial Johnson & Johnson® kit (Amersham Pharmacia Biotech, UK). Concentration limits of the assay were within the range of 25 to 1000 pg/ml.

Plasma corticosterone was extracted with methylene chloride (Merck, Germany) and analyzed by RIA using specific antibodies (Sigma-Aldrich, Germany) and [1, 2, 6, 7-^{3}H]-corticosterone (Amersham Pharmacia Biotech, UK). Free and bound hormones were

separated by dextran-coated charcoal. Coefficient for intra-assay variance was 2.3% and inter-assay variance was 5.6%.

Plasma CRP was estimated by a double antibody ELISA using a commercial kit (Immunology Consultants Laboratory, Inc. Oregon, USA).

Concentration of albumin in plasma was measured spectrophotometrically using an Albumin (BCG SYS 1 BMI Hitachi® kit (Boehringer, Germany).

3.6 QUANTITATIVE real-TIME PCR

Poly (A) RNA was isolated from nArc with the use of Chemagic® mRNA Direct Kit (Chemagen Biopolymer- Technologie AG, Beasweiler, Germany). Extracted Poly (A) RNA was reversely transcribed into cDNA with the use of Omniscript® RT Kit (Quiagen Inc., Valencia, California, USA). The reaction mixture contained: RNase inhibitor (Takara® Holdings Inc., Shiga, Japan), pd$(N)_6$ random hexamer primers (Amersham Biosciences, Piscataway, NJ).

Total RNA was isolated separately from the right and left hippocampus with the use of RNAqueous®- 4PCR kit (Applied Biosystems, Czech Republic). DNase1 treatment was included to prevent DNA contamination. Total RNA was reversely transcribed into cDNA using the High-Capacity® cDNA Reverse Transcription kit (Applied Biosystems, Czech Republic).

Both cDNAs were used for PCR estimations. PCR reactions were performed using TaqMan® gene expression products (Applied Biosystems, Czech Republic). The multiplex PCR reaction mix contained cDNA, universal PCR master mix and TaqMan® probes. Eucaryotic 18 S RNA was used as endogenous control, and was labeled with VIC reporter

dye. Target genes (BDNF, CRH, IL-1β, IL-6, NOX1, NOX2 -gp91^{phox}, and iNOS, NPY) were labeled with FAM reporter dye. Samples run in triplicate. Thermal cycling was performed according to the manufacturer's instructions with 2 initial setup steps: 2 minutes at 50 °C and 10 minutes at 95 °C, and 40 cycles at 95 °C for 15 seconds and at 60 °C for 1 minute. Expression of target gene mRNA was quantified using an ABI Prism® 7000 Sequence Detector and software (Applied Biosystems, Czech Republic). Multiplex PCR reaction mix contained: cDNA, TaqMan® Universal PCR Master Mix, TaqMan® primers and probes (Applied Biosystems, Czech Republic). As an endogenous control eucaryotic 18S RNA was used. All samples run in triplicate. Thermal cycling proceeded according to manufacturer´s protocol. Data were analyzed using ABI Prism® 7000 Sequence Detection System software (Applied Biosystems, Czech Republic). The results were calculated with a relative comparative method, and are expressed as the ratio the mRNAs of interest to18S RNA.

3.7 STATISTICAL ANALYSES

In the series 1, body weight, withdrawal latencies, concentration of hormones, and imunological parameters in plasma were analyzed by one-way ANOVA followed by Bonferroni multiple comparison t test. GraphPad® software (GraphPad.com) was used for all analyses.

In the series 2 spatial learning data were analyzed by two-way ANOVA (factor day and treatment) with repeated measures. Quantitative differences between experimental groups were assessed by day-treatment interaction tests. The chi-square (χ^2) test of homogeneity followed by the analysis of adjusted standardized residuals (Z-values) was

used to estimate search strategies during spatial learning. SPSS 17.0 (SPSS Inc, Chicago, IL), Statistica® software (StatSoft Inc,Tulsa, OK) was used for statistical analyses.

In the series 3 behavioural data were analyzed by two-way ANOVA with repeated measures. The differences between experimental groups on both factors (treatment and day) were assessed by the day-treatment interaction effects. Statistica® software (StatSoft Inc,Tulsa, OK) was used for statistical analyses.

In the series 4 the differences between groups were assessed by Bonferroni multiple comparison t test. In all tests, the difference was considered significant if $p < 0.05$*. GraphPad® software (GraphPad.com) was used for statistical analyses.

4. RESULTS

4.1 SERIES 1: *CLINICAL FEATURES OF EARLY ADJUVANT ARTHRITIS*

ANOREXIA

In all measured intervals of early AA the body weight was significantly decreased in AA rats compared to control rats. In AA rats body weight decline was accompanied by decreases in food intake with a maximal decline on day 2 of AA (C2 vs. AA2: 23.79 ± 0.03 vs. 12.33 ± 0.27). Since the total food consumption value presented is a mean value of a cage (four rats), the statistical evaluation was not appropriate in this case (Figure. 4.1.1).

Circulating leptin levels decreased borderline significantly in AA rats on the 2^{nd} and the 4^{th} day. Opposite to leptin, concentration of ghrelin increased in plasma on day 2 of early arthritis significantly (C vs. AA2: 2.066± 0.058 vs. 2.516± 0.149) (Figure.4.1.2).

Simultaneously to increases of ghrelin concentrations, mRNA of NPY in nArc was up- regulated in the same disease intervals (C vs. AA2: 1± 0.054 vs. 6.303± 0.606). Furthermore, expression of IL-1β mRNA in nArc was increased in AA rats from day 2, and remained elevated since day 4 of early arthritis (C vs. AA2: 1± 0.056 vs. 3.85 ± 0.086, C vs. AA4: 1± 0.056 vs. 1.521± 0.077) (Figure. 4.1.3).

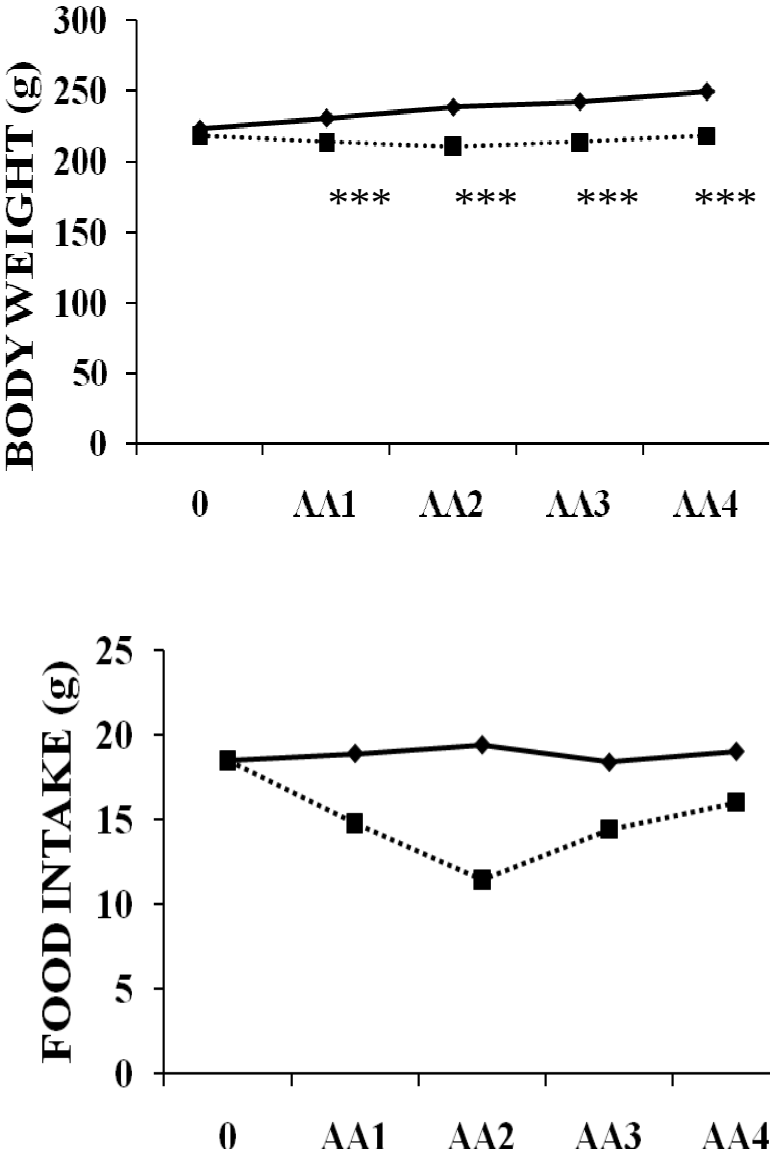

Figure 4.1.1: Early adjuvant arthritis: food intake and body weight are depicted. The black line shows control rats. The dotted line shows early arthritic rats. The body weight values are means of 8 rats ± SEM. The food intake value is a mean value of a cage. Numbers under x-axis are arthritis days, day zero is the day of cFA administration. Abbreviations: AA= early AA rats. Statistical significance: *C vs. AA: $p < 0.001$* *** in all measured intervals of early arthritis. Statistical evaluation: see page 43.

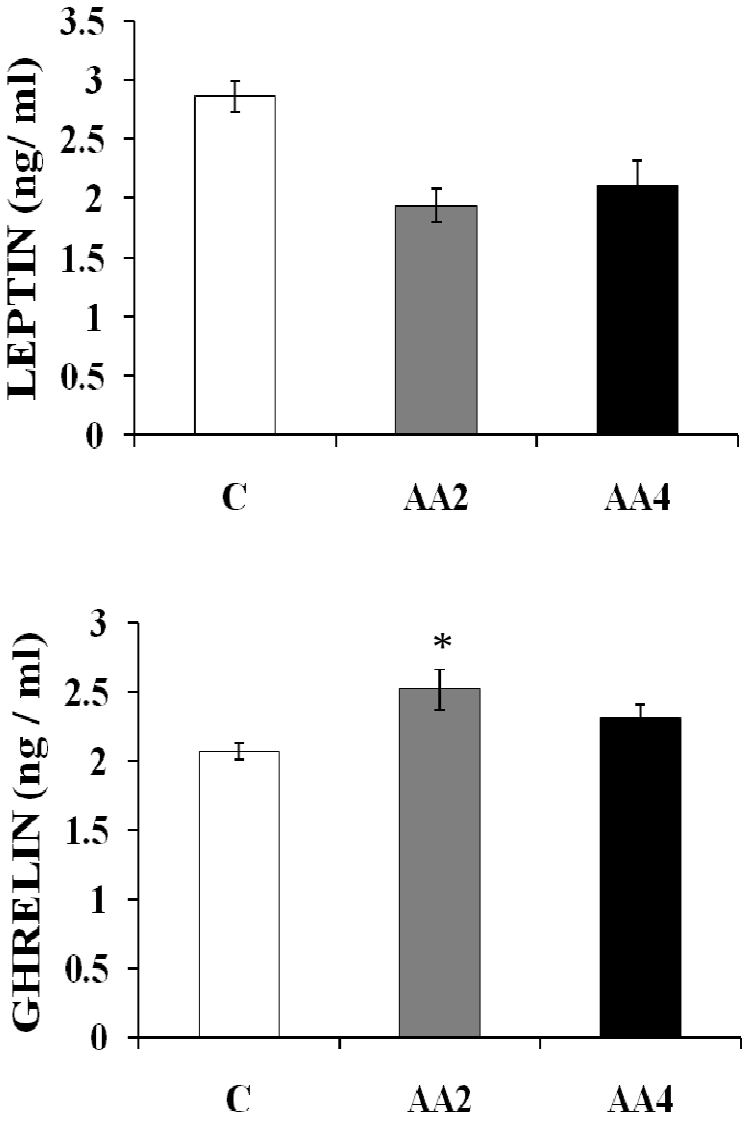

Figure 4.1.2: Early adjuvant arthritis: plasma leptin and ghrelin are depicted. The values are a mean of 8 rats ± SEM. The white bars show control rats, and the grey and black bars show early AA rats. The numbers represent days after arthritis inoculation. Abbreviations: C= control rats, AA= early arthritic rats. Statistical significance*: C vs. AA: $p < 0.05$ **.* Statistical evaluation: see page 43.

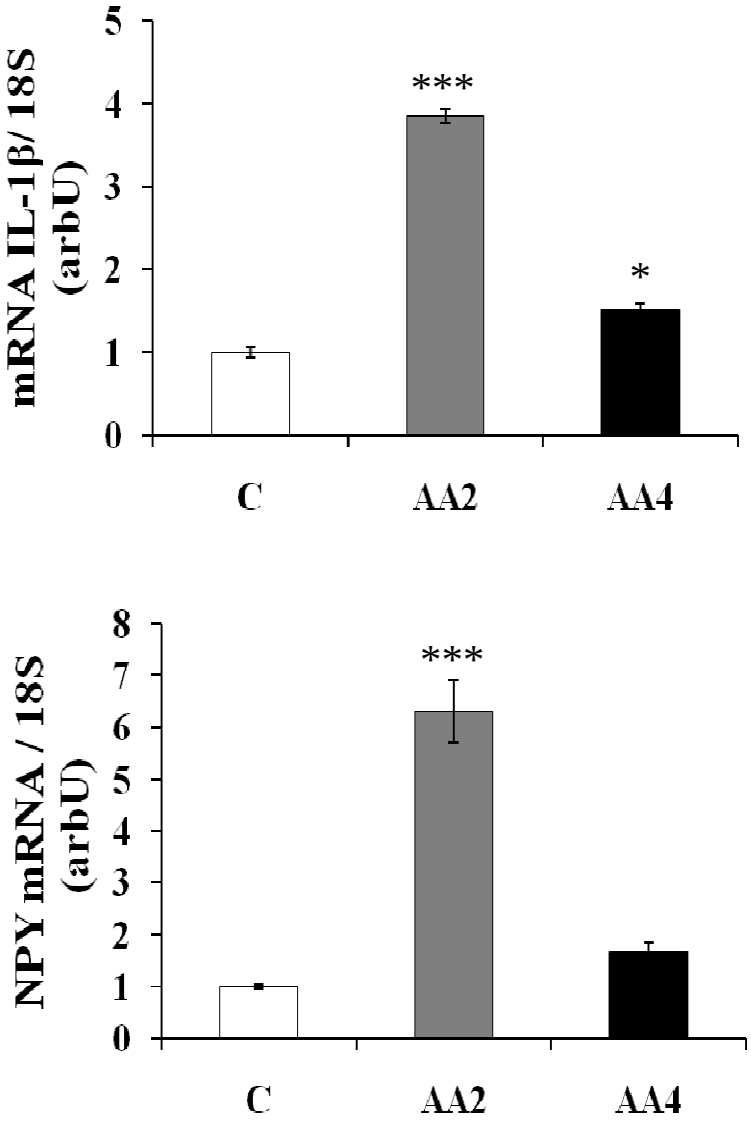

Figure 4.1.3: Early adjuvant arthritis: expression of NPY and IL-1β mRNA in nArc are depicted. The mRNA expression value is a ratio of target mRNA (IL-1β, NPY) to endogenous control (18S). Each value represents a mean of 8 rats ± SEM. The white bars show control rats, and the grey and the black bars show early arthritic rats. The numbers represent days after arthritis inoculation. Abbreviations: C= control rats, AA= early arthritic rats. Statistical significance*: C vs. AA: * p<0.05, *** p<0.001*. Statistical evaluation: see page 43.

PAIN REACTIVITY

The withdrawal latency given as mean of all four limbs (Figure. 4.1.4) decreased significantly on day 2 of AA only, thus indicating transiently enhanced pain reactivity (C vs. AA2: 9.28± 0.58 vs.7.41±0.29). Transient hyperalgesia was also present on the tail. Pain thresholds on tail decreased in cFA rat group compared to control rats on day 2 of AA (C vs. AA2: 8.61±0.48 vs. 6.95±0.52).

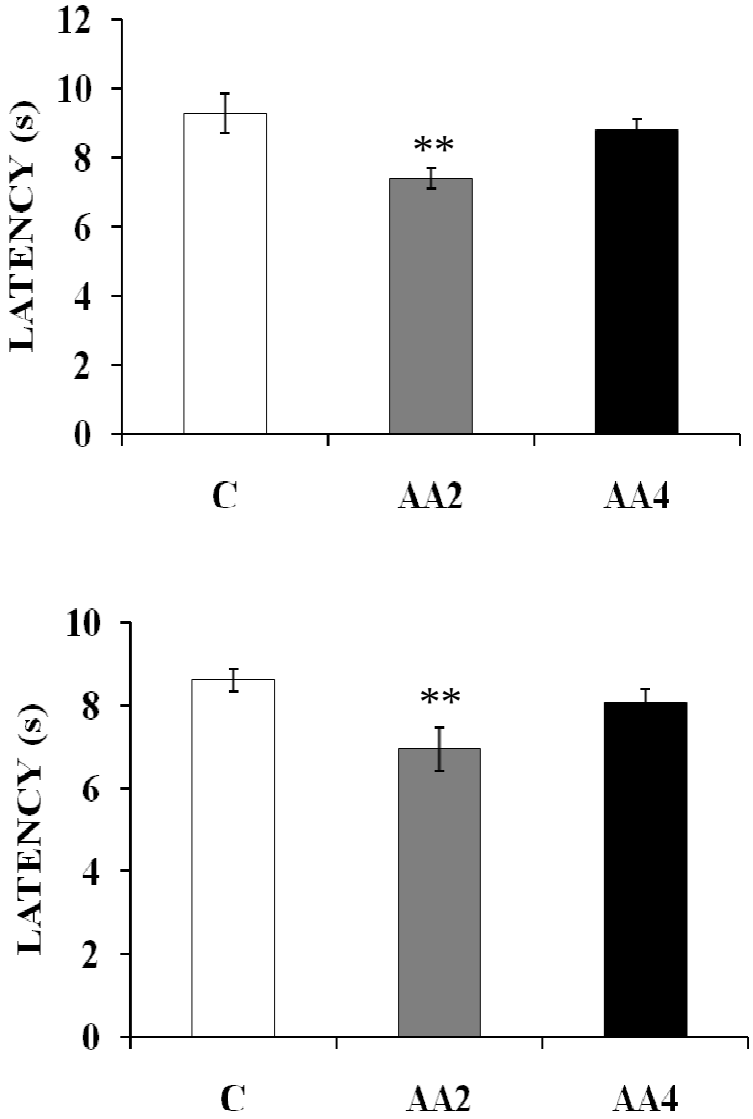

Figure 4.1.4: Early adjuvant arthritis: pain reactivity. Withdrawal latencies of limbs (up in the picture) and tail (down in the picture) are depicted. The white bars represent control rats, the grey and black bars represent early arthritic rats. Numbers under x-axis are days after arthritis induction. The values are means of 8 rats ± SEM. Abbreviations: C= controls, AA= early arthritic rats. Statistical significance: ***p*< *0.01*. Statistical evaluation: see page 43.

SYSTEMIC INFLAMMATORY CHANGES

In the early phase of AA the systemic inflammatory changes were clearly manifested by weight changes of immune organs. The thymus weight decreased significantly on day 2, and continued in that trend by day 4 (C: 476.7±7.39 vs. AA2: 380.0±31.4, and C: 467.7±17.1 vs. AA4: 300.88±21.0). In contrast, the weight of spleen was increased on both early arthritis days (C: 476.7±7.4 vs. AA2: 529.9±11.7, and C: 476.7±7.4 vs. AA4: 608.9±22.5) (Fig. 4.1.5).

The specific inflammatory indicator, CRP, was enhanced on both examined days of arthritis (C vs. AA2, and AA4: 573.68± 25.12 vs. 1662.59± 132.94, and 1462.33± 117.38). The inflammatory process was also reflected by a reduction of the negative indicator of the hepatic acute phase response, albumin on both early arthritis days compared to controls (C vs. AA2, and AA4: 33.74± 0.55 vs. 31.18± 0.346, and 27.4± 0.24) (Fig.4.1.6).

Plasma levels of ACTH and corticosterone were equally elevated on days 2 and 4. Elevated levels of ACTH in cFA rats (C vs. AA2, and AA4: 86.04± 2.29 vs. 116.25 ±3.94, and 106.25± 5.05) were followed by enhanced corticosterone levels (C vs. AA2, AA4: 0.44± 0.11 vs. 3.02± 0.97, and 3± 1.23) (4.1.7).

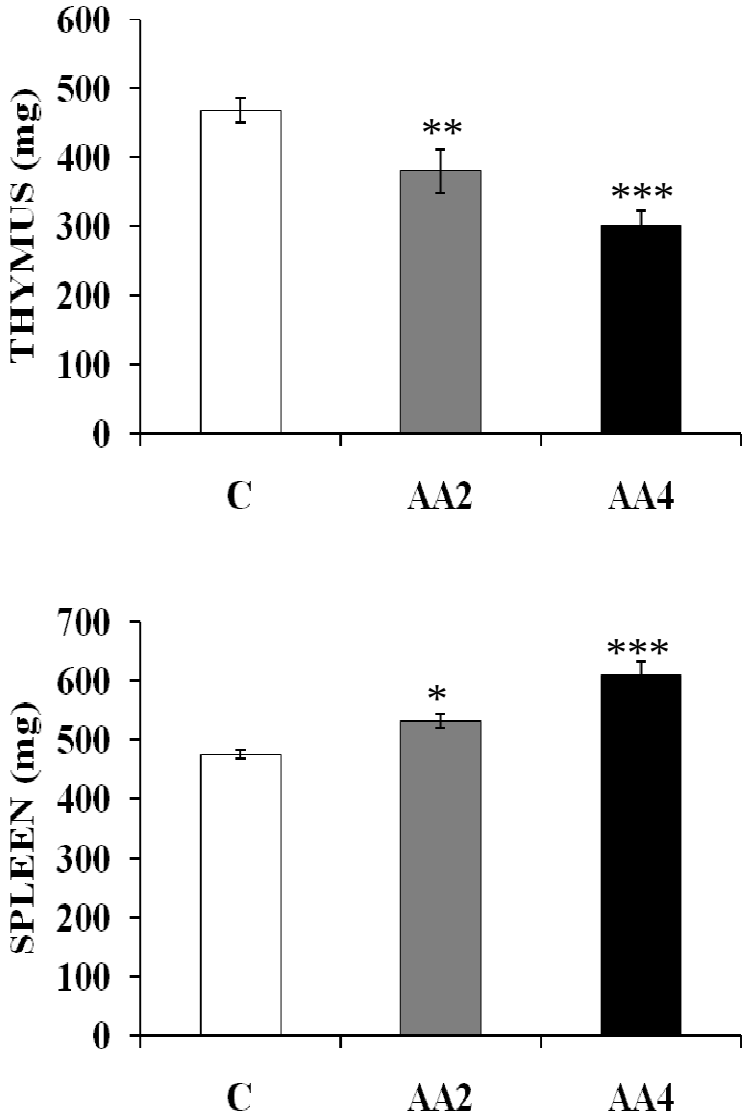

Figure 4.1.5: Early adjuvant arthritis: systemic inflammatory changes -weight of thymus and spleen are depicted. The white bars represent control rats; the grey and the black bars represent early arthritic rats. The values are means of 8 rats ± SEM. Abbreviations: C= control rats, AA= early arthritic rats. Numbers under x-axis are days after arthritis induction. Statistical significance: C vs. AA: *$p < 0.05$ *; $p < 0.01$ **; $p < 0.001$* ***. Statistical evaluation: see page 43.

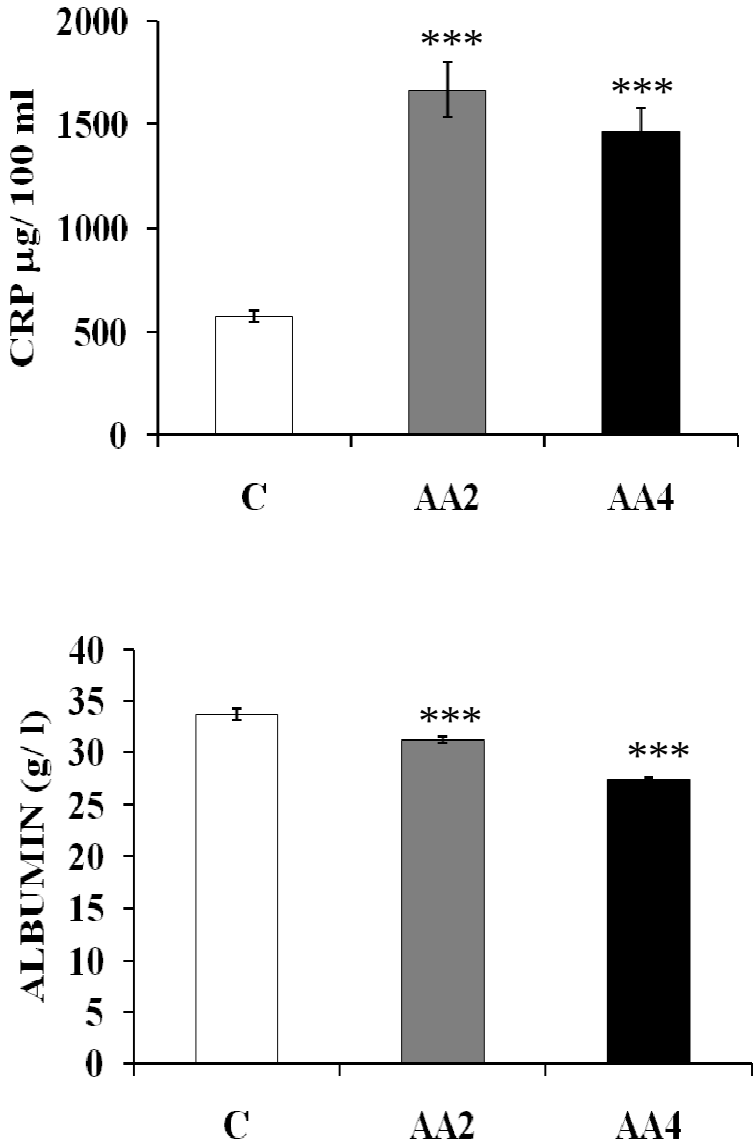

Figure 4.1.6: Early adjuvant arthritis: systemic inflammatory changes - plasma levels of albumin and C-reactive protein are depicted. The white bars are control rat, the grey and black bars are early arthritic rats. The values are means of 8 rats ± SEM. Abbreviations: C= control rats, AA= early arthritic rats. Numbers under x-axis are days after arthritis induction. Statistical significance: C vs. AA: $p < 0.001$ ***. Statistical evaluation: see page 43.

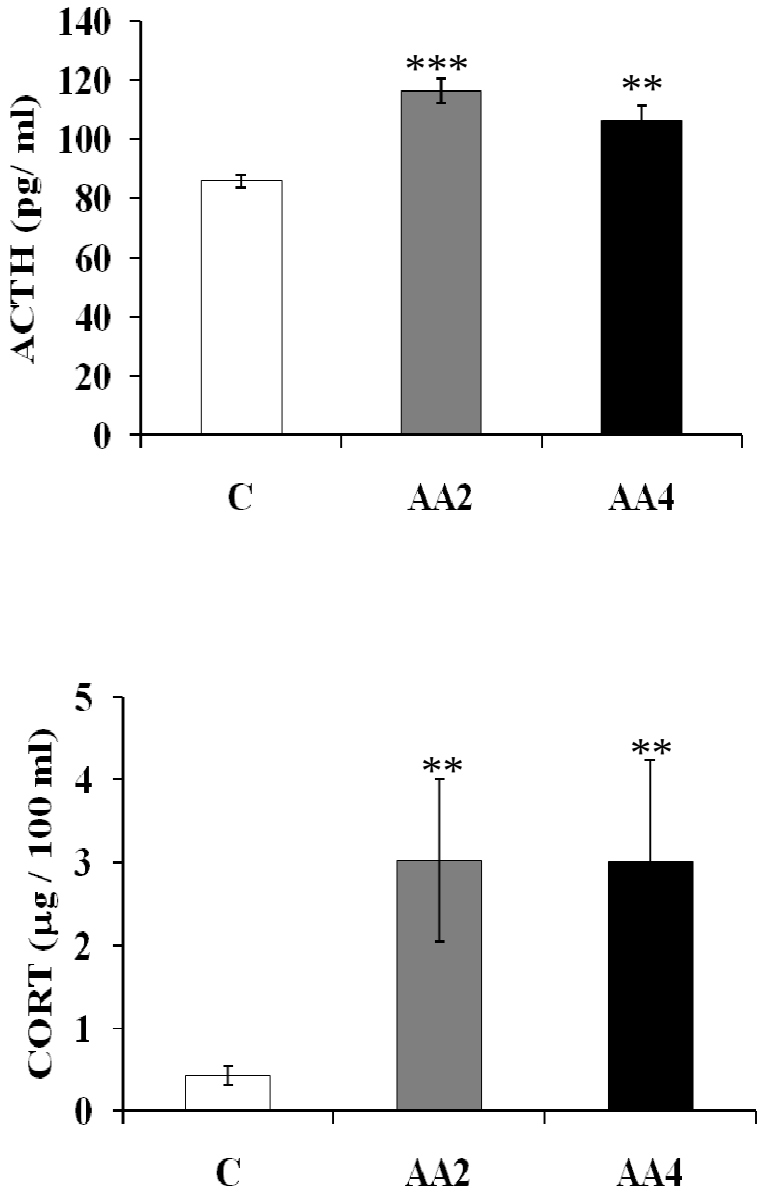

Figure 4.1.7: Early adjuvant arthritis: systemic inflammatory changes - plasma levels of ACTH and corticotrophin depicted. The white bars are control rats, and the grey and black bars are early arthritic rats. The numbers under x-axis depict days after arthritis induction. Values in each column are a mean of 8 rats± SEM. Abbreviations: C= control rats, AA= early arthritic rats. Statistical significance: ** *p<0.01;* *** *p<0.001*. Statistical evaluation: see page 43.

4.2 SERIES 2: *COGNITION IN THE EARLY ADJUVANT ARTHRITIS*

SPATIAL LEARNING

When evaluating 4 days of AA the findings indicated that there were substantial differences among days, the main effect of day being statistically significant for all variables measured – latency ($F_{(3,60)}$= 73.54; p<0.001), distance ($F_{(3,60)}$= 47.02; p<0,001), speed ($F_{(3,60)}$= 51; p<0.001), as well as thigmotaxis ($F_{(3,60)}$= 67.16; p<0.001) (Fig. 4.2.1). The values for latency, distance, and thigmotaxis decreased, while those for speed increased over days, presumably due to learning effects. The between-subject interaction (treatment*day) for latency, distance, swim speed, and thigmotaxis parameters were not statistically significant over the four 4 arthritic days. However, when evaluating the first two days of the disease, the time needed to find the hidden platform - latency was significantly longer for the rats with AA than for control rats (interaction: treatment*day, $F_{(1,20)}$= 3.08, p<0.05). Similarly, the distance moved by the rats with AA was significantly longer, (interaction: treatment*day, $F_{(1,20)}$= 6.47, p<0.01) as was the time spent in the thigmotaxis zone (interaction: treatment*day, $F_{(1,20)}$= 7.12, p<0.01). No difference in swim speed was observed between healthy and arthritic rats in that time interval.

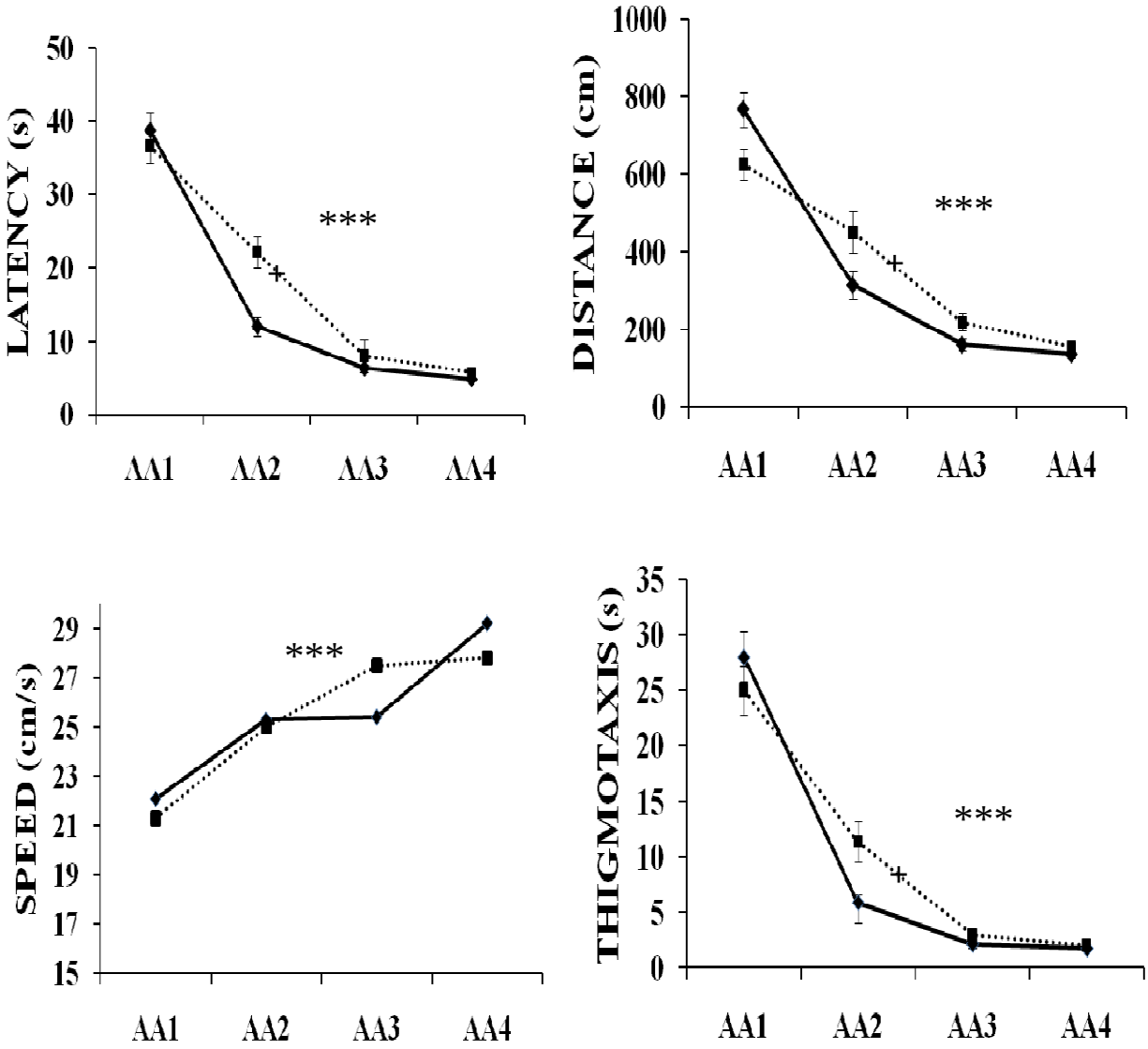

Figure 4.2.1: Early adjuvant arthritis: spatial learning. Values of the latency, distance, speed, and thigmotaxis parameters are depicted. Numbers under lines represent arthritis days. Solid lines are saline injected controls; dotted lines are early arthritic rats. Each value represents the mean of 11 values ± SEM. Abbreviations: C= control rats, AA= early arthritic rats. Significance: *** *p< 0.001 (among days)*; + *p< 0.05(interaction: day X treatment).* Statistical evaluation: see page 43.

SWIMMING NAVIGATION

Table 1 shows the frequencies of individual swim strategies used by animals during the spatial learning process in the MWM. The differences between control and early arthritic rats were significant on day 3 (chi-square= 30.41; $p<0.001$) and day 4 (chi-square= 22.49; $p<0.01$). Comparison of swim strategies between control and early arthritic rats on individual days of spatial learning revealed that control rats preferred the direct swim strategy on days 3 and 4 (day 3: $Z= 5.2$; $p<0.001$; day 4: $Z= 2.8$; $p<0.01$). Early arthritic rats had higher incidence of focal searching in the incorrect quadrant than control rats (day 2: $Z = 3.0$; $p<0.01$; day 3: $Z = 3.3$; $p<0.001$; day 4: $Z = 3.5$; $p<0.001$). These results indicate that early arthritic rats used a different and less effective strategy when solving the spatial learning task from day 2 to 4 of AA. A representative trajectory of one healthy and one rat with AA during days 2 to 4 of AA testing is given in Figure 4.2.2.

DAY	1		2		3 ***		4 **	
STRATEGY	C	AA	C	AA	C	AA	C	AA
THIGMOTAXIS	23	21	4	8	1	2	1	0
RANDOM	28	34	10	10	4	7	1	3
SCANNING	5	4	2	2	2	3	2	1
CHAINING	14	15	10	11	5	12	10	5
CIRCLING	8	5	17	12	7	12	6	13
CF	1	1	6	6	2	7	1	4
IF	1	1	2	++ 13	1	+++ 13	1	+++14
DIRECT SWIM	8	7	37	26	+++ 66	32	++ 66	48

Table 1: **Early adjuvant arthritis: incidence of swim strategies:** Table 1 gives the frequencies of individual search strategies in controls (n = 11) and AA rats (n = 11) during spatial learning on days 1 through 4 of the experiment. Statistical significance: *** $p<0.001$; ** $p<0.01$; between control and AA rats is based on chi-square statistics; +++$p<0.001$; ++$p<0.01$ for a given strategy is based on Z-values of adjusted standardized residuals. Abbreviations: CF: focal correct, IF: focal incorrect.

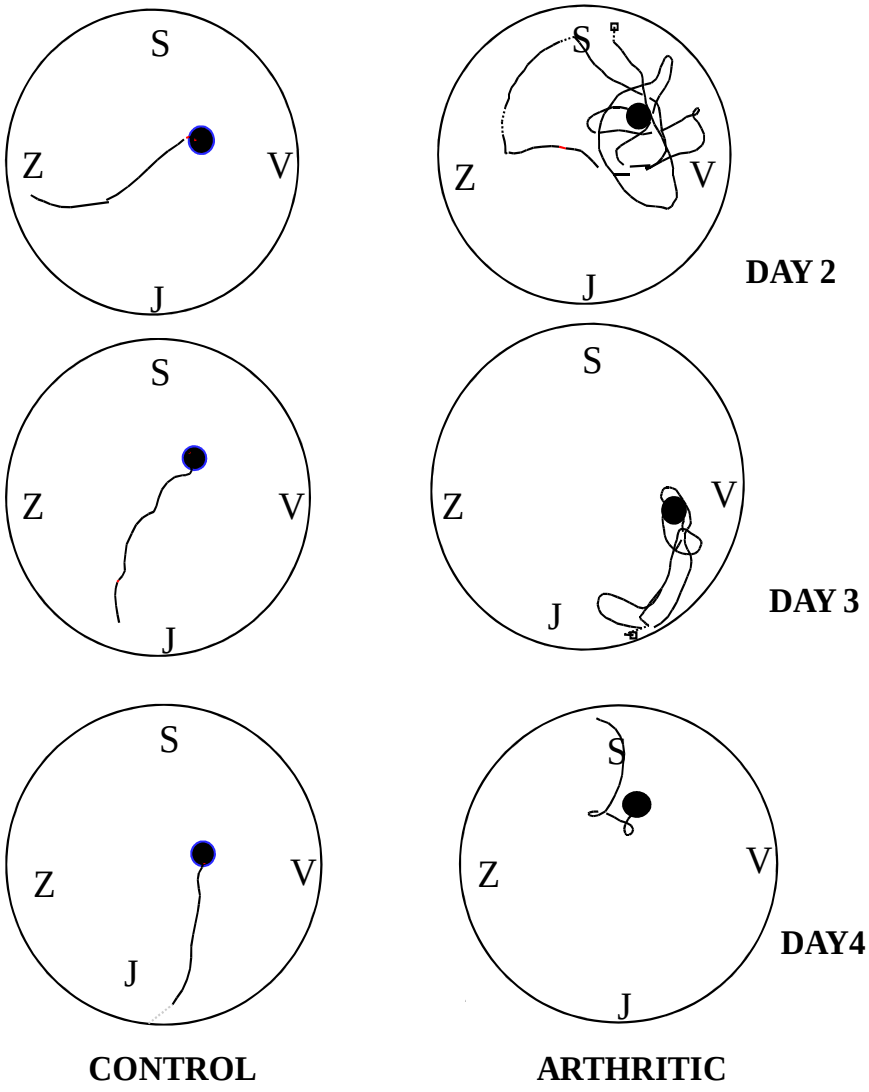

Figure 4.2.2: Early adjuvant arthritis: representative swim strategies. Each picture represents the most preferred swim strategy used by the group on day 2, 3, 4 of arthritis. Control and arthritic are depicted. The highest proximity of distance to the average of the group was considered by the strategy selection. The rats have started from different positions.

4.3 SERIES 3: *ANXIETY BEHAVIOUR IN THE EARLY ADJUVANT ARTHRITIS*

ELEVATED PLUS MAZE

Both control and early arthritic rats showed a significant reduction of CAE ($F_{(1,25)} = 10.11$; $p < 0.005$) and OAE ($F_{(1,25)} = 9.14$; $p < 0.01$) over time (interaction treatment*day, N. S.), presumably due to the habituation. However, early arthritic rats showed a lower number of OAE compared to control animals ($F_{(1,25)} = 9.14$; $p < 0.01$), while the number of CAE did not differ between the groups. Consequently the total ambulatory activity of early arthritic rats was significantly reduced ($F_{(1,25)} = 6.40$; $p < 0.02$). The percentage of OAE was lower in early arthritic rats ($F_{(1,25)} = 7.14$; $p < 0.02$), as was the ratio of OAE/CAE ($F_{(1,25)} = 4.53$; $p < 0.05$). As for the time spent in individual arms, early arthritic rats spent less time in the open arms of the maze ($F_{(1,25)} = 4.63$; $p < 0.05$), and more time in closed arms ($F_{(1,25)} = 8.14$; $p < 0.01$) than control rats did. The total time spent in the arms was not different between the groups. Furthermore, the percentage of the OAT for early arthritic rats was significantly reduced ($F_{(1,25)} = 5.21$; $p < 0.05$). (Figure. 4.3.1).

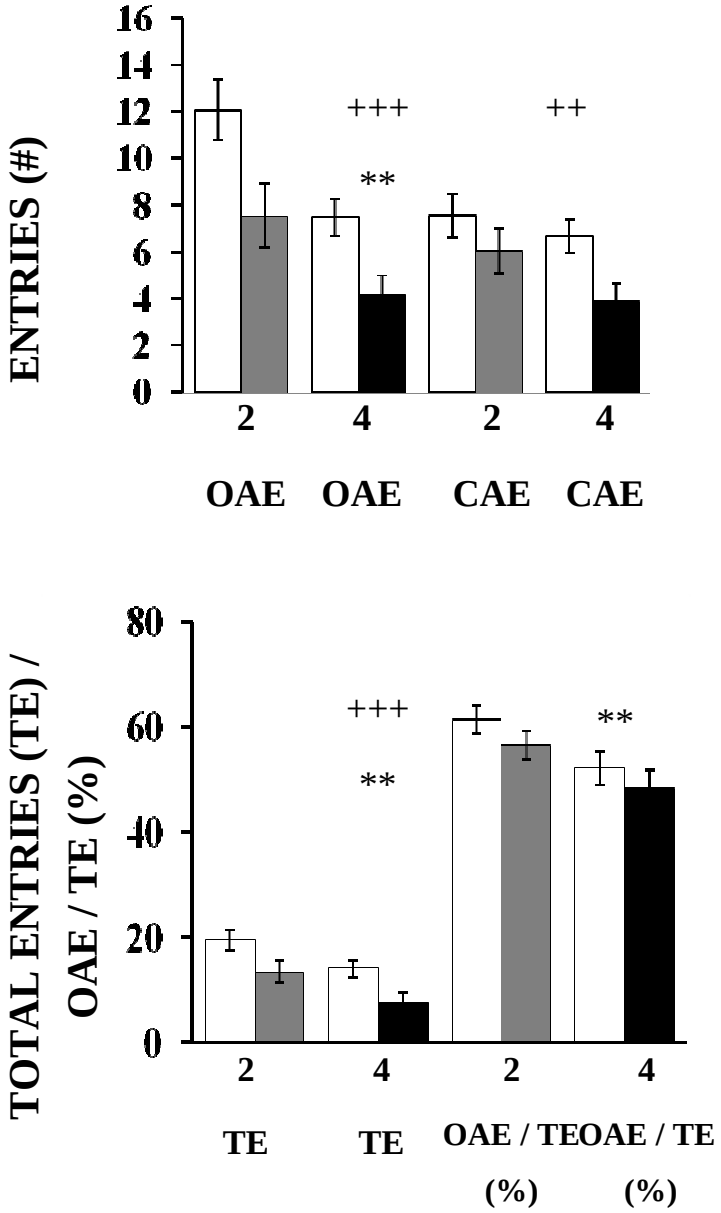

Figure 4.3.1: Anxiety behaviour in EPM on days 2 and 4 of AA:number of entries. The upper part of the picture shows the numbers of entries into individual arms of the elevated plus-maze. The lower part of the picture shows number of entries into both arms of the elevated plus-maze, and percentage of the open arm entries. Empty bars are for control rats; shaded or black bars are early arthritic rats. The numbers under the columns represent days of adjuvant arthritis. Abbreviations: OAE – open arm entries, CAE – closed arm entries, TE – total entries. Each column represents the mean of 14 values for controls and 13 values for early arthritic rats ± S.E.M Significance: asterisks represent significant differences among treatments; crosses represent significant differences among days **p< 0.01; $^{++}$p<0.01; $^{+++}$p<0.001. Statistical evaluation: see page 44.

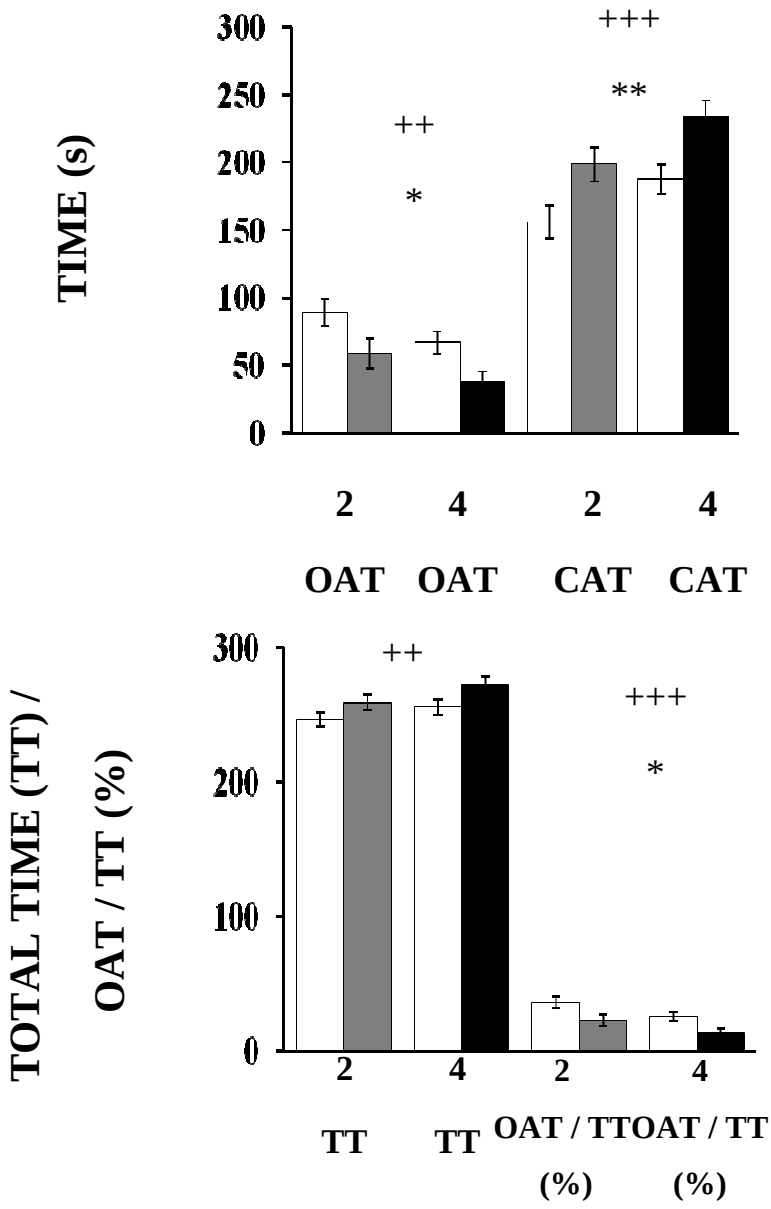

Figure 4.3.2: Anxiety behaviour in the EPM on days 2 and 4 of AA: time spent in the arms. The upper part of the picture shows the time spent in individual arms of the elevated plus-maze. The lower part of the picture shows total time spent in both arms of the elevated plus-maze, and percentage of the open arm time. Empty bars are control rats; shaded or black bars are early arthritic rats. The numbers under the columns represent days of adjuvant arthritis. Abbreviations: OAT – open arm time, CAT – closed arm time, TT – total time. Each column represents the mean of 14 values for controls and 13 values for cFA rats ± S.E.M Significance: asterisks represent significant differences among treatments; crosses represent significant differences among days: *p< 0.05 **p< 0.01; $^{++}$p<0.01; $^{+++}$p<0.001. Statistical evaluation: see page 44.

OPEN FIELD

The number of central entries (Figure 4.3.2) decreased in both control and early arthritic rats over days ($F_{(1,11)}$ = 32.93; p< 0.001), presumably due habituation to testing conditions. However, when central time was analyzed, early arthritic rats spent less time in the central area of the OF than control rats ($F_{(1,11)}$ =5,865 ; p<0.05) on both examined intervals of early AA ($F_{(1,11)}$ = 8,992; p<0.01), and there was a significant interaction (treatment * day: $F_{(1,11)}$ = 6.328; p< 0.05). Ambulation activity in the central circle of the OF decreased over days in both experimental groups over days ($F_{(1,11)}$ = 6.959; p<0.05), and border line significantly in cFA rats ($F_{(1,11)}$ = 4.509; p= 0.057). Opposite to central circle, ambulation in the peripheral area of the OF was unchanged between groups on both examined days intervals of early AA.

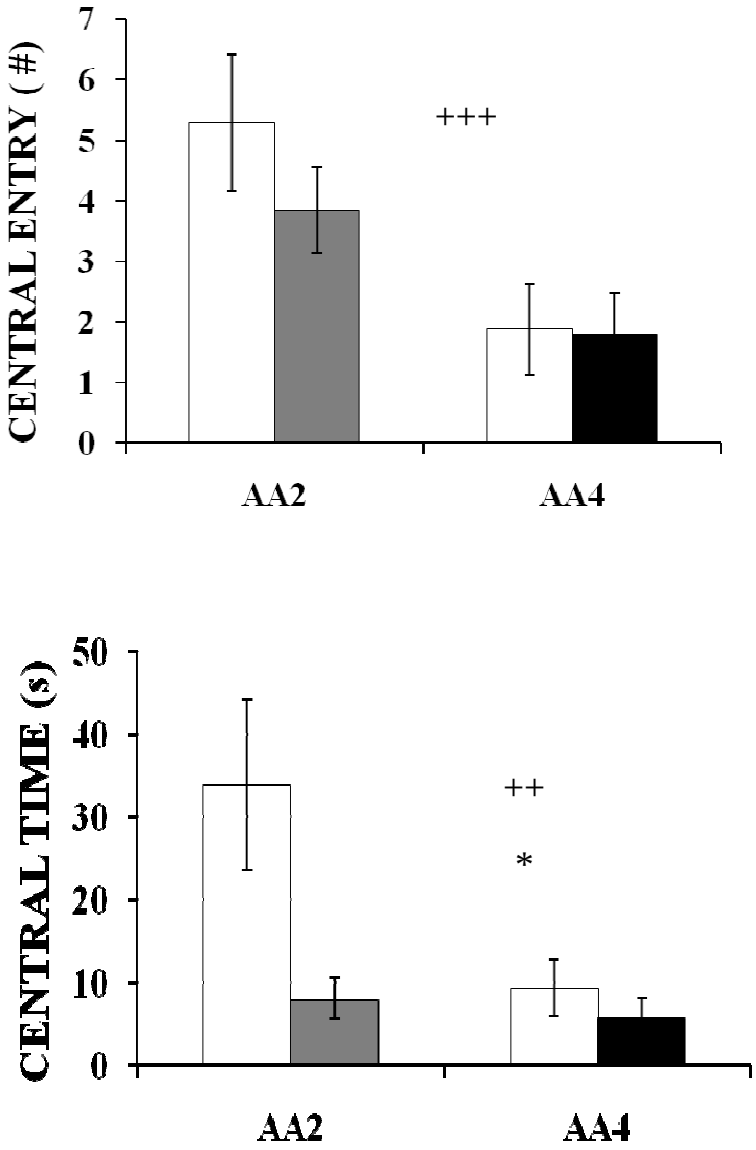

Figure 4.3.2: Anxiety behaviour in the open field on days 2 and 4 of AA: central entries, time are depicted. The black bars are control rats the coloured bars are cFA rats. Each column represents the mean of 6 values for controls and 7 values for early arthritic rats ± SEM. Abbreviations AA= early arthritic rats. Numbers indicate arthritis days. Statistical significance: asterisk represents significant differences among treatments, and crosses represent significant differences among days: * $p<0.05$, +++ $p<0.001$. Statistical evaluation: see page 44.

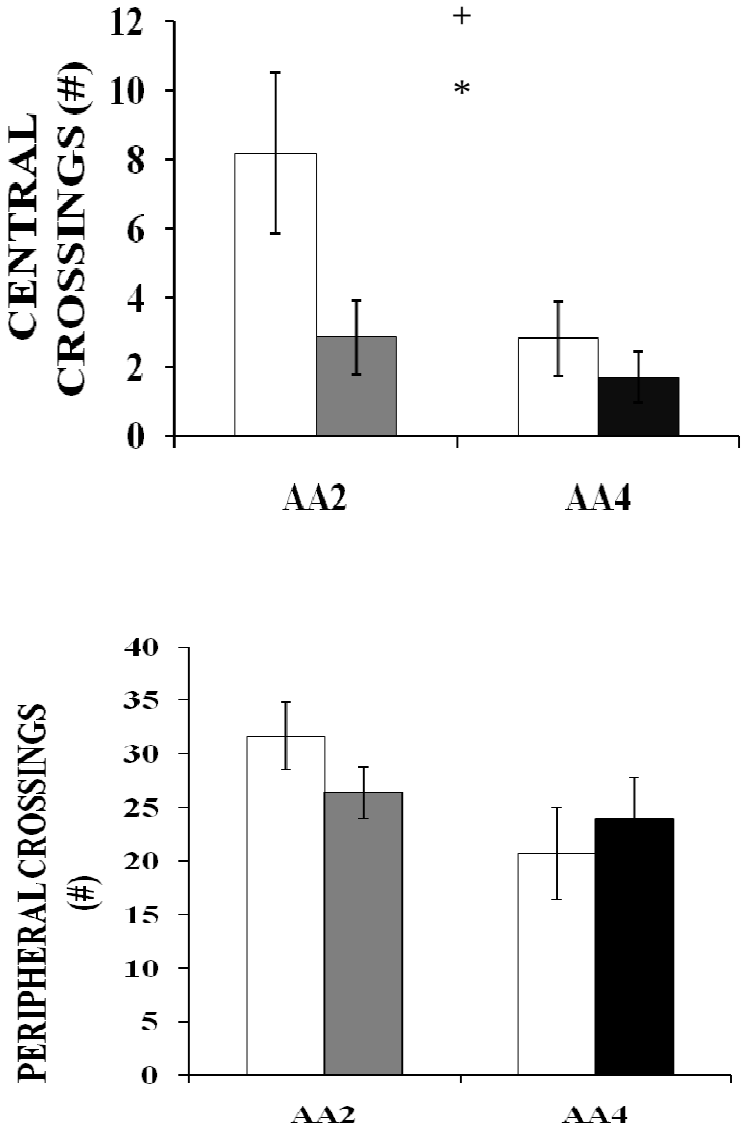

Figure 4.3.3: Anxiety behaviour in the open field on days 2 and 4 of AA: crossings are depicted. The black bars are control rats the coloured bars are cFA rats. Each column represents the mean of 7 values for controls and 7 values for early arthritic rats ± SEM. Abbreviations AA= early arthritic rats. Numbers indicate arthritis days. Statistical significance: asterisks represent significant differences among treatments, and crosses represent significant differences among days: * $p<0.05$, $p<0.05$+. Statistical evaluation: see page 44.

4.4 SERIES 4: *HIPPOCAMPAL NEURO-INFLAMMATORY/OXIDATIVE CHANGES IN THE EARLY ADJUVANT ARTHRITIS*

The mRNA for IL-1β (Figure 4.4.1) showed a mild, but significant up-regulation on day 2 (C vs. AA2: 1± 0.13 vs. 1.34± 0.11) and a more pronounced increase on day 4 (C vs. AA 4: 1±0.13 vs. 3.01± 0.37). IL-6 mRNA (Figure 4.4.1) was increased significantly on day 4 (C vs. AA 4: 1±0.86 vs. 6.4± 0.67). CRH transcripts (Figure 4.4.3) were lower on day 4 in AA rats compared to controls (C vs. AA4: 1±0.14 vs. 0.63± 0.13). The mRNA parameters of oxidative stress changed in a different manner: while mRNA expression of NOX2 (Figure 4.4.2) remained unaffected by the inflammatory process, expression of NOX1 (Figure 4.4.2) was up-regulated in both studied intervals (C vs. AA2, and AA4: 1± 0.14 vs. 1.36± 0.17, and 1.56± 0.21). The mRNA expression of iNOS (Fig. 20) was up regulated on day 4 of AA (C vs. AA2, and AA4: 1± 0.24 vs. 1.32± 0.2 vs. 4.28±0.76). The mRNA of BDNF (Figure 4.4.2) remained unaffected throughout early arthritis.

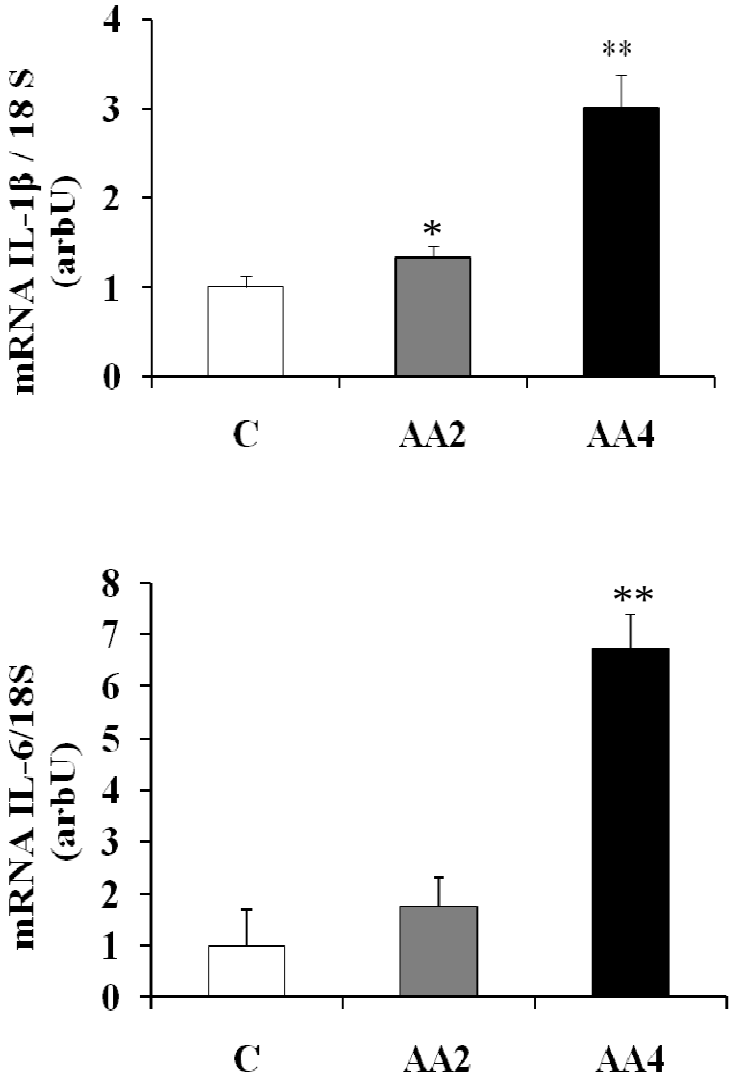

Figure 4.4.1 : Early adjuvant arthritis: mRNA expressions of IL-1β, IL-6 in the hippocampus are depicted. The white bars represent control rats, and the grey and the black bars represent arthritis rats. The numbers under column represent days of arthritis. The values are pooled values of right and left hippocampus. The results are expressed in arbitrary units for the ratio of the target gene mRNA to endogenous control mRNA (eucaryotic 18S RNA). Abbreviations: C=control rats, AA= early arthritic rats. Each column is the mean of 8 animals ± SEM. Statistical significance: * *p<0.05*. Statistical evaluation: see page 44.

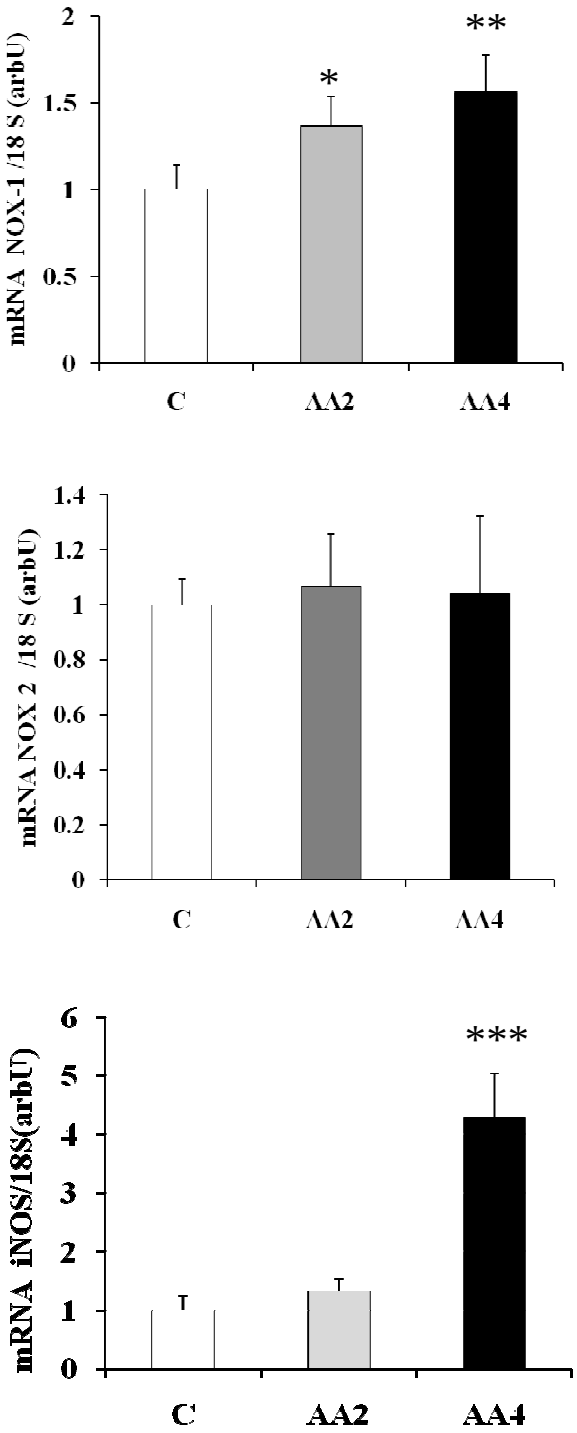

Figure 4.4.2: Early adjuvant arthritis: mRNA expressions of NOX-1, NOX-2, and iNOS in the hippocampus are depicted. The white bars represent control rats, and the grey and the black bars represent arthritic rats. The numbers under column represent days of arthritis. The values are pooled values of right and left hippocampus. The results are expressed in arbitrary units for the ratio of the target gene mRNA to endogenous control mRNA (eucaryotic 18S RNA).Abbreviations: C= control rats, AA= early arthritic rats. Each column is the mean of 8 animals ± SEM. Statistical significance: * $p<0.05$. Statistical evaluation: see page 44.

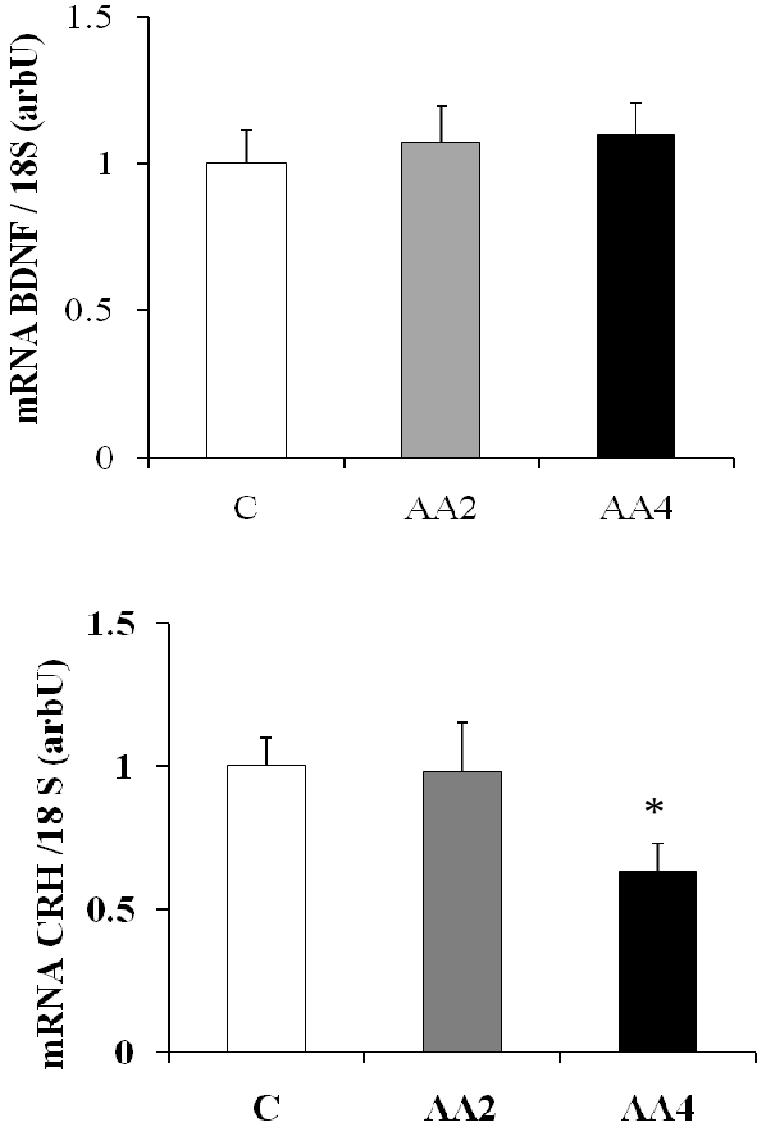

Figure 4.4.3: Early adjuvant arthritis: mRNA expressions of BDNF, and CRH in the hippocampus are depicted. The white bars represent control rats, and the grey and the black bars represent arthritic rats. The numbers under column represent days of arthritis. The values are pooled values of right and left hippocampus. The results are expressed in arbitrary units for the ratio of the target gene mRNA to endogenous control mRNA (eucaryotic 18S RNA). Abbreviations: C= control rats, AA= early arthritic rats. Each column is the mean of 8 animals ± SEM. Statistical significance: * *p<0.05*. Statistical evaluation: see page 44.

5. DISCCUSSION

5.1 SERIES 1: *CLINICAL FEATURES OF EARLY ADJUVANT ARTHRITIS*

Early phase of AA manifested with anorexia, transient hyperalgesia, and systemic inflammatory changes.

CRP is an important and sensitive indicator of active inflammatory states in various diseases (Hirschfield and Pepys, 2003). In the study of Cai et al. (2006) CRP plasma level was found to be increased from day 6 of AA onwards. In our study, plasma level of CRP has increased as early as on day 2 of AA. Also plasma albumin has decreased indicating ongoing inflammation. Moreover, reduction of thymus weight and enlargement of spleen was apparent on both examined days (day 2 and day 4) of early AA. Since glucocorticoids are known to negatively affect thymus function (Jondal et al., 1993), the thymus involution may be, at least in part, a consequence of endogenous corticosterone enhancement visible in this stage of the disease (Skurlova et al., 2011). The spleen, a secondary lymphoid organ, is a major site of immune surveillance, antigen recognition, activation, and of clonal proliferation in chronic inflammatory diseases (Kimpel et al., 2003). Spleen enlargement is very common in established RA, and it is supposed to be related to the sequestration of circulating white blood cells (Nishiya et al., 2000). Leucocytes accumulate into plasma from day 4 of AA (Johnston et al., 2007). It is possible that sequestration of leucocytes contribute to the observed spleen enlargement in the early arthritis. Alterations in weight of immune organs, increases in CRP plasma levels and decreases in albumin levels reflect systemic inflammation in early AA, which did not follow the transient pattern.

Anorexia in arthritic rats was most profound on day 2 of the disease. Anorexia is one of features of acute inflammation, and is regulated peripherally as well as centrally (Langhans, 2007). Leptin, a cytokine-like hormone and a known anorexigenic peptid, is a mediator of acute LPS induced anorexia possibly through the hypothalamic IL-1β- dependent mechanism. It was found that this LPS effect was attenuated by leptin antiserum administration (Sachot et al. 2004). Opposite to leptin, gastric hormone ghrelin stimulates food intake and reduces energy expenditure. Ghrelin has stimulated the activity of hypothalamic orexigenic neurons, and icv injection of ghrelin has up-regulated orexigenic factors such as neuropeptide-Y (Gil-Campos et al., 2006).

In our experimental series plasma leptin levels were mildly depressed on both examined days of early arthritis. Opposite to leptin, plasma ghrelin levels have increased on day 2 of AA. Moreover, expression of NPY mRNA was also increased on that day. The overexpression of NPY mRNA in the nARC as found on day 2 in this study in AA rats can be ascribed to the effect of enhanced circulating ghrelin levels. Since the diminished inhibitory input from leptin is known to induce the activation of NPY (Ahima et al. 1999), moderate leptin reduction on day 2 of AA might contributed to the NPY overexpression.

An important finding of our study is that despite activation of the orexigenic peripheral and central factors on the 2nd arthritis day, food intake was decreased. Furthermore, the food intake decline culminated on that day. Moreover, since day 2 of AA, IL-1β mRNA in nArc was up-regulated. Observations by several investigators have suggested that IL-1β is one of the strong signals negatively altering energy homeostasis. Intracerebroventricular infusion of the IL-1–receptor antagonist improved food intake after ip administration of LPS. Furthermore, the treatment did not alter peripheral levels of IL-1β. These findings indicate that central IL-1β contributes to anorexia during acute

inflammation. Acute anorexia in infection was ascribed to overexpression of hypothalamic IL-1β mRNA, too (Gayle et al., 1997). The IL-1β overexpression and also the highly probable overproduction in the nArc may be one of the factors responsible for inflammation-induced anorexia in spite of the activated NPY orexigenic pathway in the early arthritis. This mechanism has been proposed also in anorexia during the fully developed phase of AA (Stofkova et al., 2009).

Pain thresholds as measured on limbs and on tail decreased on day 2 of early arthritis only. It has been widely accepted that pain sensation increases under acute inflammatory conditions (Hori et al., 2000). Hyperalgesia could be elicited by LPS administration (Kanaan et al., 1996) and/or by other proalgetic immune mediators like IL-1β cytokine (Cunha et al., 2000). Bacteria and its components play a critical role in eliciting and processing inflammatory pain. Experimentally, cFA administration increases spontaneous activity of peripheral nociceptors as well as of pain transmitory Aδ and C nerve fibers (Xiao and Bennett, 2007; Djouhri et al., 2006). In this study, cFA administration to Lewis rats induced hyperalgesia on day 2 of AA, which was in parallel with increased plasma CRP levels. Furthermore, increased CRP level was shown to be associated with wrist pain thresholds in arthritic patients (Lee et al., 2009b). Systemic inflammation, as indicated by CRP plasma elevations, is a possible trigger of acute hyperalgesia on day 2 of early adjuvant arthritis. Thermal and/ or joint hyperalgesia are features of arthritic pain with differential temporal pattern throughout the disease development. As shown by Nagakura et al. (2003), in monoarthritis pain model, in the ipsilateral paw the thermal hyperalgesia occurred on the first post inoculation day, and recovered by day 3. In the contralateral paw thermal hyperalgesia did not occur in this time interval of the disease. The authors concluded that observed transient thermal hyperalgesia was a feature of cFA- induced acute inflammation. Furthermore, they argued that thermal

hyperalgesia was not movement- limiting, because no ankle stiffness and oedema had been observed in this disease interval. In our laboratory we have not observed c-fos activated neurons in lumbar spinal cord pain areas on day 9 of AA (unpublished results) suggesting no pain signals are generated from the paws before development of oedema. Therefore we assume that transiently enhanced pain sensivity on day 2 of AA occurs as a result of acute sytemic inflammation and not of arthritic pain.

The finding on thermal hyperalgesia on day 2 of AA, which recovered on the 4th arthritis day seem to be confined to the onset of autoimmune proces. Previous results from our laboratory showed analgesia in the later phase of AA when measured by tail flick (Jurcovicova et al, 2001). Similarly, peripheral administration of LPS has been shown to cause changes in pain sensation: hyperalgesia, which occurred initially after this immune challenge was later followed by analgesia (Hori et al., 2000). Inflammatory pain is counteracted by endogenous analgesic mediators including opioid peptides. Both neutrophils and monocytes contain opioid peptides (Brack et al., 2004), and they are the predominant leukocyte subpopulations during the first 4 days of cFA induced inflammation (Johnston et al., 1998). It is of speculate that recovery from hyperalgesia on the 4th arthritis day was mediated by neutrophils´ opiod mechanism.

Summarized, systemic inflammatory changes are characteristic for early phase of AA. This phase also manifests with anorexia, which occurs under still functional peripheral and central orexigenic mechanisms. The anorectic effect is most probably caused by the activation of central IL-1β. Acute systemic inflammation alters pain sensitivity in the early adjuvant arthritis. Initial hyperalgesia may be a consequence of acute phase response when the organism encounters the foreign

antigen, similarly as was observed after LPS administration. That is followed by a short recovery period. The activation of the HPA axis occurs as a feedback mechanism to compensate the inflammatory challenge.

5.2 SERIES 2*: COGNITION IN THE EARLY ADJUVANT ARTHRITIS*

Early AA impaired learning abilities in Lewis rats. The observed cognitive deficit was mostly evident on the 2nd arthritis day, and was followed by impaired spatial navigation on day 3, and day 4 of early AA.

On the 2nd training day the MWM data of arthritic group revealed longer latency and distance, while the swim speed did not differ between normal and arthritic rats. Both parameters, latency and distance, have been frequently used to evaluate spatial learning ability (Lindner, 1997). Earlier studies (Arai et al., 2001; Oitzl et al., 1993) have demonstrated increases in latency and distance parameter in LPS and/or IL1β treated mice and rats, and concluded that acute inflammation may impair cognition. On the other hand, these studies also reported decreases in swim speed under acute inflammatory conditions. It is well known that acute infections and/or inflammation induce locomotor hypoactivity which is also considered a sign of sickness (Cunningham and Sanderson, 2008). While the swim speed did not differ between arthritic Lewis rats and the controls on day 2, the observed increases in escape latency and distance parameters indicate rather to cognitive impairment on that day of AA.

During repeated exposure to the MWM on days 3 and 4 of AA, both control and arthritic rats learned equally, and cognitive impairment was no more visible on the basis of MWM tests. When analyzing search strategies, from the 2nd training day, early arthritic

Lewis rats preferred the “focal search in incorrect quadrant” strategy. The strategy has been cathegorized as the nonspatial strategy (Janus, 2004) and its higher incidence in arthritic rats may indicate less effective spatial learning in these rats. Similarly, in a mouse model of Alzheimer´s cognitive impairment, mice learned to locate the escape platform with a nonspatial strategy called “chaining” (Janus, 2004).

Moreover, arthritic rats spent significantly more time in the thigmotaxis zone on day 2 of AA. Thigmotaxis, (swimming near the maze wall) is one of the behavioural factors influencing swim navigation. Thigmotaxis is considered an index of timidity and anxiety-like behaviour (Treit and Fundytus, 1988) as it has been effectively suppressed with anxiolytics. Thigmotaxis swimminig has been found in autoimmune mice throughout the acquisition phase (Sakić et al., 1992) of spatial learning process, too. It is possible that enhanced emotionality, presented as thigmotaxis swimming in our experiments, negatively influenced swim navigation in early arthritic rats on the 2nd training day.

Summarized, spatial learning abilities are impaired in the early adjuvant arthritis. The impaired learning in the MWM manifested on day 2 of the disease, is accompanied and followed by using less effective swim strategies. It is possible that enhanced emotionality negatively influenced spatial learning process and navigation in early arthritic Lewis rats.

5.3 SERIES 3: *ANXIETY BEHAVIOUR IN THE EARLY ADJUVANT ARTHRITIS*

The results from the elevated plus maze and open field tests confirmed anxiety behaviour in the early arthritic Lewis rats.

Aversion of open spaces in the EPM is the main sign of anxiety behaviour (Pellow et al., 1985). The results of this experimental series has shown that the open arm entries number together with open arm time decreased in arthritic rats on both examined disease intervals. Decreased anxiety index, reflecting higher anxiety in arthritic rats, was accompanied by reductions in locomotor activity. Our observations in early arthritic Lewis rats are similar to previous findings in LPS and/or IL-1β treated mice and rats (Swiergel and Dunn, 2007; Koo and Duman, 2009). The latter authors (Koo and Duman, 2009) using interleukin-1 receptor null mice demonstrated anxiety behavior without accompanying decreases in locomotor activity indicating a specific anxiogenic effect of IL-1β in these mice. Emotional disorders are features of acute systemic inflammation as revealed from clinical and experimental studies (Maier and Watkins, 1998). Under experimental conditions IL-1β and LPS administration increased anxiety as demonstrated in different anxiety tests. Both acute inflammatory agents reduced number of entries and time spent in the open arm of the EPM as well as increased avoidance of central area of the OF (Song et al., 2004). Moreover, LPS administration decreased exploration in the illuminated part of the light- dark box and also decreased number of entries and time spent in the open arm of the EPM (Lacosta et al. 1999).

The distinct locomotor impairment we observed in EPM tests in arthritic Lewis rats might have occurred as part of the sickness and was not caused by gross motor alterations, since the rats did not show any signs of pain or muscle weakness in this initial phase of

AA. Additionally, it was reported that central administration of IL-1β or IL-6 significantly attenuated voluntary-wheel running in rats (Harden et al., 2008). Similarly, LPS- treated rats were lethargic and less involved into voluntary wheel running (Harden et al., 2010).

The behaviour in the peripheral zone (along the walls) of the OF reflects potential locomotion deficit (Belzung and Griebel, 2001; Prut and Belzung, 2003). In our study, the ambulatory activity in the peripheral zone of the OF was not different between the groups on both examined days of AA (day 2 and day 4). However, arthritic rats had less interest for the central area of the OF, because they spent less time in the central circle, and ambulated in the area less intensively than the control rats. Similarly, anxiety, and depression behaviour occurred in mice in the early phase of the autoimmune lupus disease before joint deformities development (Sakić et al., 1993).

Summarized, early adjuvant arthritis evokes anxiety behaviour as revealed in EPM and OF tests. These changes are interpreted as feature of the sickness behaviour of ongoing autoimmune inflammation and not as a gross motor deficit. Anxiety behaviour was evident on both days of early AA indicating to ongoing character of sickness behaviour independent from thermal hyperalgesia observed on day 2.

5.4 SERIES 4: *HIPPOCAMPAL NEURO-INFLAMMATORY/OXIDATIVE CHANGES IN THE EARLY ADJUVANT ARTHRITIS*

In hippocampus, mRNA gene expression of pro-inflammatory cytokine IL-1β, and the marker of oxidative stress NOX1 increased from day 2 of AA, and IL-6 and iNOS from day 4 of AA, indicating to neuro- inflammatory /oxidative changes in the early AA.

It has been shown that acute peripheral inflammation potentiated immune-to-brain signalling resulting in activation of specific brain areas and production of central pro-inflammatory cytokines. LPS and/or pro-inflammatory cytokines, administered peripherally, induced transient c-fos immunoreactivity in hypothalamic, autonomic, and limbic brain regions, which returned to basal levels within 24-48 hours after the treatment (Lacroix and Rivest, 1997). The mechanisms of hippocampal activation under acute inflammatory conditions involve the rise of circulating pro-inflammatory cytokines, which directly or by peripheral afferent nerve stimulation induce synthesis of pro-inflammatory cytokines centrally (Carter et al., 2010). *In vivo,* bacterial LPS challenge increases hippocampal IL-1β levels 6 hours after its administration (Csölle and Sperlágh, 2009). Elevation of IL-1β specifically in the hippocampus has been observed after infection, and in elderly rats which additionally was long lasting (Barrientos et al., 2009). Similarly, cFA administration evokes robust and lasting expression of c-Fos in the hippocampus (Carter et al., 2010). The main finding of this study was the up-regulation of the IL-1β mRNA since day 2 after cFA immunization followed by up-regulation of IL-6 mRNA on day 4 of AA. The results demonstrate the induction of brain tissue inflammation at the onset of autoimmune process.

Neuroinflammation refers to a specific brain process comprising activation of the brain-resident “immune” cells, namely microglia and astrocytes. In the neuroinflammatory context, several cytokines and chemokines are produced in the brain tissue, along with reactive oxygen and/or nitrogen species (Spooren et al., 2011). As revealed from the experiments, the early arthritis up-regulated the NOX1 mRNA since day 2 with subsequent up-regulation of iNOS mRNA from day 4 of AA. The NOX1 oxidase is an enzyme, which biological function is the generation of reactive oxygen species (Bedard et al., 2007). NOX1 mRNA up-regulation in the early arthritis may be an indirect proof of oxidative stress changes in the early phase of autoimmune inflammation. Moreover, the expression of CRH mRNA decreased on day 4 of early AA. As CRH has neuro-protective effects against oxidative stress (Lezoualch et al., 2000), its mRNA down-regulation in the early arthritis may indicate to insufficient protective effect against oxidative stress in that phase of the disease. Furthermore, up-regulation of iNOS mRNA could indicate to increased NO-production, and to exaggerated inflammation in the hippocampal area in the course of early AA.

It has been suggested that NOX1 triggers IL-1β and NO- radical production. Chéret et al. (2008) found that icv LPS administration induced IL-1β and NO-radical production via NOX1. These authors also demonstrated on NOX1 and NOX2 knock-out mice, that NOX1 induced IL-1β production after LPS, while both NOX1 and NOX2 are involved in NO-radical production. Our results on the mutual activation of NOX1, iNOS and IL-1β mRNA in the hippocampus suggest similar mechanism in the early phase of AA and also underline the importance of NOX1 in these events.

It has been shown that anxiety behaviour in mice was suppressed by inhibition of NADPH- oxidases confirming the crucial role of oxidative stress in the development of affective disorders (Masood et al., 2008). Oxidative stress in hippocampus decreased time and number of refusals into light-compartment of the light-dark box what may indicate to anxiety behaviour (de Oliveira et al., 2007). And, moreover inhibition of NO-radical production in the dorsal hippocampus diminished anxiety behaviour which became evident by increased exploratory activity in the EPM test (Spolidório et al., 2007).

BDNF has been implicated in synaptic transmission. Existing research focusing on the effects of acute inflammation on BDNF expression has demonstrated dose related changes in BDNF expression after LPS administration: While single dose of LPS did not cause any effect (Shaw et al., 2001), repeated high doses of LPS downregulated BDNF (Schnydrig et al, 2007). It has been shown that severe endogenous corticosterone elevation (several hours immobilization stress) resulted in BDNF downregulation in the hippocampus which consequently contributed to worsening of memory consolidation (Schaaf et al., 2000). The authors further showed significantly elevated corticosterone levels also in the MWM, but without any inhibition of BDNF. In spite of moderate, but sustained corticosterone elevation in the early arthiris, which was shown in the series 1, the BDNF mRNA expression in the hippocampus remained unchaned. Taken together, the starting autoimmune process does not affect BDNF expression as it does under more severe immune challenge (LPS) (Schnydrig et al, 2007).

Summarized, the peripheral immune signal, cFA administration, triggers neuro-inflammatory/oxidative changes in the hippocampus in the initial phase of autoimmune inflammation. Hippocampal neuroinflammation in the early AA manifests with mRNA up-regulation of IL-1β, and IL-6. Increases of hippocampal

mRNA gene expression of NOX1, iNOS together with decreased mRNA expression of CRH are indirect markers of oxidative stress in that brain area in the early AA. The hippocampal neuro- inflammation/oxidative stress does do not influence synaptic transmission when measured at the level of BDNF mRNA expression.

6. CONCLUDING THOUGHTS

Inflammatory- based sickness behaviour are clearly distinguishable from other features of acute inflammation, for instance hypolocomotion, and pain. Arai (Arai et al., 2001) confirmed cognitive impairment in LPS- treated mice on longer time spent in MWM, and on less correct choices made in Y maze. No alteration of grip tone, motor activity or swimming speed was observed in these mice (Arai et al., 2001). Similarly, in this study, early arthritic Lewis rats were cognitively impaired, and anxious. Thermal hyperalgesia, a feature of arthritic pain, was also evident in these rats. Moreover, observed thermal hyperalgesia was transient, and did not reduce locomotion in MWM and OF tests as reflected on stable swim speed and unchanged ambulatory activity in the peripheral area of the OF.

Hippocampal neuro-inflammation/ oxidative stress and sickness behaviour are interconnected in inflammatory diseases including autoimmune diseases (Sakíc et al., 1997). Depressive behaviour induced by LPS administration paralleled with up-regulation of TNF-α and IL-6 in the hippocampus (Tonelli et al., 2008). Crupi et al. (2010) demonstrated that anxiety in autoimmune lupus mice, presented by less time spent in the central area of the OF and decreased percentage of open arm entries in the EPM, correlated to microglia activation in the hippocampus (Crupi et al., 2010). Oxidative stress in the hippocampus was related to memory deficits throughout early phase of systemic inflammation (Cassol- Jr. et al., 2010).

In this study, spatial learning deficit, mostly evident on day 2 of early AA, was accompanied and followed by impaired swim navigation up to day 4 of AA. Moreover, anxiety behaviour emerged on both examined days of early AA reflecting lasting character of sickness behaviour in the early AA. In the hippocampus, mRNA gene expression of pro-inflammatory cytokine IL-1β, and the marker of oxidative stress NOX1 increased from day 2 of AA, and of IL-6 and iNOS from day 4 of AA.

The study suggests that hippocampal neuro-inflammation / oxidative stress interfere with the development of sickness behaviour in the early AA rather than thermal hyperalgesia. Moreover, this affirmation is encouraged by finding that hippocampal neuro-inflammation / oxidative stress are lasting opposite to thermal hyperalgesia, which showed transient pattern.

2 CONCLUSIONS

7.1 CLINICAL CHARACTERIZATION OF THE EARLY ADJUVANT ARTHRITIS

Early AA manifests clinically with systemic inflammatory changes, anorexia and transient hyperalgesia, as well as with activation of HPA axis. Anorexia is most profoundly visible on day 2 of AA, and is probably mediated by enhanced anorectic IL-1β in nArc of the hippocampus. Hyperalgesia observed in the early arthritis is transient, and may be a consequence of acute host defence when the organism encounters the foreign antigen, similarly as in the reactions to LPS. The acute host defence also activates HPA axis to couterregulate the inflammation.

7.2 COGNITION IN THE EARLY ADJUVANT ARTHRITIS

Early AA induces cognitive impairment manifested by insufficient spatial learning abilities, and less effective swim navigation. Transient thermal hyperalgesia did not influence spatial navigation, since no differences in swim speed were observed. It is possible that enhanced emotionality, presented as thigmotaxis swimming, negatively influenced swim navigation on day 2 of AA.

7.3 ANXIETY BEHAVIOUR IN THE EARLY ADJUVANT ARTHRITIS

Early AA induces anxiety behaviour in Lewis rats. Anxiety behaviour was observed on both examined day wihtout showing any transient character. Because of unaltered ambulatory activity in the peripheral area of OF on both days, thermal hyperalgesia is not probable to be involved in the enhanced anxiety behaviour.

2.3 NEURO-INFLAMMATORY/OXIDATIVE CHANGES IN THE HIPPOCAMPUS

In the early AA, systemic inflammation triggers neuro-inflammatory/oxidative alterations in the hippocampus as revealed by enhanced mRNA expressions of IL-1β, IL-6, NOX1, iNOS and diminished mRNA expression of CRH. These hippocampal changes correspond with cognitive deficit and anxiety behaviour in early arthritis. It suggests that neuroinflammatory/oxidative stress may represent the background of sickness behaviour which occurs at the beginning of the process of AA, before any manifestation of oedema or joint deformities.

8. FUTURE PERSPECTIVES

The given study investigated aetiology of sickness behaviours in the early inflammatory phase of autoimmune inflammation. Observed sickness behaviour (impaired spatial learning, less effective swim navigation, and anxiety behaviour) occurred in parallel with neuro-inflammatory/ oxidative stress changes in the hippocampus. Unfortunately, only mRNA alterations at the level of hippocampus were presented what does not really reflect concentrations of the parameters measured, and may constitute some limitations to the study. On the other hand, alteration of hippocampal mRNA gene expression reflects changes in this particular brain area. Protein levels of measured parameters are needed to be estimated in future.

In this study, neuroinflammation was suggested to trigger autoimmune sickness behaviour. The aetiology of neuroinflammation has been partly clarified by showing that pro-inflammatory cytokine IL-1β starts the process of microglial activation. Moreover, the cytokine expression precedes iNOS expression. NF- κB is a ubiquitous transcription factor, which initiates synthesis of IL-1β *de novo* (Laflamme et al., 1999). Colocalization between IL-1β and nuclear factor κB (NF- κB) in glial cells strengthens this possibility. The future research focusing on the mechanism of neuro-inflammation-based autoimmune sickness behaviour should be orientated: (1) on the development of anti-IL-1β strategies, and (2) on the development of anti-NF- κB strategies followed by testing of sickness behaviour.

Also in this study, oxidative stress in the hippocampus was suggested to trigger autoimmune sickness behaviour. The markedly enhanced redox status was found in the hippocampal glial cells in animal models of anxiety. Further, it was shown that oxidative stress- based anxiety may be NADPH-oxidase mediated. To confirm involvement of oxidative stress in mediation of autoimmune sickness behaviour, the research should be orientated on: (1) the development of effective NADPH- mediated antioxidant therapy followed by examination of sickness behaviour.

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11. AUTHOR´S PUBLICATIONS AND ABSTRACTS

11.1 PUBLICATIONS RELATED TO THE TOPIC OF THE WORK

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