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DISSERTATION

The role of 11β-hydroxysteroid dehydrogenase type 1 in the regulation of the hypothalamic-pituitary-adrenal axis in immune-mediated arthritis

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Abbreviations

11β-HSD	11β-hydroxysteroid dehydrogenase
11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
AA	Acute arthritis mouse model
AB	Antibody
ACTH	Adrenocorticotropic hormone
AVP	Arginine vasopressin
CA	Chronic arthritis mouse model
CBG	Corticosteroid-binding globulin
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
CRHR1	Corticotropin-releasing hormone receptor 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoid
GH	Growth hormone
GR	Glucocorticoid receptor
H6PD	Hexose-6-phosphate dehydrogenase
HPA axis	Hypothalamic-pituitary-adrenal axis
IHC	Immunohistochemistry
KO	Knockout
MR	Mineralocorticoid receptor
PCR	Polymerase chain reaction
PG	Pituitary gland
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus
qPCR	Real time quantitative polymerase chain reaction
RA	Rheumatoid arthritis

Abstract

Background

Patients with rheumatoid arthritis exhibit abnormal hypothalamic-pituitary-adrenal (HPA) axis activity. The basis for this abnormality is not known. Rheumatoid arthritis is associated with increased extra-adrenal synthesis of active glucocorticoids by the 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) enzyme. 11β-HSD1 is expressed in the central nervous system, including regions involved in HPA axis regulation. This study examined whether altered 11β-HSD1 expression within these regions contributes to HPA axis dysregulation during arthritis.

Methods

The expression of 11β-HSD1, and other components of glucocorticoid signalling, were examined in various brain regions and the pituitary gland of mice with experimentally-induced arthritis. Two arthritis protocols were employed: The K/BxN spontaneous arthritis model for chronic arthritis and the K/BxN serum transfer arthritis model for acute arthritis.

Results

11β-HSD1 was expressed in the hippocampus, hypothalamus, cortex, cerebellum and pituitary gland. Hypothalamic 11β-HSD1 expression did not change in response to arthritis in either model. Pituitary 11β-HSD1 expression was however significantly increased in both chronic and acute arthritis models. Hippocampal 11β-HSD1 was decreased in acute but not chronic arthritis. Chronic, but not acute, arthritis was associated with a reduction in hypothalamic corticotropin-releasing hormone and arginine vasopressin expression. In both models, serum adrenocorticotropic hormone and corticosterone levels were no different from non-inflammatory controls.

Conclusion

These findings demonstrate inflammation-dependent regulation of 11β-HSD1 expression in the pituitary gland and hippocampus. The upregulation of 11β-HSD1 expression in the pituitary during both chronic and acute arthritis, and thus an increase in glucocorticoid negative feedback, could contribute to the abnormalities in HPA axis activity seen in immune-mediated arthritis.

Kurzdarstellung

Einleitung

Patienten mit rheumatoider Arthritis (RA) weisen Auffälligkeiten in der Aktivität ihrer Hypothalamus-Hypophysen-Nebennierenrinden-Achse (HHN-Achse) auf. Der Grund für diese Auffälligkeiten ist bisher unbekannt. RA ist assoziiert mit einer Zunahme der extraadrenalen Glucocorticoid-Synthese durch das Enzym 11β-Hydroxysteroid-Dehydrogenase 1 (11β-HSD1). 11β-HSD1 wird im zentralen Nervensystem exprimiert, unter anderem in Regionen die an der Regulation der HHN-Achse beteiligt sind. In dieser Studie wurde untersucht, inwieweit eine veränderte Expression von 11β-HSD1 in diesen Regionen zu der beschriebenen HHN-Achsen-Dysregulation in Patienten mit RA beiträgt.

Methodik

Die Expression von 11β-HSD1 und anderen Komponenten der Glucocorticoid-Signalübertragung wurde in verschiedenen Hirnarealen sowie der Hypophyse untersucht in Mäusen mit experimentell induzierter Arthritis. Hierfür wurden zwei Protokolle verwendet: Das K/BxN-Spontan-Arthritis-Modell als eine chronische Form der Arthritis und das K/BxN-Serumübertragungs-Arthritis-Modell, als eine akute Form der Arthritis.

Ergebnisse

Die Expression von 11β-HSD1 wurde nachgewiesen in Hippocampus, Hypothalamus, Cortex, Cerebellum und Hypophyse. In beiden Mausmodellen kam es zu keiner Veränderung der 11β-HSD1-Expression im Hypothalamus in den arthritischen Mäusen. Im Gegensatz dazu war die 11β-HSD1-Expression in der Hypophyse signifikant erhöht in den arthritischen Mäusen beider Modelle. Die 11β-HSD1-Expression im Hippocampus war erniedrigt in den akuten, jedoch nicht in den chronischen Arthritis-Mausmodellen. Chronische, jedoch nicht akute Arthritis, war assoziiert einer reduzierten Expression von Corticotropin-releasing Hormone und Arginin-Vasopressin im Hypothalamus. In beiden Mausmodellen unterschieden sich die Serumspiegel von Adrenocorticotropin und Corticosteron nicht zwischen den arthritischen Mäusen und ihren Kontrollen.

Schlussfolgerungen

Diese Ergebnisse zeigen eine entzündungsabhängige Regulation der 11β-HSD1-Expression in der Hypophyse und dem Hippocampus. Die Hochregulierung der 11β-HSD1-Expression in der Hypophyse in chronischer und akuter Arthritis, und ein dementsprechend verstärktes negatives Feedback durch Glucocorticoide, könnten zu den HHN-Achsen-Auffälligkeiten beitragen, die in rheumatoider Arthritis beobachtet werden.

1 Introduction

Rheumatoid arthritis (RA) is a common and potentially debilitating disease, that critically impacts on the life of affected patients. Despite decades of basic and clinical research, the pathophysiology of the disease is not fully understood and a cure for RA has not been found. The pharmaceutical treatment is restricted to immunosuppressive therapy, which involves the risk of adverse effects. During acute inflammation, there is typically an activation of the hypothalamic-pituitary-adrenal axis (HPA axis) resulting in increased levels of the body's own glucocorticoids (GCs). These GCs exert an important anti-inflammatory effect. As opposed to acute inflammation, patients with RA show a failure of the HPA axis to increase in activity in response to high levels of inflammation (Chikanza, Petrou et al. 1992, Crofford, Kalogeras et al. 1997). This failure has been linked to the chronicity of RA in the affected individuals. The reason for the relative nonresponsiveness of the HPA axis to RA is not clear, however, an impaired GC-negative feedback mechanism of the HPA axis has been proposed (Edwards 2012). In addition to systemic GC production via the HPA axis, there is also extra adrenal GC production via the 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) enzyme at a cellular level. The overall activity of 11β-HSD1 is increased in response to RA (Hardy, Rabbitt et al. 2008). As this enzyme is expressed in many parts of the central nervous system (CNS), an increase of 11β-HSD1 activity in the central components of the HPA axis could lead to an increased GC-negative feedback there. This could explain the failure of HPA axis activation in RA patients. By examining that hypothesis, this study could crucially contribute to the understanding of the development of RA. This understanding could open the door to the development of disease-specific drugs, which could save the patients from the adverse effects of an immunosuppressive therapy.

2 Literature review

2.1 Rheumatoid arthritis

RA is a chronic, progressive autoimmune disease, that affects about 1% of the world's population (Lee and Weinblatt 2001, Gabriel and Michaud 2009). RA patients that are under insufficient disease control, suffer from a variety of clinical symptoms and signs. Primarily, they exhibit symmetrical swelling of the small joints, associated with pain, stiffness and loss of function (Arnett, Edworthy et al. 1988). Furthermore, extraarticular manifestations, including cardiovascular, pulmonary and psychological disorders, can lead to an increased mortality in these patients (Wolfe, Mitchell et al. 1994). Despite the high prevalence and adverse outcomes of RA, decades of basic and clinical research have not revealed the complete underlying pathophysiology yet. However, some features have been delineated, that take part in the initiation and perpetuation of the disease process.

The development of RA cannot be explained on a monocausal basis. In fact, there is a complex interaction between a susceptible genotype of the individual, like the so-called shared epitope on the HLA-DRB1 alleles (Gregersen, Silver et al. 1987), and environmental factors like cigarette smoking (Silman, Newman et al. 1996) and composition of the oral and intestinal microbiota (Scher and Abramson 2011). In patients that develop RA, a combination of these factors leads to the loss of immunotolerance towards certain endogenous antigens and therefore creates autoimmunity within the body (McInnes and Schett 2011). For reasons that are not completely understood, the autoimmune process primarily affects the synovial tissue and leads to an invasion of leucocytes into the synovial compartment, where a so-called pannus is formed (Walsh and Gravallese 2010). The inflammatory changes there initiate a vicious circle, including destruction and reorganization of the local tissue structures on the one side and activation of the innate and adaptive immune system on the other. The inflammation is perpetuated by a multitude of members of the immune system, including T-cells, B-cells and proinflammatory cytokines such as TNF-α, IL-6 and IL-1 (Dayer 2004, Scott, Wolfe et al. 2010). Even though the first clinical manifestations of the disease take place in the joints, RA must be considered a systemic disease. Circulating

proinflammatory cytokines affect additional organs, which can lead to the development of secondary diseases, as shown in Figure 1.

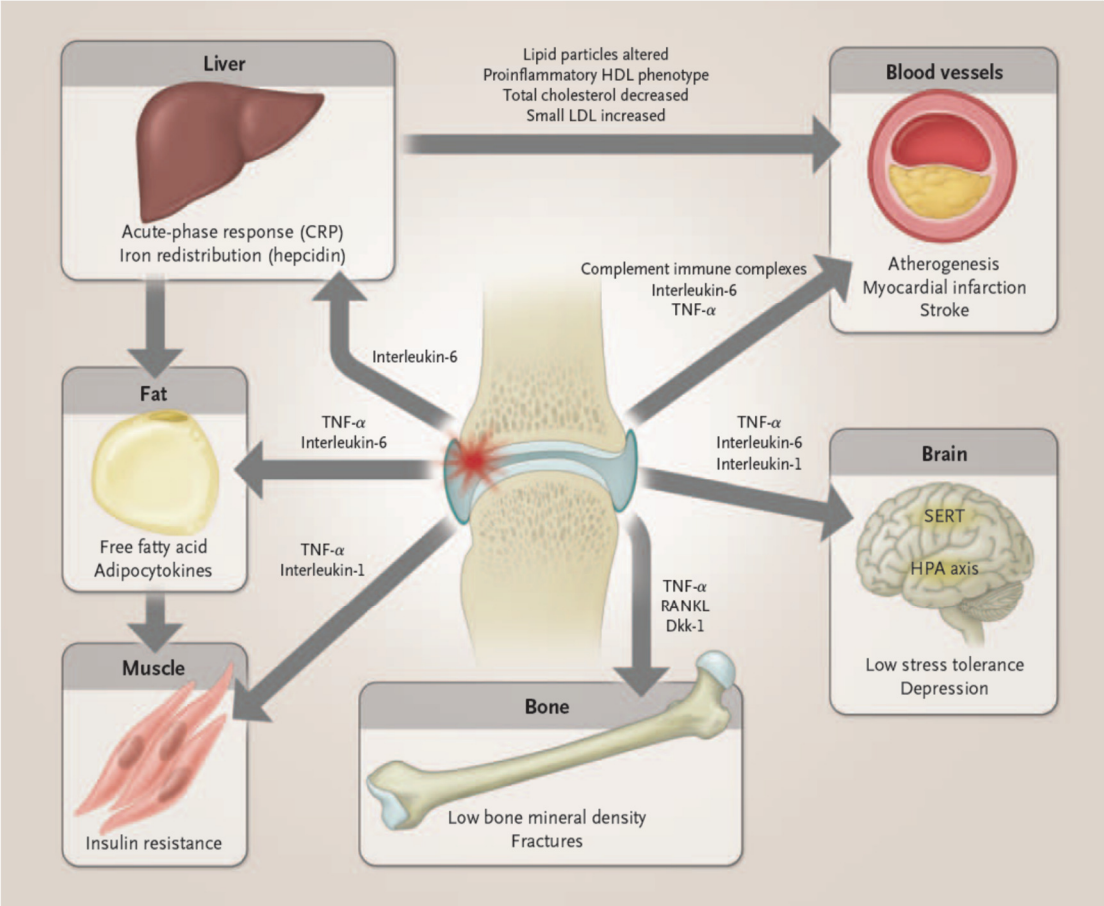

Figure 1: Extraarticular manifestations of rheumatoid arthritis (McInnes and Schett 2011)

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So far, no cure has been discovered for RA. However, medical management of patients with RA has made tremendous progress in recent years. The medical treatment strategies aim to keep the inflammatory processes under control by suppressing parts of the body's immune system. Modern RA treatment includes biological as well as conventional and targeted synthetic disease modifying drugs (Smolen, Landewé et al. 2017). Besides these drugs, RA therapy still includes the use of GCs. Being the first immune suppressive drug used in the treatment of RA, GCs have not lost any of their importance over the decades and there are still new developments, aiming to optimize

their treatment effect and reduce the side effects for the patients (Strehl, van der Goes et al. 2017).

In 1949, Hench and colleagues observed for the first time that RA patients showed a dramatic improvement of their symptoms when treated with GCs (Hench, Kendall et al. 1949). These findings drew the attention of RA researchers towards the HPA axis and the GCs. The underlying theory was that if the application of exogenous GCs could alleviate the symptoms of RA patients, the disease could be caused by an impaired endogenous GC production.

2.2 Glucocorticoids

GCs are steroid hormones that are produced as an endocrine response to stress and regulate a plethora of mechanisms in mammals. They are, for example, involved in energy metabolism, hematopoietic differentiation and bone homeostasis. Moreover, they are important mediators of anti-inflammatory action. In humans, cortisol is the main GC, the equivalent in rodents is corticosterone. Because of their lipophilic structure, GCs can freely diffuse through the cell membrane and enter the cytosol, where they can bind to their low-affinity glucocorticoid receptor (GR) and high-affinity mineralocorticoid receptor (MR). The ligand-receptor complex then becomes an activated transcription factor and translocates into the nucleus. There are two main mechanisms of action by which the ligand-receptor complex acts: Firstly the “classical” way of modulating genomic transcription, in which the complex binds directly to DNA. Secondly, by direct protein-protein interactions not involving nuclear DNA (Rhen and Cidlowski 2005, Kassel and Herrlich 2007). The second mechanism has been thought of as the more important one concerning the anti-inflammatory effects of GCs (Smoak and Cidlowski 2004). GCs suppress the transcription factors NF-κB and AP-1. As a result, the expression and production of a whole set of proinflammatory cytokines is reduced, amongst these are TNF-α, IL-1β and IL-6 (Almawi and Melemedjian 2002). In this way, GCs can counteract inflammation, for example in patients with RA.

Cellular levels of GCs are determined mainly by the level of GCs circulating in the blood stream and the intracellular metabolism of GC via the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes.

2.3 The HPA axis

Systemic production of cortisol is regulated by the HPA axis. A variety of external and internal stimuli trigger the production and the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) in the hypothalamus. These hormones induce the release of adrenocorticotropic hormone (ACTH) from the pituitary gland (PG) into the circulation. ACTH in turn, stimulates the production of cortisol in the adrenal glands. Through negative feedback control, cortisol inhibits the release of CRH and ACTH, and thus tightly regulates its own production (Figure 2).

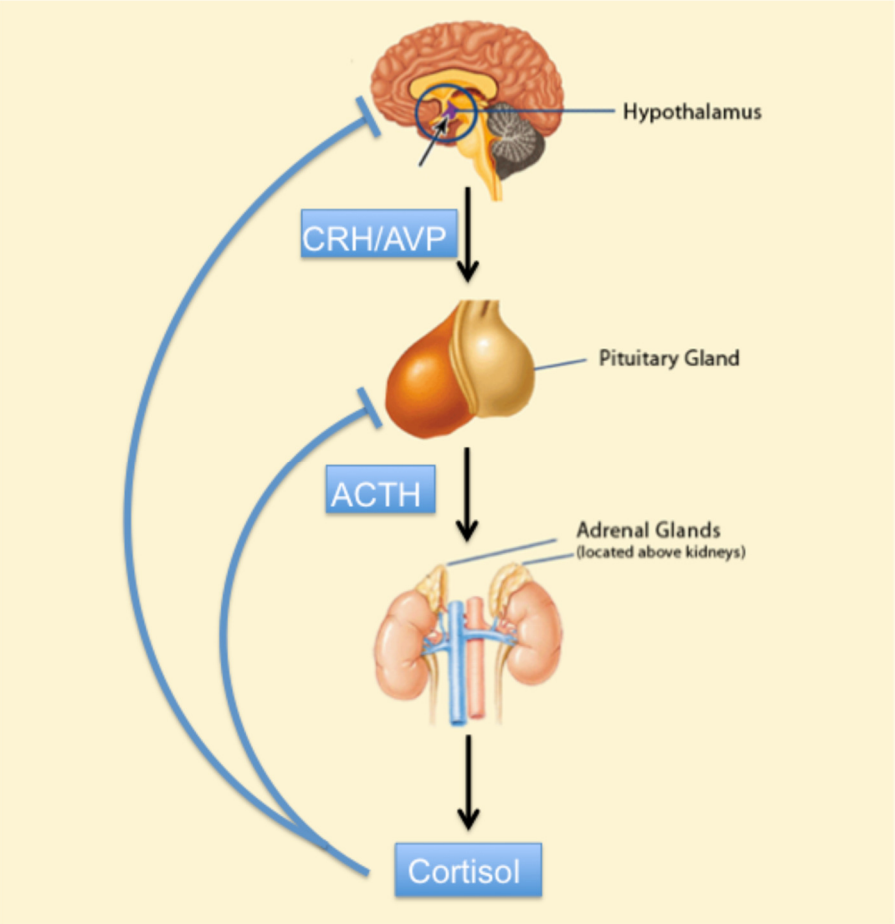

Figure 2: The HPA axis (Tu 2014)

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The hypothalamus is the integrator of a range of information from different body regions that indicate a threat to the body's homeostasis (Herman and Cullinan 1997). Direct neural connections are from visceral afferents, nociceptors and the circumventricular

organs (Herman, Figueiredo et al. 2003). Additional information from the limbic system is integrated as well, for example from the hippocampus (Jacobson and Sapolsky 1991). The hypothalamus responds to the input information by adapting its output levels of PG secretagogues that eventually lead to the production of cortisol.

The main hypothalamic HPA axis secretagogue is CRH. Within the hypothalamus, CRH is expressed mainly in the parvocellular division of the paraventricular nucleus (PVN) (Swanson, Sawchenko et al. 1983). In most of these cells, CRH is coexpressed with AVP (Whitnall, Mezey et al. 1985), which is considered another activator of the HPA axis (Makino, Smith et al. 1995, Chikanza and Grossman 1998). Neurons from the PVN stretch into the area of the median eminence, where they secrete CRH and AVP into a portal vein system that is connected to the anterior lobe of the PG (Antoni 1986). There, the secretagogues trigger the release of ACTH into the systemic circulation (Proulx-Ferland, Labrie et al. 1982), as well as the upregulation of proopiomelanocortin (POMC) mRNA expression. POMC is the precursor protein of ACTH (Bruhn, Sutton et al. 1984, Höllt and Haarmann 1984). ACTH stimulates the production and release of cortisol in the zona fasciculata of the adrenal glands. In a classical negative feedback manner, cortisol is a strong inhibitor of HPA axis activity by suppressing production and release of CRH in the hypothalamus and ACTH in the PG.

2.4 The HPA axis in acute inflammation

Important activators of the HPA axis are proinflammatory cytokines. This effect has been reviewed extensively (Chrousos 1995, Turnbull and Rivier 1999). The administration of cytokines like IL-1 α and β, IL-6 and TNF-α leads to increased ACTH and/or cortisol serum levels in humans (Crown, Jakubowski et al. 1991, Smith, Urba et al. 1992, Mastorakos, Chrousos et al. 1993, Nolten, Goldstein et al. 1993). In many situations of systemic inflammation, this activating effect of proinflammatory cytokines on the HPA axis can be observed as well. Increased ACTH and GC blood levels have been described, for example, in animal models infected with the Newcastle disease virus (Besedovsky, del Rey et al. 1986), in lipopolysaccharide-induced inflammation, a model that mimics features of a bacterial infection (Rivier, Chizzonite et al. 1989) and animals with induced local inflammation by turpentine injection (Turnbull and Rivier 1996).

Even though these experiments on acute inflammation show a stimulatory effect of proinflammatory cytokines on the HPA axis, these findings cannot be automatically translated to patients with RA, which represents a state of chronic inflammation.

2.5 The HPA axis in immune-mediated arthritis

In spite of the aforementioned findings, the HPA axis regulation by proinflammatory cytokines seems to be deficient in RA patients. Even though RA is a condition of systemic inflammation and high levels of proinflammatory cytokines are found in the blood circulation of the patients (Feldmann, Brennan et al. 1996), systemic ACTH and cortisol levels typically stay within the physiological range (Chikanza, Petrou et al. 1992, Crofford, Kalogeras et al. 1997, Straub, Paimela et al. 2002). Some studies even showed decreased night-time levels of serum ACTH and cortisol in RA patients compared to healthy individuals (Zoli, Lizzio et al. 2002, Straub, Weidler et al. 2004). In addition, the HPA axis is inhibited in its response to new inflammatory stimuli, such as surgical intervention (Chikanza, Petrou et al. 1992). Taking into account the high levels of circulating proinflammatory cytokines in RA patients, it has been proposed, that the HPA axis in rheumatic patients is deficient, precisely because its activity remains normal, if not subnormal, under the inflammatory condition (Straub and Cutolo 2001).

Animal models for immune-mediated arthritis show a similar picture to that in patients with RA. In a study on rats with induced streptococcal cell wall arthritis, a model that shares many features with RA, the arthritis susceptible rat strain showed an attenuated ACTH and GC response to arthritis compared to more resilient strains (Sternberg, Hill et al. 1989). This attenuation is paralleled by downregulated hypothalamic CRH expression and release (Sternberg, Young et al. 1989).

Therefore, it might be rewarding to research the mechanism of this relative HPA axis deficiency in RA patients. To approach that issue, it is necessary to firstly have a look at another regulator of cortisol production; the 11β-HSD enzymes.

2.6 11β-Hydroxysteroid dehydrogenase type 1

Besides the systemic cortisol production via the HPA axis, there is also cortisol production and metabolism on a cellular level. In the 1980s, Carl Monder and his team isolated an enzyme group in the rat, that regulates the pre-receptor metabolism of GCs, called the 11β-HSD enzymes (Lakshmi and Monder 1985, Lakshmi and Monder 1985, Lakshmi and Monder 1988, Agarwal, Monder et al. 1989). Humans carry equivalents of these enzymes (Tannin, Agarwal et al. 1991, Maser, Völker et al. 2002). The 11β-HSD enzymes interconvert biologically active cortisol and biologically inert cortisone in humans (corticosterone and dehydrocorticosterone respectively in rodents). First thought of as one enzyme, a second isoform of that enzyme has been identified later (Brown, Chapman et al. 1993, Rusvai and Naray-Fejes-Toth 1993). 11β-HSD2 works unidirectionally as a dehydrogenase, deactivating cortisol to cortisone. The directionality of 11β-HSD1, in contrast, is tissue-specific and depends on the ratio of surrounding cofactors (Chapman, Holmes et al. 2013). Amongst these, the NADPH/NADP+ ratio, which is regulated by hexose-6-phosphate dehydrogenase (H6PD), seems to be most crucial (Dzyakanchuk, Balázs et al. 2009). Despite the bidirectionality of 11β-HSD1, several studies indicate, that 11β-HSD1 works mainly as a reductase in most tissues in vivo, hence activating intrinsically inactive cortisone to cortisol (Tomlinson, Walker et al. 2004). GC production via 11β-HSD1 plays an important role for the serum levels of GC. In humans, 11β-HSD1 in the splanchnic bed alone, contributes about 30-40% to the systemic levels of cortisol (Chapman, Holmes et al. 2013). Moreover, 11β-HSD1 works in an intracrine manner, enhancing local action of cortisol via cortisone rescue. “Thus, any tissue expressing 11β-HSDs can regulate the exposure of that tissue to “active” GCs” (Tomlinson, Walker et al. 2004).

2.7 11β-HSD1 distribution in the CNS

11β-HSD1 is expressed with a similar distribution in rodents and humans, with the highest expression levels in the liver, adipose tissue and gonads (Tomlinson, Walker et al. 2004, Chapman, Holmes et al. 2013). Interestingly, 11β-HSD1 is also expressed at high levels in the rat CNS (Moisan, Seckl et al. 1990, Lakshmi, Sakai et al. 1991, Sakai, Lakshmi et al. 1992), remarkably, in the PVN (Seckl, Dow et al. 1993) and the PG

(Moisan, Seckl et al. 1990, Whorwood, Sheppard et al. 1993). More recently, the presence of 11β-HSD1 was described in the human CNS (Sandeep, Yau et al. 2004), in particular in the PVN (Bisschop, Dekker et al. 2013) and in the PG (Korbonits, Bujalska et al. 2001). In the human PVN, 11β-HSD1 is colocalized with CRH and AVP (Bisschop, Dekker et al. 2013). The expression of 11β-HSD1 in the central components of the HPA axis could suggest a role in regulating HPA axis activity.

2.8 11β-HSD1 and the HPA axis

It has been proposed that 11β-HSD1 expression in the components of the HPA axis can directly influence the GC-negative feedback and therefore modulate HPA axis activity (Seckl, Dow et al. 1993, Seckl 1997, Harris, Kotelevtsev et al. 2001, Edwards 2012). Insights into the relationship between 11β-HSD1 and the HPA axis arose from animal models with global 11β-HSD1 or H6PD knockout (KO) and 11β-HSD inhibition as well as pharmacological studies regarding the effects of 11β-HSD1 inhibitors in humans (Harno and White 2010). Harris et al. examined a mouse model with global 11β-HSD1 KO. They found that basal and stress-induced plasma levels of corticosterone and ACTH were elevated (Harris, Kotelevtsev et al. 2001) and adrenal gland size was enlarged (Kotelevtsev, Holmes et al. 1997, Abrahams, Semjonous et al. 2012), indicating an enhanced HPA axis activity. Increased ACTH and corticosterone levels were also reported in rats treated with glycyrrhizic acid, a strong inhibitor of 11β-HSD enzymes (Hanafusa, Mune et al. 2002). These findings were paralleled by observations in a mouse model of H6PD deficiency. Via reduction of the NADPH/NADP+ ratio, the decrease in H6PD activity indirectly inhibits 11β-HSD1 reductase activity or even changes the reaction direction to a net dehydrogenase activity (Lavery, Walker et al. 2006). In the mouse model of H6PD deficiency, increased levels of ACTH and corticosterone could be seen (Rogoff, Ryder et al. 2007), as well as increased corticosterone response to ACTH (Abrahams, Semjonous et al. 2012) and increase of adrenal gland size (Lavery, Walker et al. 2006).

In pharmacological studies on healthy humans examining the effect of orally administered 11β-HSD1 inhibitors, a rise of plasma ACTH levels could be seen in individuals treated with the inhibitors compared to the control groups, whereas cortisol

levels remained substantially unchanged (Rosenstock, Banarer et al. 2010, Webster, McBride et al. 2017).

One way to explain those findings is that a lack of 11β-HSD1 reductase activity in the central components of the HPA axis leads to a decreased GC-negative feedback and hence a disinhibited central production of ACTH and GCs. Therefore, these reports suggest a role of 11β-HSD1 in the regulation of the HPA axis. To see if HPA axis activity can be modulated in arthritis by changes in 11β-HSD1 activity, the relationship between inflammation and 11β-HSD1 expression/activity has to be examined.

2.9 11β-HSD1 in inflammation

There are many studies showing upregulation of 11β-HSD1 expression in response to inflammation (Chapman, Holmes et al. 2013). Exposure of human cells to the inflammatory cytokines IL-1β and TNF-α in vitro increases their expression and reductase activity of 11β-HSD1 (Escher, Galli et al. 1997, Cooper, Bujalska et al. 2001, Tomlinson, Moore et al. 2001, Hardy, Filer et al. 2006). In vivo studies in humans have shown that in inflammatory diseases, the plasma cortisol/cortisone ratio increases compared to healthy individuals (Ichikawa, Yoshida et al. 1977), implicating an overall dominance of the reductase over the dehydrogenase activity of the 11β-HSD enzymes. More recently, these findings could be examined specifically in patients with RA. The group around Mark Cooper found an increased cortisol/cortisone ratio in urine samples of the RA patients compared to patients with osteoarthritis, and this was paralleled by increased cortisol production in synovial tissue of the inflamed joints, indicating increased 11β-HSD1 activity (Hardy, Rabbitt et al. 2008). The cortisol to cortisone metabolite ratio correlated in magnitude with levels of inflammation markers such as the level of the erythrocyte sedimentation rate (Hardy, Rabbitt et al. 2008) as well as cellularity and T cell density (Schmidt, Weidler et al. 2005), and this ratio could be normalized by anti TNF-α therapy (Nanus, Filer et al. 2014). Similar findings could be seen in vivo in a rat model for RA, the so-called adjuvant-induced arthritis model. The arthritic rats exhibited upregulation of 11β-HSD1 mRNA expression and reductase activity in the synovial tissue compared to their healthy littermates (Ergang, Leden et al. 2010).

The increase of peripheral 11β-HSD1 activity in inflammation, especially in inflamed joints, seems to be part of the body's response to keep the inflammation locally under control. When this response is blocked, as can be observed in K/BxN serum-induced arthritis mice with KO of 11β-HSD1, animals exhibit an earlier onset and slower resolution of their arthritis (Coutinho, Gray et al. 2012).

While all these studies on peripheral tissues or overall body activity showed an upregulation of 11β-HSD1 mRNA and an increase of 11β-HSD1 activity in inflammation, studies looking at 11β-HSD1 in nervous cells or components of the HPA axis in conditions of inflammation have been scarce. As for ethical reasons, these types of study cannot be performed in humans, animal models have become increasingly popular in the research of the pathophysiology of RA.

2.10 Mouse models for immune-mediated arthritis

There is a multitude of animal models that have been used for research on the pathogenesis and treatment of RA (Asquith, Miller et al. 2009). Generally, they can be categorized into induced arthritis and spontaneous arthritis models. For this study, one model of each group was used.

2.10.1 The K/BxN spontaneous arthritis mouse model

Mice from the KRN-C57BL/6 transgenic line host a T cell receptor that recognizes a bovine pancreas ribonuclease peptide in the context of the MHC II I-Ak molecule (Peccoud, Dellabona et al. 1990). Crossed with mice from the NOD strain (KRNxNOD), T cells of the transgene-positive offspring (K/BxN) autoreact with the enzyme glucose-6-phosphate isomerase and induce a cascade of immune responses which leads to spontaneous joint inflammation of the host at the age of 4 to 5 weeks (Kouskoff, Korganow et al. 1996). This joint inflammation shares pathological features with RA like "leukocyte invasion, synovitis, pannus formation, cartilage and bone destruction, and anarchic remodeling" (Korganow, Ji et al. 1999). Furthermore, immunologic abnormalities such as "polyclonal B cell activation, hypergammaglobulinemia and

autoantibody production" resemble the ones seen in RA patients, with the limitation, that no rheumatoid factor is produced (Ditzel 2004).

Apart from being an animal model for RA itself, serum from these mice can induce arthritis in other mice too, a feature which is utilized in the creation of another arthritis mouse model as described below.

2.10.2 The K/BxN serum-induced arthritis mouse model

Transfer of K/BxN mouse serum reliably induces arthritis in the recipients in most mouse strains (Korganow, Ji et al. 1999, Ji, Gauguier et al. 2001). In contrast to the K/BxN spontaneous arthritis mouse model, this model bypasses many of the initial immunological events leading to the development of the disease by directly delivering high titers of arthritogenic antibodies. It therefore works independently of the T and B cell response (Korganow, Ji et al. 1999). Instead, in the K/BxN serum-induced arthritis mouse model, the joint inflammation is mediated by parts of the innate immune system, such as neutrophils (Wipke and Allen 2001), macrophages (Solomon, Rajasekaran et al. 2005), mast cells (Shin, Nigrovic et al. 2009) and FcR (Wipke, Wang et al. 2004).

The usage of the two different mouse models has the advantage, of being able to break down in which phases of RA development which pathologic processes take place (Ji, Gauguier et al. 2001). Furthermore, it makes it more likely that findings from the experiments will be generalizable to RA if both models show the same results.

3 Aims and hypothesis

Taking into consideration that,

1) there is a relative lack of HPA axis activity in patients with RA,
2) 11β-HSD1 is expressed in the central components of the HPA axis,
3) changes in 11β-HSD1 expression can alter HPA axis activity, and
4) 11β-HSD1 activity is increased in conditions of inflammation,

there are substantial indications that the lack of HPA axis responsiveness in patients with RA could occur due to an increased GC-negative feedback secondary to 11β-HSD1 upregulation in components of the HPA axis.

The hypothesis for this project was that an increased activity of 11β-HSD1 in the PVN and the PG during RA leads to an increased GC-negative feedback at these locations which could explain the absent elevation of cortisol production in patients with RA.

Therefore, critical aspects of GC signaling and metabolism in the hypothalamus and the PG were examined in two murine models of immune-mediated arthritis.

4 Materials and methods

4.1 Mouse models

4.1.1 Animal welfare and housing

Mice were kept at the Molecular Physiology Unit of the ANZAC Research Institute in accordance with institutional animal welfare guidelines and in agreement with the Animal Welfare Committee of the Sydney Local Health District under protocol 2008/042 and extensions. The mice had access to food and water ad libitum. Diurnal rhythm was simulated by a 12-hours light-/dark cycle. The temperature was kept constantly at 21-23°C.

4.1.2 K/BxN spontaneous arthritis mice (chronic arthritis mouse model)

The genetic background of the animals was determined by my colleague Ling Zhuang with conventional polymerase chain reaction (PCR), using RNA from toe clips of the mice. The following nucleotide sequences were used as primers:

QM1 Forward (5' – 3' sequence): AGGTCCACAGCTCCTTCTGA
QM2 Reverse (5' – 3' sequence): GTATTGGAAGGGGCCAGAG

The transgene-negative offspring lack the arthritic phenotype and were used as control. Mice were examined and tissue samples harvested at the age of 60 days. As these mice showed clinical signs of arthritis for the comparatively long time of 30 days, they will be referred to as chronic arthritis mice (CA).

4.1.3 K/BxN serum-induced arthritis mice (acute arthritis mouse model)

Pooled serum of 60 days old K/BxN mice was used to induce arthritis in healthy, 9-week-old male C57BL/6 mice. The mice received an intraperitoneal injection of 150 µl K/BxN serum for the arthritic group or 150 µl normal saline for the control group. This

injection was repeated two days later. The development of inflammation was followed up by clinical scoring on at least every second day. Previous studies have shown that features of immune-mediated arthritis peak at day 7 after the first injection (Buttgereit, Zhou et al. 2009). Therefore, this time point was chosen for tissue sample harvesting. As these mice showed clinical signs of arthritis for the comparatively short time of less than 7 days, they will be referred to as acute arthritis mice (AA).

4.2 Clinical assessment of arthritis

For both mouse models, clinical scoring of the severity of joint inflammation was performed as described previously (Lee, Zahra et al. 2006, Buttgereit, Zhou et al. 2009). The limbs of the mice were examined and scored according to the number of affected joints and the inflammation severity. Each limb was categorized as follows: 0 = normal, 1 = mild to moderate swelling, 2 moderate swelling, 3 = marked swelling. Adding up the score of every limb, each mouse ranked on an inflammation intensity scale from 0 to 12 points.

4.3 Tissue collection and storing

At the assigned time points, mice were transferred from the Molecular Physiology Unit to the laboratory for the sample collection. To avoid variations due to the diurnal hormonal changes, the tissue harvest time points were scheduled at noon, which corresponds to the late nadir phase of the rodent circadian GC production rhythm. Mice were anaesthetized with a mixture of 0.1 ml xylazine and 0.75 ml ketamine in 10 ml saline at a dosage of 0.12 ml/10 g body weight. Blood samples were collected from the eye vein, allowed to clot, and spun down at 4°C. The supernatant serum was collected and stored at -80°C until further use. Then mice were euthanized by cervical dislocation. Brains were quickly dissected from the scull base as a whole. Brain samples assigned for gene expression studies were stored in RNAlater (Life Technologies, Carlsbad, CA, USA). Brain samples for histology studies were stored in paraformaldehyde. The PGs, which remained on the skull base after the brain removal, were taken out and immediately frozen down in liquid nitrogen before they were stored at -80°C until further use.

4.4 Gene expression studies

4.4.1 Brain dissection

To obtain a firm consistency for further dissection and at the same time protect the tissue RNA from degrading, brains were stored overnight in RNAlater at 4°C after the tissue harvest. 24 h later, the brains were further dissected into distinct regions. Hippocampus, cerebellum and cortex were dissected according to a protocol of S. Spijker (Spijker 2011). From the remaining brain tissue, the hypothalamus was dissected out with a method adapted from Baker et al (Baker, Joh et al. 1983). The macroscopic landmarks used as borders were as follows (Figure 4 and Figure 3):

Rostral: optical chiasma and anterior commissure
Caudal: interpeduncular fossa
Lateral: choroidal fissure

The samples were then stored at -80°C until processing.

4.4.2 RNA isolation

RNA was isolated from each sample individually except for the PGs. In a pilot trial with different numbers of PGs pooled together, we found that a minimum of two pooled samples was required to yield sufficient amounts of RNA for further analysis. Whenever possible, these two samples were matched according to litter and gender. The two pooled samples are referred to as one sample number in this thesis.

RNA was isolated from the tissue samples using a TRIzol RNA extraction protocol from the Bone Biology Group of the ANZAC Research Institute in combination with a commercial RNA extraction kit (Nucleospin RNA by Macherey Nagel, Dueren, Germany). Samples were immersed in beads tubes (lysing matrix D, MP Biomedicals, Santa Ana, CA, USA) filled with 1 ml TRIzol and then homogenized using a Powerlyzer machine (MO BIO Laboratories, Carlsbad, CA, USA). The company's protocol for nervous system tissue was used, shaking the tubes for 2 times 45 s at 3,500 rpm, with a pause of 30 s. Then, homogenates were incubated for 5 min at room temperature before they were centrifuged for 10 min at 12000 rpm at 4°C. The supernatant was

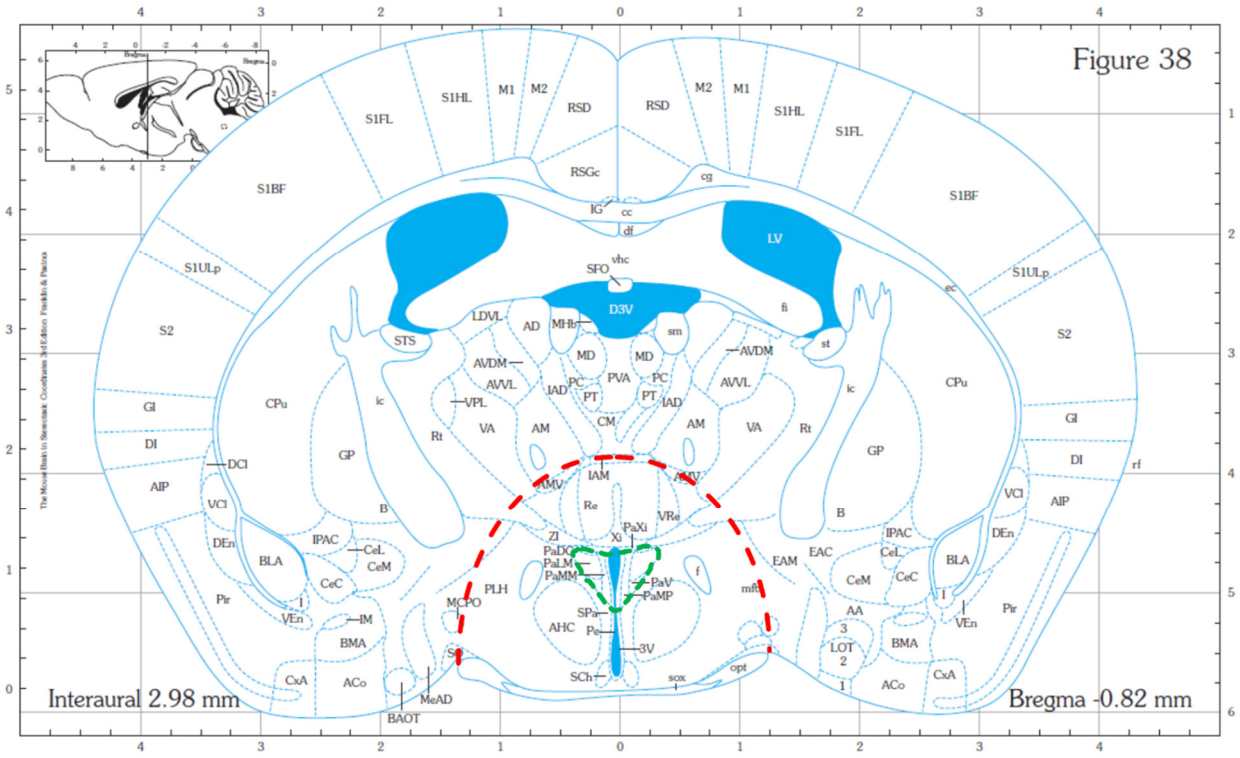

Figure 4: Coronal section of the mouse brain.

Level of the paraventricular nucleus (PVN). Red line: The gross dissection line for the hypothalamus. Green line: The PVN. This figure was published in “The mouse brain in stereotactic coordinates third edition” (Franklin and Paxinos 2007), page 38, Copyright Elsevier (2007). Reproduced with permission from the publisher.

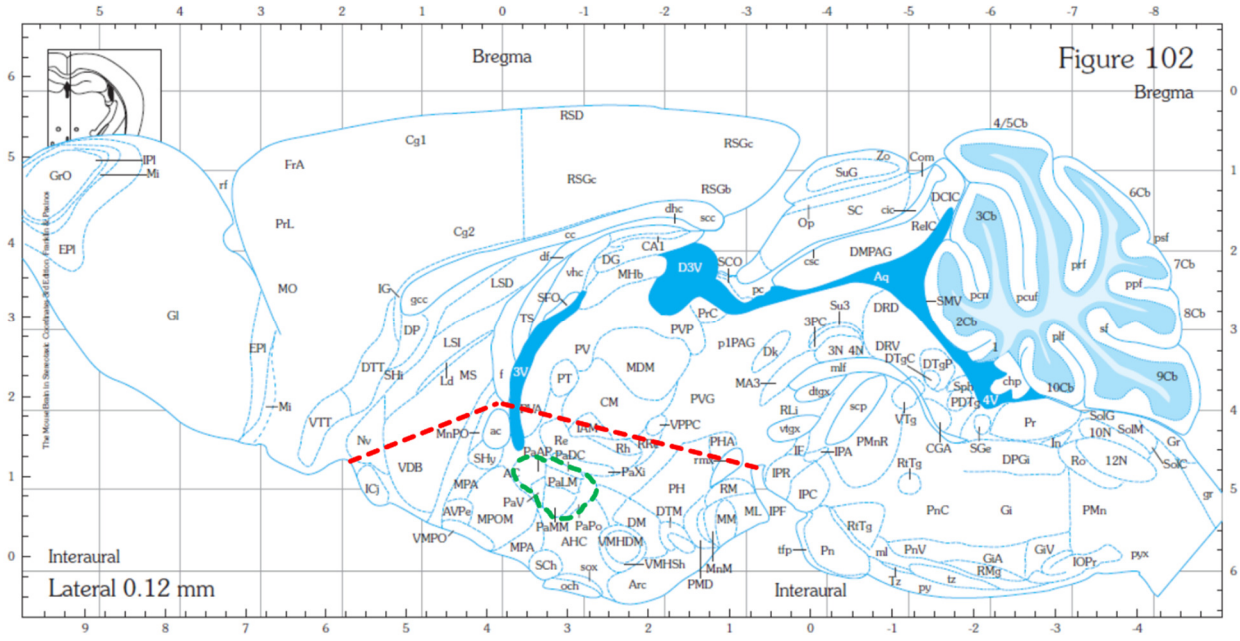

Figure 3: Sagittal section of the mouse brain.

Level of the paraventricular nucleus (PVN). Red line: The gross dissection line for the hypothalamus. Green line: The PVN. This figure was published in “The mouse brain in stereotactic coordinates third edition” (Franklin and Paxinos 2007), page 102, Copyright Elsevier (2007). Reproduced with permission from the publisher.

transferred into an RNase-free tube and 200 µl of chloroform was added, followed by 15 s of vigorous shaking. After another 5 min of incubation at room temperature, the homogenates were spun down for 15 min at 12000 rpm at 4°C and the aqueous phase was transferred into a new RNase-free tube. 500 µl of 2-propanol was added and the tubes inverted several times before the samples were incubated for 2 h on ice. Subsequent steps followed the protocol of the company's RNA extraction kit. Briefly: Samples were mixed with 350 µl lysis buffer and 3.5 µl ß-mercaptoethanol and filtered through the filter columns provided. The RNA binding conditions were adjusted by the addition of 350 µl 70% ethanol. Through centrifugation, the RNA was bound to the column membrane. The membrane was then desalted by adding 350 µl membrane desalting buffer. Contaminative DNA was removed from the membrane by adding 95 µl of DNase for 15 min. The membrane was then washed and dried by the addition and centrifugation of a series of wash buffers. Finally, the RNA was collected in RNase-free water. After isolation, RNA was assessed for quality (Chapter 4.4.3) and either directly analyzed further or stored at -80°C until further processing.

4.4.3 RNA quality assessment

The quality of the extracted RNA was verified by gel electrophoresis and spectrophotometry. For the gel, 0.8 g of agarose powder was mixed with 80 ml of Tris/Borate/EDTA buffer and 8 µl of sybr safe stain (Thermo Fisher, Waltham, MA, USA). 2 µl of RNA sample was combined with 1.5 µl of loading dye and diluted in 10 µl of RNase-free water. The mixture was pipetted into the pockets of the gel and run in a horizontal gel tank (Galileo Bioscience, Cambridge, MA, USA) for 30 min at 120 V. The gel was then evaluated with an imaging system (Bio Rad, Hercules, CA, USA). Inclusion criteria for the samples were the presence of clearly delineated 28S and 18S bands on the gel image and the absence of degradation smears. Purity of the samples from protein contamination as well as other residues of reagents from the RNA extraction process was assessed by spectrophotometry (Nano Photometer, Implen, München, Germany). An a260/a280 ratio greater than 1.8 was considered a sufficient purity. Additionally, the photometry results were used to determine if sufficient amounts of RNA were isolated to perform reverse transcription. Samples with very low yields of less than

20 ng RNA/µl indicated suboptimal RNA extraction and were excluded from further processing.

4.4.4 Reverse transcription

RNA was converted to cDNA with a commercial reverse transcription kit (SuperScript III First-Strand Synthesis SuperMix for qRT-PCR, Life Technologies, Carlsbad, CA, USA), following the company's protocol. RNA samples were mixed with diethylpyrocarbonate-treated water at a ratio calculated for each sample individually depending on the amount of RNA. This was to make up for variations in RNA concentration that were determined by spectrophotometry. The kits' reverse transcription reaction mix and the enzyme mix were added. Samples were first incubated for 10 min at 25°C then for 30 min at 50°C. After terminating the reaction for 5 min at 85°C, samples were quickly chilled on ice. E. coli RNase was added to each sample and they were incubated for another 20 min at 37°C. Finally, the cDNA samples were diluted in RNase-free water and either instantly used for real time quantitative polymerase chain reaction (qPCR) or stored at -20°C until further use.

4.4.5 Real time quantitative polymerase chain reaction

The most reliable reference gene was determined using the GeNorm software as described by Vandesompele et al (Vandesompele, De Preter et al. 2002). Amongst several tested candidate genes, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) proved to be the most stable one in our experimental setting and was therefore used as reference gene. Primers for qPCR were designed by Professor Hong Zhou (Table 1). Samples and primers were mixed with Sybr Green Supermix (Bio Rad, Hercules, CA, USA) and amplified with a qPCR machine (Bio Rad, Hercules, CA, USA). For the reference gene, sample triplicates were examined to make up for measurement inaccuracies. For the genes of interest, sample duplicates were examined. The protocol for the qPCR machine runs was set as follows: 95°C for 3 min, 40 cycles 95°C for 10 s + 60°C for 15 s + 72°C for 30 s. Data were acquired in Ct, as cycle numbers when the color reaction exceeded the set threshold. For further calculations, the mean Ct value of each duplicate and triplicate respectively was used. To transfer the Ct values into

relative expression values, the ΔΔ-Ct method was applied as described previously (Livak and Schmittgen 2001).

Gene	Forward (5' – 3' sequence)	Reverse (5' – 3' sequence)
Avp	AGGATGCTCAACACTACGCT	TCTTGGGCAGTTCTGGAA
Crh	CAAATGCTGCGTGCTTTC	CCTTTCTCTTCAGTCTCTCAACG
Crhr1	TTTCTACGGTGTCCGCTACA	CTTTGCTCTTCTTCTCTTCGTTG
Gapdh	TCCATGACAACTTTGGCATT	CAGATCCACGACGGACACA
Gr	GTTCATGGCGTGAGTACCTC	AGAGTTTGGGAGGTGGTCC
11β-HSD11b1	GGAGCCGCACTTATCTGAA	GACCTGGCAGTCAATACCA
Mr	TGCCGTCTTCAGTATGCAG	GACTTGGAGGGCTGGAAAT
Pomc	TCCAATCTTGTTTGCCTCTG	ATCTCCGTTGCCAGGAAA

Table 1: Primer nucleotide sequences used for qPCR analysis.

Avp = arginine vasopressin, Crh = corticotropin-releasing hormone, Crhr1 = corticotropin-releasing hormone receptor 1, Gapdh = glyceraldehyde 3-phosphate dehydrogenase, Gr = glucocorticoid receptor, 11β-HSD11b1 = 11β-hydroxysteroid dehydrogenase type 1, Mr = mineralocorticoid receptor, Pomc = proopiomelanocortin.

4.5 Histology studies

4.5.1 Sample preparation

In preparation for histological examination, samples were stored in 4% paraformaldehyde at 4°C for 24 h. Then the medium was changed to phosphate-buffered saline until further processing. The dehydration steps were performed with the aid of an automated tissue processing machine (Shandon Excelsior ES, Thermo Fisher Scientific, Australia). Samples were dehydrated in a series of increasing ethanol concentrations (50% 4 h, 70% 4 h, 95% 4 h, 95% 4 h, 100% 2 h, 100% 2 h, 100% 2 h in vacuum) and then cleared in xylene (three times 2 h in vacuum). After that, samples were embedded in paraffin wax (Paraplast Tissue Embedding Medium, Tyco Healthcare Group, Mansfield, MA, USA). A manual Microtome (Leica Microsystems, Wetzlar, Germany) was used to cut 6 µm thick sections of samples.

4.5.2 H&E staining

As a control for the dissection technique, some of the dissected brain parts were examined by H&E staining. First, paraffin was removed from the sections by melting them on a heat plate. For further removal of the wax, samples were immersed in three changes of xylene for 5 min each. Then they were hydrated in three changes of 100% ethanol. After washing, they were stained in hematoxylin (type Lillie Mayer's) for 7 min. After another washing step, the stain in eosin followed for another 11 min. Sections were then rehydrated in 3 changes of 100% ethanol for 10 dips each and then cleared in xylene for three times 2 min. Finally, they were covered with a cover slip attached with DEPEX mounting media.

4.5.3 Immunohistochemistry

4.5.3.1 Orientation

Whole brain samples were cut in coronal orientation for immunohistochemical (IHC) examination. To determine the correct level of the PVN, test samples were examined regularly. These were collected starting at the level of the fusion of the right and left sides of the anterior commissure. They were stained with toluidine blue and the position was determined under the microscope. After reaching the level of the PVN, sections were collected for immunostaining.

4.5.3.2 Antibodies and staining system

For the immunostaining, primary ABs against 11β-HSD1 and GR were used. The 11β-HSD1 AB (Cayman Chemicals, Ann Arbor, MI, USA) is a polyclonal AB raised in rabbits that binds to the following antigen domain: CLELGAASAHYIAGT, for example in mice. A dilution of 1:100 was used. The GR antibody (AB) (GR antibody (M-20): sc-1004, Santa Cruz Biotechnology, Dallas, TX, USA) is also a polyclonal AB raised in rabbits. It binds to the N-terminus of each the α and β subtype of the GR in mice. A dilution of 1:400 was used for that AB. Both ABs were used with a biotinylated goat anti-rabbit secondary AB (Vectastain ABC-Peroxidase Kits, Vector Laboratories, Burlingame, CA, USA). In that system, an Avidin-Biotin-Complex system is used to link biotinylated peroxidase enzymes with the secondary AB. Then this peroxidase activity is visualized by a diaminobenzidine staining kit (Vector Laboratories, Burlingame, CA, USA).

4.5.3.3 Staining protocol

Firstly, paraffin was removed by heating the slides on a heat plate, followed by immersion into xylene in three subsequent tanks for 5 min each. Sections were then rehydrated in ethanol in decreasing concentrations (Three times 100%, then 95%, 70% for 3 min each). Antigen sites were retrieved in hot citrate buffer for 2 h. Endogenous peroxidase activity was quenched by incubating the samples in 3% hydrogen peroxide for 20 min. To suppress non-specific AB binding, the samples were incubated in 5% goat serum for 30 min. Samples were then incubated overnight with the primary AB in 2% goat serum. For each run, one sample was incubated in 2% goat serum without primary AB as a negative control. On the next day, the samples were incubated with the secondary AB for 1 h. After preparing the samples with the Avidin-Biotin-Complex reagents for 30 min, the diaminobenzidine reagent was put on until a clear and specific staining could be seen under the microscope, which was after 4 min for the 11β-HSD1 AB and 1 min 20 sec for the GR AB. Samples were quickly washed in water and then counterstained with a Harris' hematoxylin 1 in 10 dilution for 3 min. They were then dehydrated in a series of ethanol (70%, 95%, three times 100%, 3 min each), and three subsequent xylene tanks before they were mounted onto object slides.

4.5.3.4 Evaluation

Under the light microscope, the PVN was visually delineated on each sample. Stained and unstained cells in this area were counted and the percentage of stained cells calculated for each sample.

4.6 Measurement of serum hormones

4.6.1 Adrenocorticotropic hormone ELISA

ACTH serum levels were measured with a commercial ELISA kit (Mouse/Rat Adrenocorticotropic Hormone ELISA, Sigma Aldrich, St. Louis, MO, USA) according to the company's protocol. Standard concentrations, calibrators, specimens and controls were distributed to a microwell plate coated with streptavidin. Two kinds of ABs were added; one AB is biotinylated and binds to the C-terminus of ACTH, another AB is labeled with horseradish peroxidase and binds to the N-terminus of the latter. The samples were covered with aluminum foil and incubated at room temperature on a

shaker for 4 h. Liquids were then removed and the wells washed. Tetramethylbenzidine substrate was added and the plate was incubated for another 30 min on a shaker. The reaction was ended with a stop solution and the plate quickly analyzed under a microplate reader (iMark microplate reader, Biorad, Waltham, MA, USA) at 450 nm wavelength. With the kits's standard concentrations, a calibration curve was created and from that ACTH concentrations of the specimens could be calculated. For each sample, duplicate samples were examined and the mean calculated as result.

4.6.2 Corticosterone ELISA

Serum samples were examined with a commercial ELISA kit (Corticosterone EIA Kit, Arbor Assays, Ann Arbor, MI, USA) using the company's protocol. A microtiter plate was prepared with a serial dilution of corticosterone concentrations, a well for non-specific AB binding, a well for maximum binding and the specimens. Assay buffer and DetectX corticosterone conjugate was added to each well. Then the DetectX corticosterone AB was added to each well except from the non-specific binding well and the plate was incubated for 1 h at room temperature on a shaker. Liquids were removed, the wells were washed and tetramethylbenzidine-solution was added for the staining. Samples were incubated for 30 min at room temperature before the reaction was ended with a stop solution. The density of the staining was then determined with a microplate reader at 450 nm wavelength. The serial dilution was used to set up a calibration curve with which the corticosterone concentrations of the specimens could be calculated. For each sample, duplicates were examined and the mean calculated as result.

4.7 Sample sizes

For different mouse models and techniques, different numbers of samples were examined. There are several reasons for the varying numbers. Firstly, the general availability of the mouse models varied due to other animal experiments in the laboratory. Secondly, the use of a sample for one experimental technique usually excluded that sample from use in another examination. Lastly, a small number of samples had to be excluded because of technical issues in the experimental process

(for example low RNA yields). Figure 5 gives an overview of the numbers of samples that were used for each mouse model and each experimental technique.

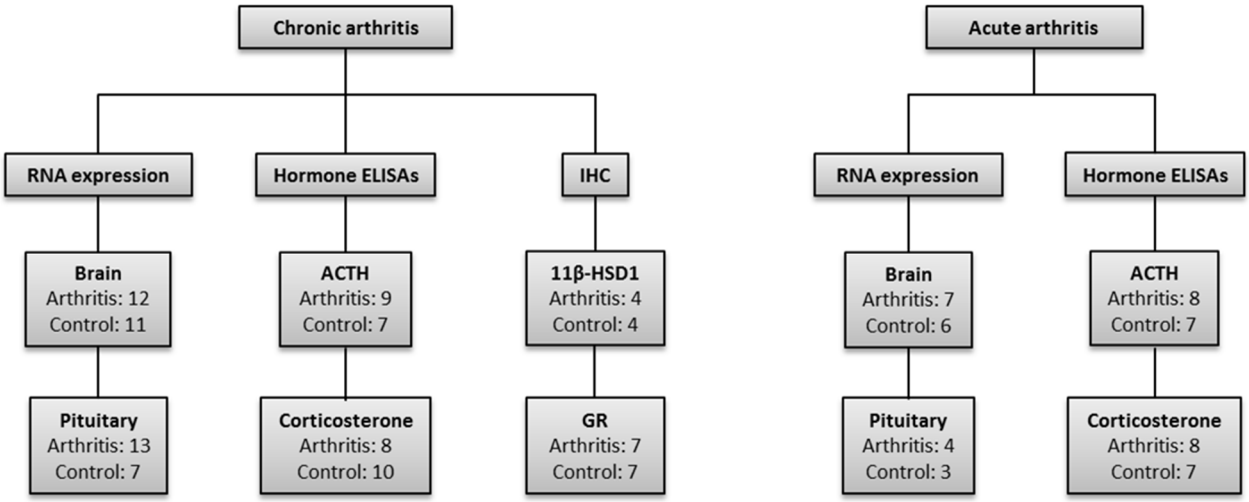

Figure 5: Sample numbers per experiment.

ACTH = adrenocorticotropic hormone, IHC = immunohistochemistry, 11β-HSD1 = 11β-hydroxysteroid dehydrogenase type 1, GR = glucocorticoid receptor. The pituitary sample numbers refer to pooled sample pairs.

4.8 Statistical analysis

To compare normally distributed values between two groups, the Student's t-test was used. A p-value of less than 0.05 was considered statistically significant. Results are shown as the mean and the standard error of mean.

5 Results

5.1 Evaluation of methods

5.1.1 Dissection technique

To confirm that the dissected brain parts for the mRNA expression studies correlate with the desired anatomical structures, test sections were cut for each dissected region and stained with H&E. Sections from the hypothalamus clearly showed the inclusion of the PVN (Figure 6). Sections from the other brain parts showed the typical structures of the examined CNS areas (Figure 7). The margins were mostly free from adjacent tissue.

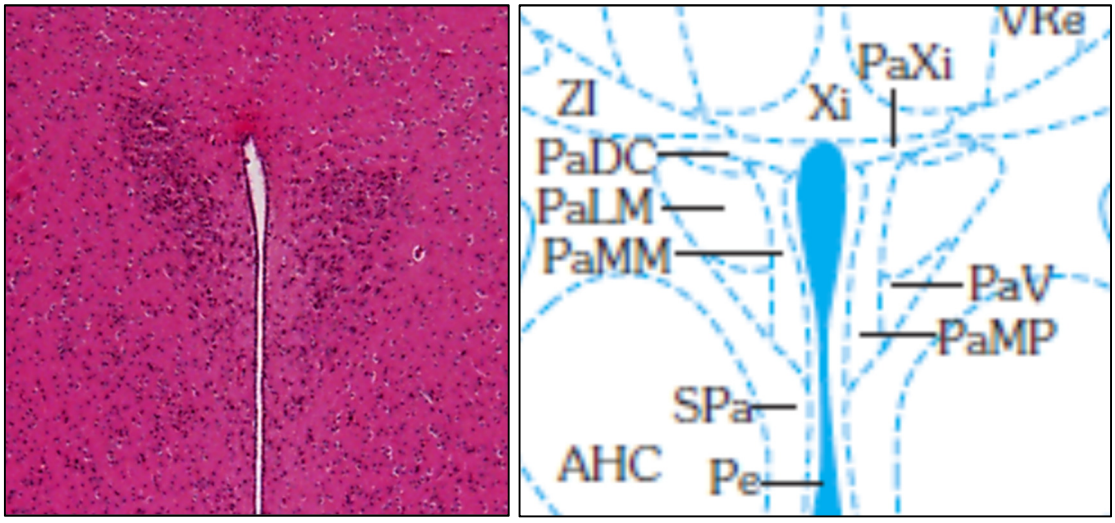

Figure 6: The paraventricular nucleus.

Left side: Coronal section, H+E staining. The paraventricular nucleus (PVN) can be delineated from its surrounding tissue. Right side: The PVN as illustrated in the mouse brain anatomy atlas. This figure was published in “The mouse brain in stereotactic coordinates third edition” (Franklin and Paxinos 2007), page 38, Copyright Elsevier (2007). Reproduced with permission from the publisher.

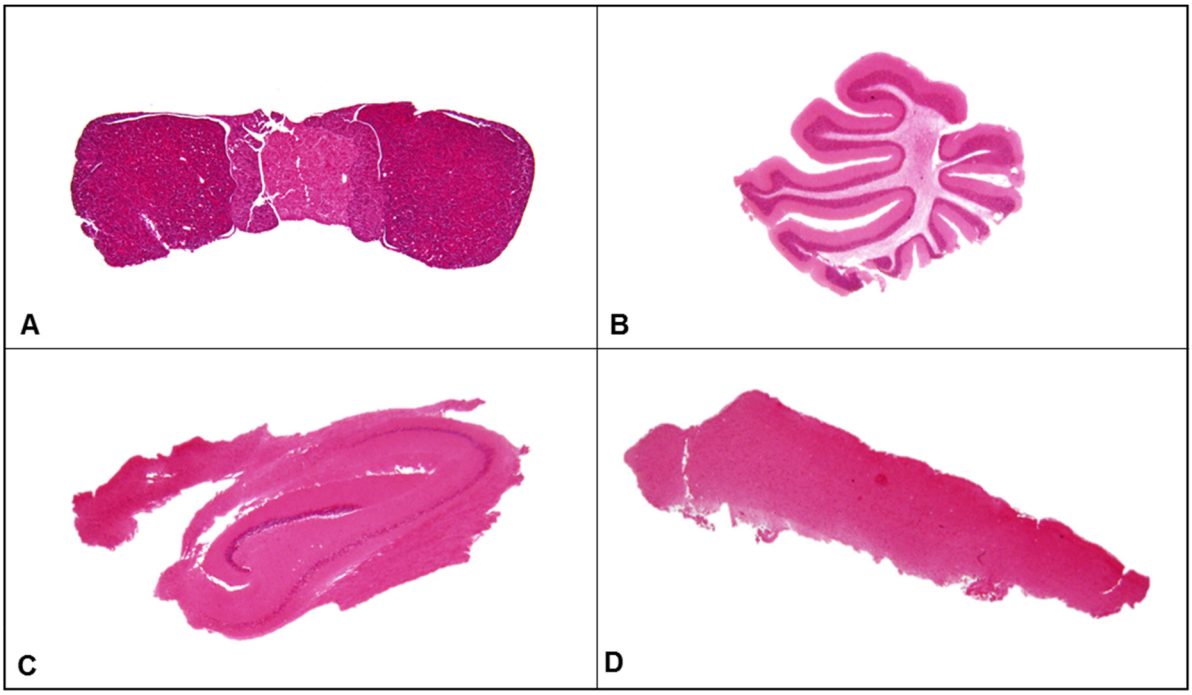

Figure 7: Dissected areas of the central nervous system.

H&E staining. A: Pituitary, coronal section. B: Cerebellum, sagittal section. C: Hippocampus, coronal section. D: Cerebral cortex, coronal section. Different scaling was used for each part.

5.1.2 RNA quality

5.1.2.1 Gel electrophoresis

The protocol for RNA extraction of the hypothalamus, hippocampus, cerebellum and cortex was suitable to yield high quality RNA. All samples met the inclusion quality criteria for the gel electrophoresis (Figure 8).

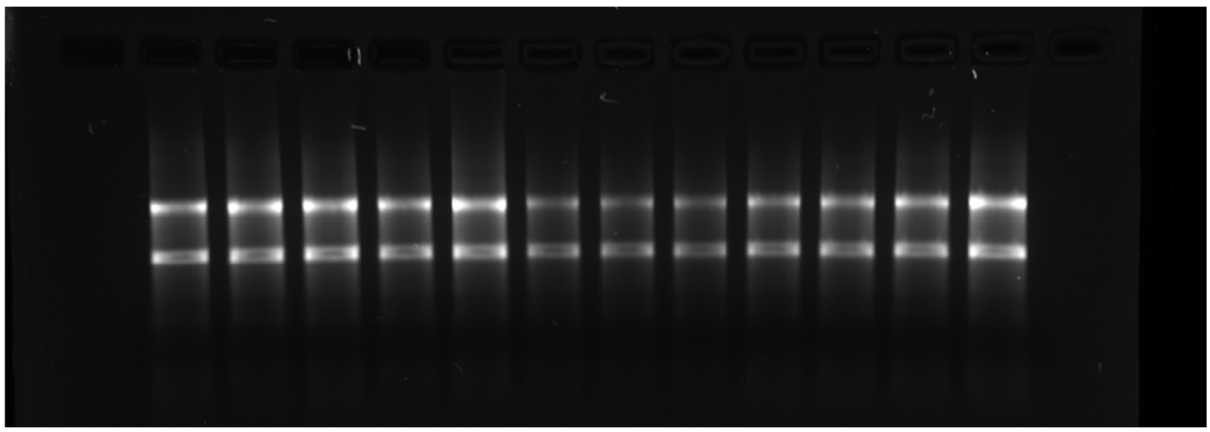

Figure 8: Gel electrophoresis picture of hypothalamic mRNA samples.

The absence of smears and the clearly delineated bands show a typical pattern for the research described in this thesis.

In the gel electrophoresis image of RNA extracted from single PGs, bands were hardly detectable. Therefore, an experimental trial was set up to determine the necessary amount of pooled PG samples to yield good quality RNA. The result was that a minimum of two pooled PG samples was required to yield adequate RNA amounts (Figure 9).

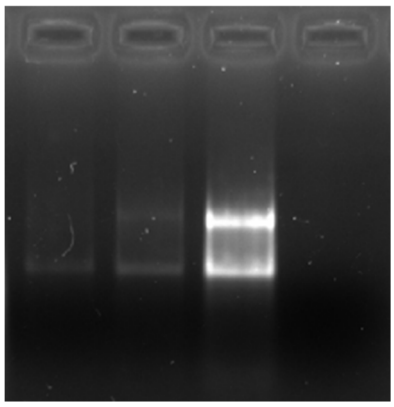

Figure 9: Gel electrophoresis picture of different amounts of pooled pituitary mRNA samples.

From left to right: 1 sample, 2 samples, 3 samples, negative control.

5.1.2.2 Nano photometry

Extracted RNA was examined with a NanoPhotometer to determine the purity as well as the amount of yielded RNA. All samples met our quality criteria for purity and showed an A260/A280 ratio of greater than 1.8. A small number of samples had to be excluded due to very low RNA yields.

5.2 11β-HSD1 expression in different parts of the CNS

Relative mRNA expression of 11β-HSD1 was examined in KRNxNOD transgene negative mice, which were also used as CA control mice, and compared between different CNS parts (Figure 10). The expression in the hypothalamus was relatively low compared to the other brain parts. However, 11β-HSD1 expression varies within different parts of the hypothalamus (Bisschop, Dekker et al. 2013) and a region-specific distribution of the 11β-HSD1 protein levels was observed in this study (Chapter 5.3.3). In contrast, the PG showed very high 11β-HSD1 expression levels compared to the other CNS parts. So far, the PG has rarely been in the focus of 11β-HSD1 research.

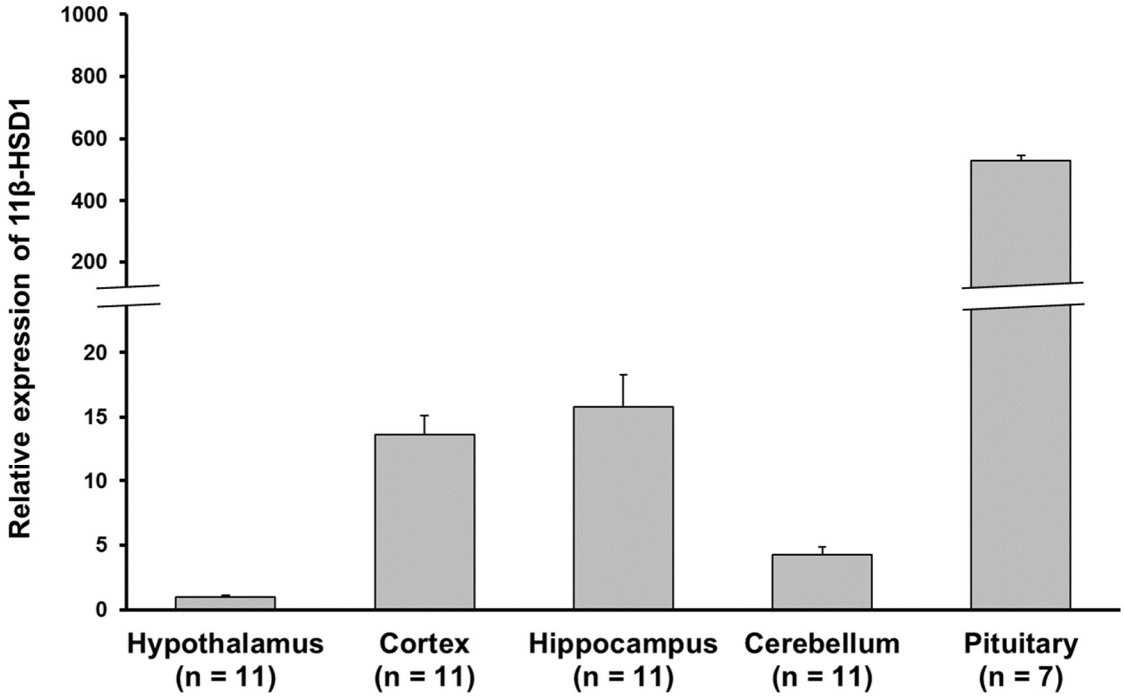

Figure 10: Relative expression of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) in different parts of the central nervous system.

Samples from non-arthritic KRNxNOD transgene negative mice. Highest expression levels were seen in the pituitary with levels 530 times as high as in the hypothalamus. This was followed by hippocampus (15.8-fold), cortex (13.7-fold) and cerebellum (4.3-fold). In the hypothalamus, there was the lowest 11β-HSD1 expression of the examined regions.

5.3 The chronic arthritis mouse model

5.3.1 Clinical scoring

On the examination day, CA mice were clinically scored before the tissue harvest. The CA mice reliably showed high joint inflammation scores with an average score of 10.74 (SE = 0.19) out of 12. Joint inflammation was absent in the control group.

5.3.2 mRNA expression studies

In the CA mouse model, parts of the CNS were examined that can influence HPA axis activity, including central parts of the HPA axis itself. mRNA expression changes between arthritic mice and their controls were examined in genes that are related to GC production, metabolism and reception.

5.3.2.1 Hypothalamus

In the hypothalamus, expression levels were examined of 11β-HSD1, GR, MR, CRH and AVP. 11β-HSD1 is the only known enzyme that can activate substantial amounts of active GCs from inert GCs (Cooper and Stewart 2009). GR and MR are crucial for GC action in the cell. Changes in the expression levels of any of these genes could influence GC-negative feedback and therefore modulate HPA axis activity. CRH and AVP are the main hypothalamic secretagogues that trigger ACTH production and release in the PG. Their expression levels are indicators of the activity of the central HPA axis parts. As shown in Figure 11, the mRNA expression of 11β-HSD1 was unchanged between arthritic and control mice. No changes could be seen in the MR mRNA expression. The GR mRNA showed a small but significant increase of the arthritic mice compared to the controls. CRH and AVP mRNA were both downregulated in arthritic mice.

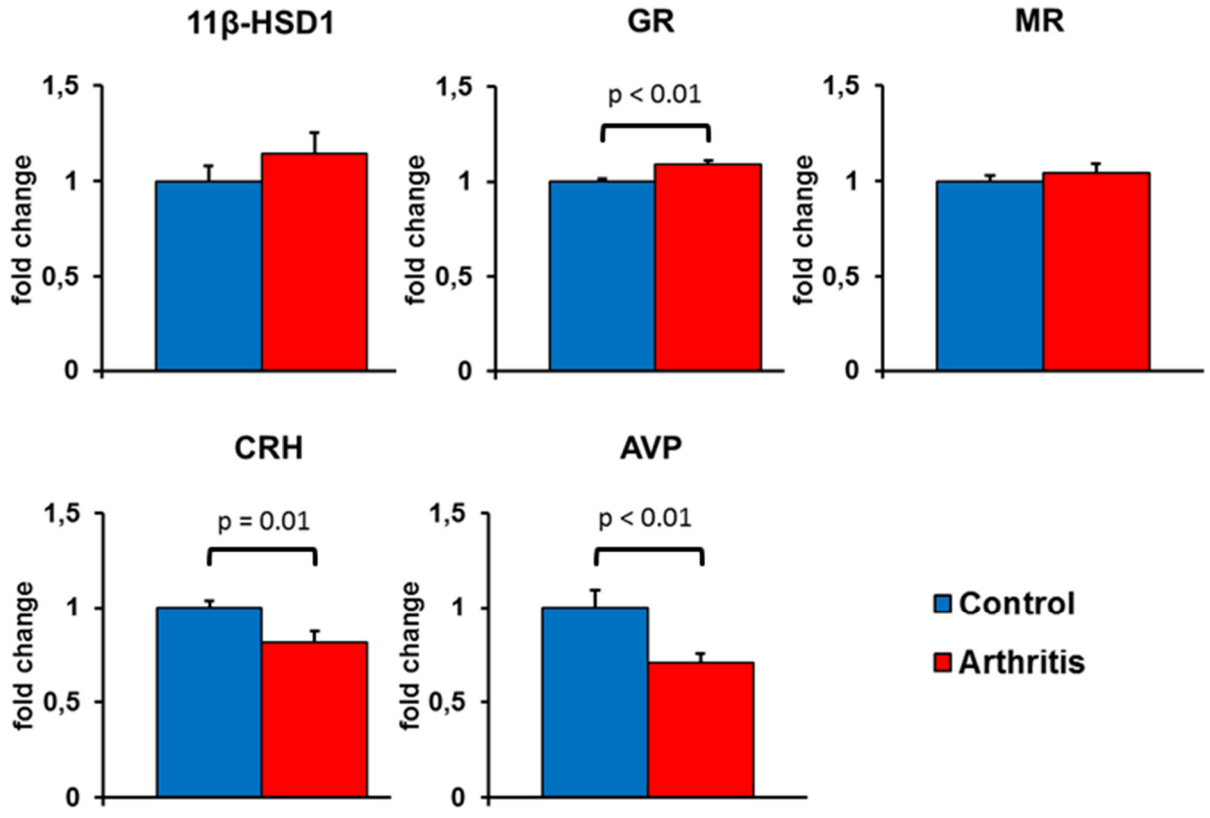

Figure 11: Comparison of mRNA expression levels in the hypothalamus between chronic arthritis mice and their controls.

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and mineralocorticoid receptor (MR) remained unchanged. Glucocorticoid receptor (GR) was upregulated by 1.09-fold ($p = 0.005$). Corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) were downregulated by 0.81-fold ($p = 0.014$) and 0.71-fold ($p = 0.008$) respectively.

5.3.2.2 Pituitary gland

In the PG, expression levels of 11β-HSD1, GR, MR, CRHR1 and POMC were examined (Figure 12). As the PG is another place for GC-negative feedback on the HPA axis, genes that can regulate GC feedback, namely 11β-HSD1, GR and MR, were examined. A high relative expression of PG 11β-HSD1, as observed in this study (Chapter 5.2), could indicate that 11β-HSD1 plays a major role in the PG. Corticotropin-releasing hormone receptor 1 (CRHR1) is a receptor regulating PG sensitivity to hypothalamic CRH stimulation (Smith and Vale 2006). POMC is the precursor protein of ACTH, which triggers the GC production in the adrenal glands. In the arthritic mice, 11β-HSD1 and MR expression was upregulated in the PG. GR expression did not change significantly. CRHR1 and POMC were upregulated significantly as well.

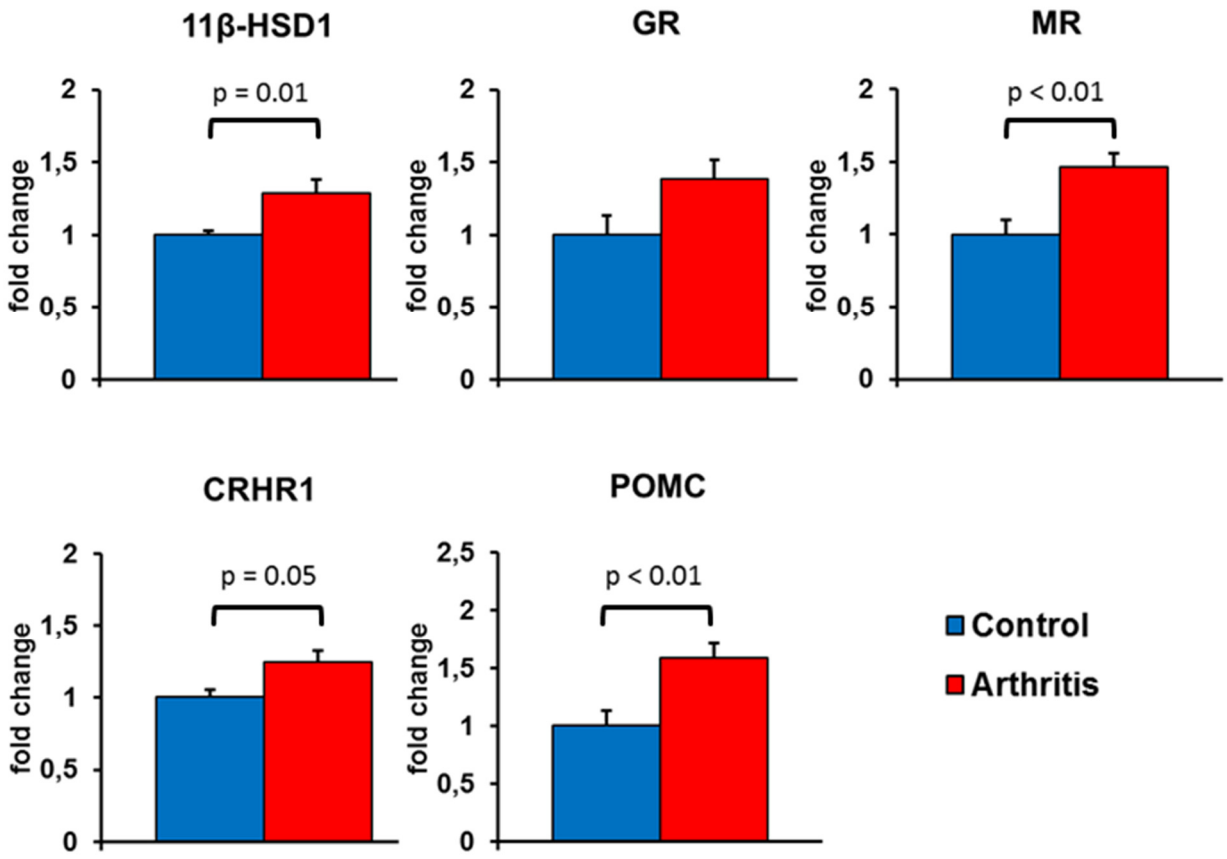

Figure 12: Comparison of mRNA expression levels in the pituitary between chronic arthritis mice and their controls.

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and mineralocorticoid receptor (MR) were upregulated in the arthritic mice by 1.29-fold ($p = 0.013$) and 1.46-fold ($p = 0.003$). Glucocorticoid receptor (GR) expression did not change significantly. Corticotropin-releasing hormone receptor 1 (CRHR1) and proopiomelanocortin (POMC) were upregulated by 1.24-fold ($p = 0.047$) and 1.59-fold ($p = 0.009$).

5.3.2.3 Other brain parts

Different parts of the brain have been reported to participate in the regulation of the HPA axis. Amongst these brain parts are the hippocampus (Jacobson and Sapolsky 1991) and the cerebral cortex (Diorio, Viau et al. 1993). Therefore, mRNA expression levels of 11β-HSD1, GR and MR were examined in these parts to see if HPA axis activity can be affected by changes in the GC-negative feedback there. Furthermore, the cerebellum was examined as a reference area, that is probably not linked to HPA axis regulation. For all examined genes and brain parts, no significant differences in the expression levels between the CA mice and their controls were observed. These results indicate that the findings in the hypothalamus and the PG are specific for these areas.

5.3.3 Immunohistochemistry

The experiments above examined mRNA expression levels in relatively large areas of the brain. This was due to the dissection technique, which did not allow for selective dissection of individual nuclei. The approach was unable to rule out the possibility of mRNA expression changes within smaller regions in response to inflammation. In particular, it was impossible to determine whether the expression of 11β-HSD1 is selectively regulated in the PVN of the hypothalamus, a region critical to the production of CRH and communication with the anterior PG. Instead, semi-quantitative IHC was used to examine for changes in 11β-HSD1 and GR expression specifically within the PVN in CA mice and their control littermates.

In the control mice, a high number of cells stained with the 11β-HSD1 AB could be detected in the PVN compared to the surrounding area (Figure 13). About 75% of the cells stained positive for 11β-HSD1. When compared to the arthritic mice, there was no significant difference in the percentage of 11β-HSD1-stained cells in the PVN, about 70% of the cells stained positive in this group (Figure 14). Examination for GR also showed no significant difference in the percentage of stained cells between the arthritic group and controls (66% versus 64%).

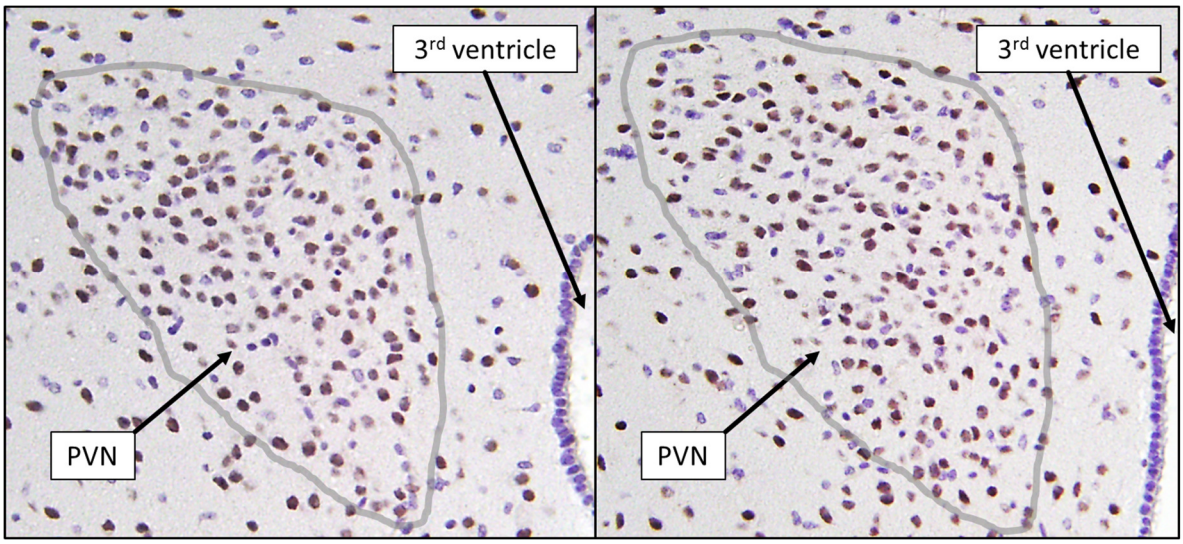

Figure 13: Representative immunohistochemistry images of the paraventricular nucleus stained with 11β-hydroxysteroid dehydrogenase type 1 antibody.

The grey line depicts the outer borders of the paraventricular nucleus. Left: Control group. Right: Chronic arthritis group.

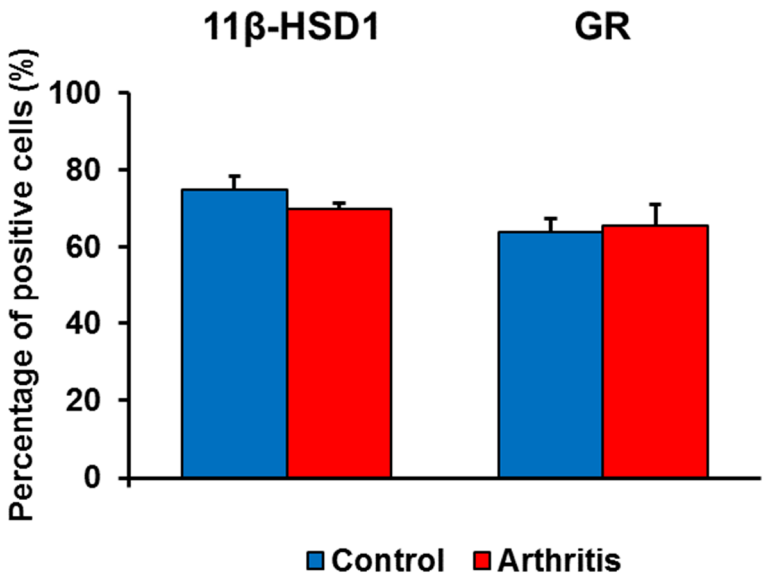

Figure 14: Comparison of positively stained cells between arthritic mice and their controls.

Staining for 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and glucocorticoid receptor (GR) in the paraventricular nucleus. For both antibodies, there were no significant differences between the groups.

5.3.4 Hormone assays

At the time point of tissue harvest, blood samples of the mice were collected from the eye vein. These samples were examined for serum levels of ACTH and corticosterone (Figure 15). This experiment was important to link the findings from mRNA expression and IHC studies to the activity of the HPA axis.

The control group showed a mean concentration of 13.9 pmol/l ACTH in the serum. Compared to previously described physiological levels of K/BxN transgene negative mice, these levels were elevated (Buttgereit, Zhou et al. 2009). The arthritic mice showed a mean concentration of 23.3 pmol/l ACTH which was not significantly different from the controls. Like ACTH, corticosterone levels in the control group were elevated compared to physiological levels (Buttgereit, Zhou et al. 2009), namely 834.8 nmol/l. In the arthritic mice, corticosterone blood levels were not higher than in the controls despite ongoing inflammation. With a mean of 786.1 nmol/l, they remained on a similar level.

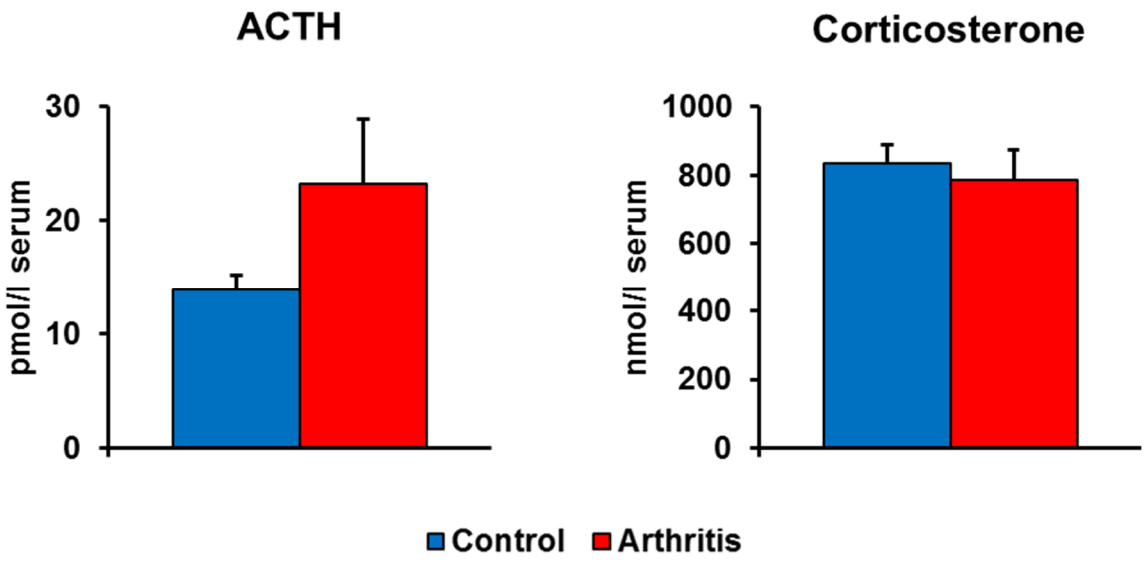

Figure 15: Comparison of serum hormone levels between chronic arthritis mice and their controls.

Adrenocorticotropic hormone (ACTH) and corticosterone remained on similar levels between the arthritic mice and their controls.

5.4 The acute arthritis mouse model

Different mouse models of RA differ in the pathophysiology and the duration of arthritis (Chapter 2.10). To determine whether the changes seen in the CA mice are consistent across different strains, similar experiments were performed in mice with a more acute form of arthritis and their controls. While in the CA model, animals had arthritis for approximately 30 days, AA mice had arthritis for a comparatively short time of less than a week.

5.4.1 Clinical scoring

AA mice were clinically scored regularly between injection of the arthritis serum or saline until the time point of tissue harvesting. Mice that received arthritis serum injections reliably exhibited clinical signs of joint inflammation (Figure 16). Control mice showed unremarkable joints at all time points.

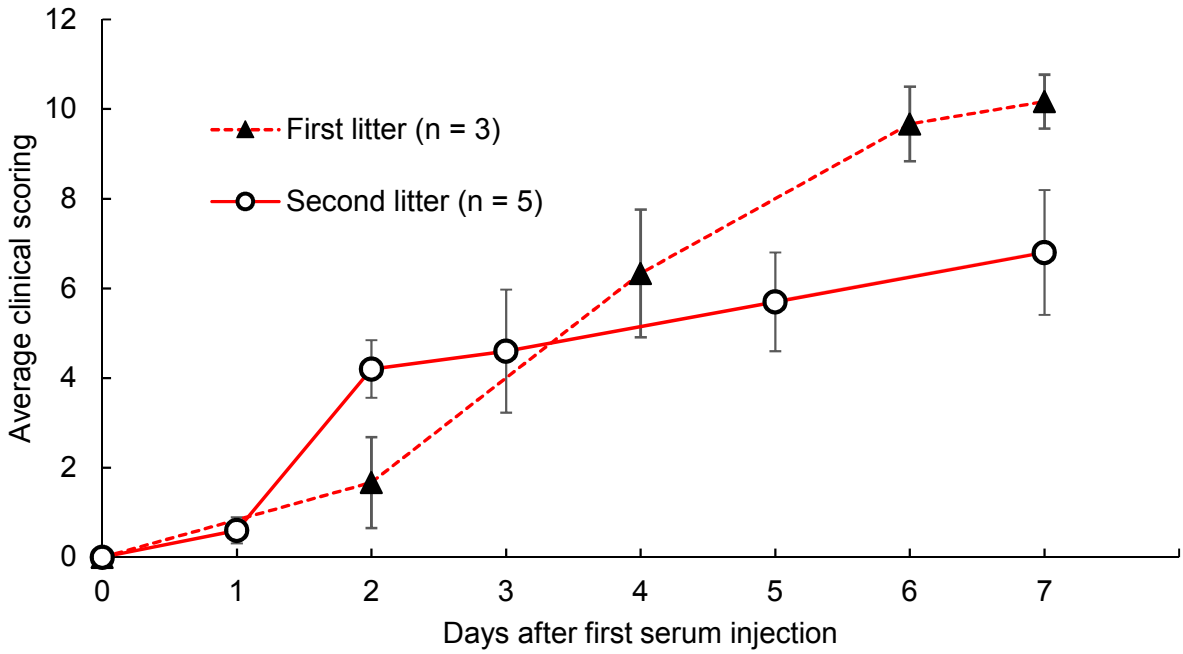

Figure 16: Mean clinical scoring of two litters of acute arthritis mice at different time points.

From the first day after the first injection, acute arthritis mice increasingly developed joint inflammation.

Clinical scores were compared between CA mice and AA mice at the time point of tissue harvest. The CA mice showed significantly higher scores than the AA mice (Figure 17).

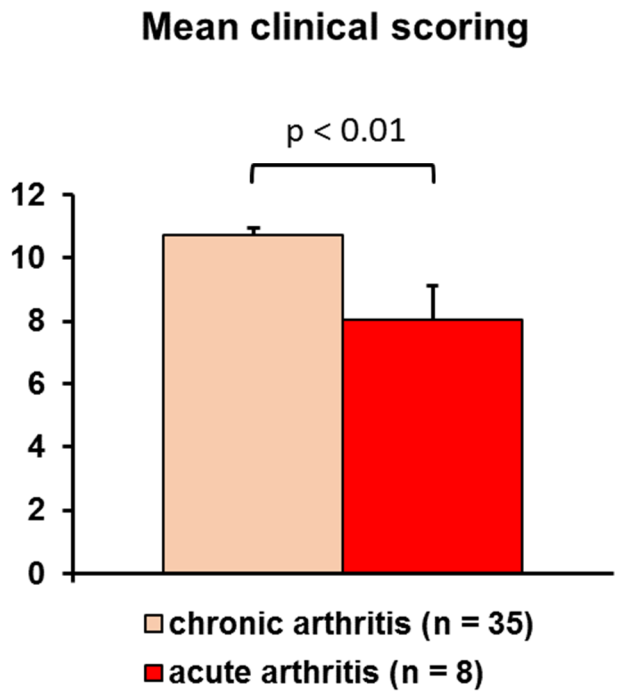

Figure 17: Comparison of clinical arthritis scores between chronic and acute arthritis mouse model.

Chronic arthritis mice showed an average score of 10.7 points at the time point of tissue harvest. Acute arthritis mice showed an average score of 8.1 points. This was significantly lower ($p = 0.0001$).

5.4.2 mRNA expression studies

5.4.2.1 Hypothalamus

As with the CA mice, the hypothalami of the AA mice and controls were examined for mRNA expression levels of 11β-HSD1, GR, MR, CRH and AVP (Figure 18). For all examined genes, the mRNA expression levels remained on similar levels between the arthritic mice and their controls. Unlike the CA mice, AA mice did not show downregulation of the CRH and AVP expression.

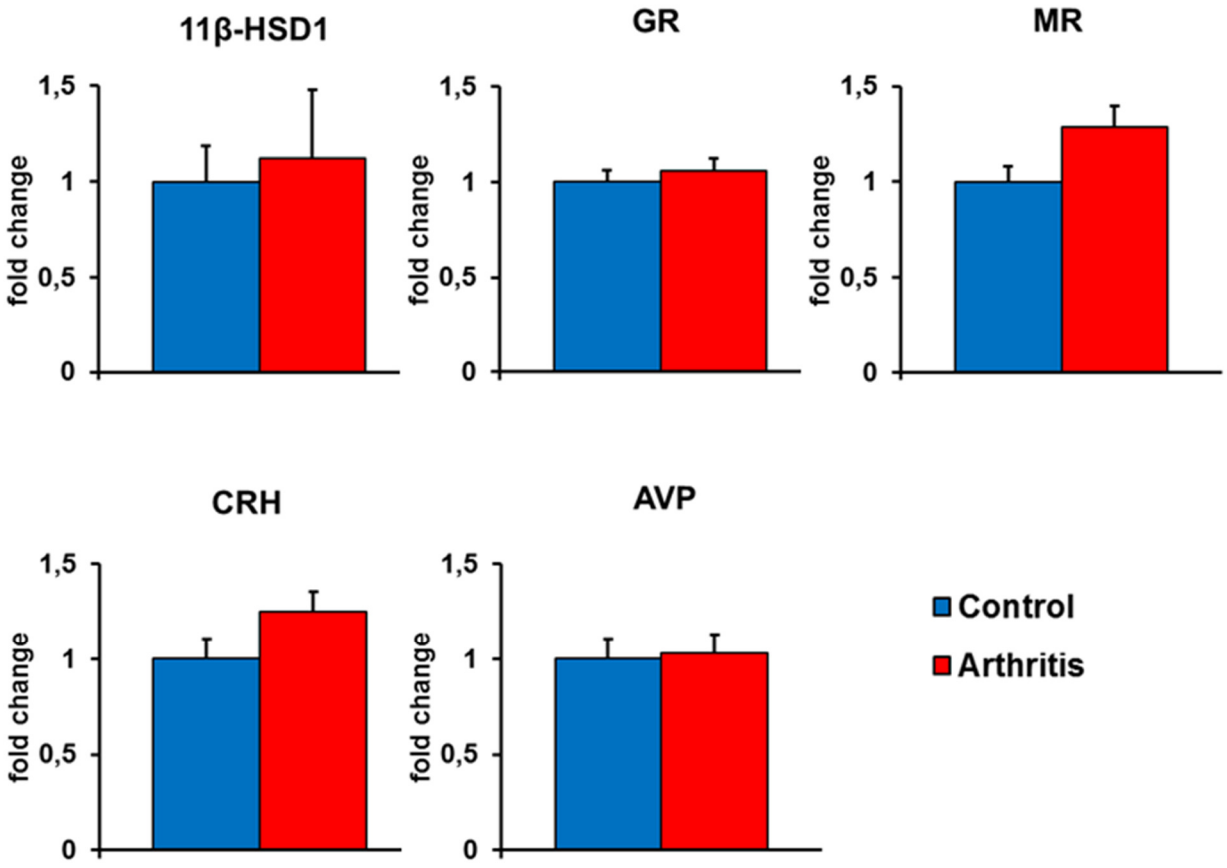

Figure 18: Comparison of mRNA expression levels in the hypothalamus between acute arthritis mice and their controls.

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP). No significant differences between the arthritic mice and their controls were observed.

5.4.2.2 Pituitary gland

PG samples of the AA mice and controls were examined for the mRNA expression levels of 11β-HSD1, GR, MR, CRHR1 and POMC (Figure 19). In line with the findings of the CA mouse model, AA mice showed an increase of 11β-HSD1 expression in the PG compared to their controls. In contrast to the CA mice, AA mice showed an upregulation of GR expression, whereas MR levels remained on a level similar to their controls (CA mice showed an upregulation of MR and no change in GR expression). CRHR1 and POMC expression was not increased in the AA mice as opposed to the CA mice.

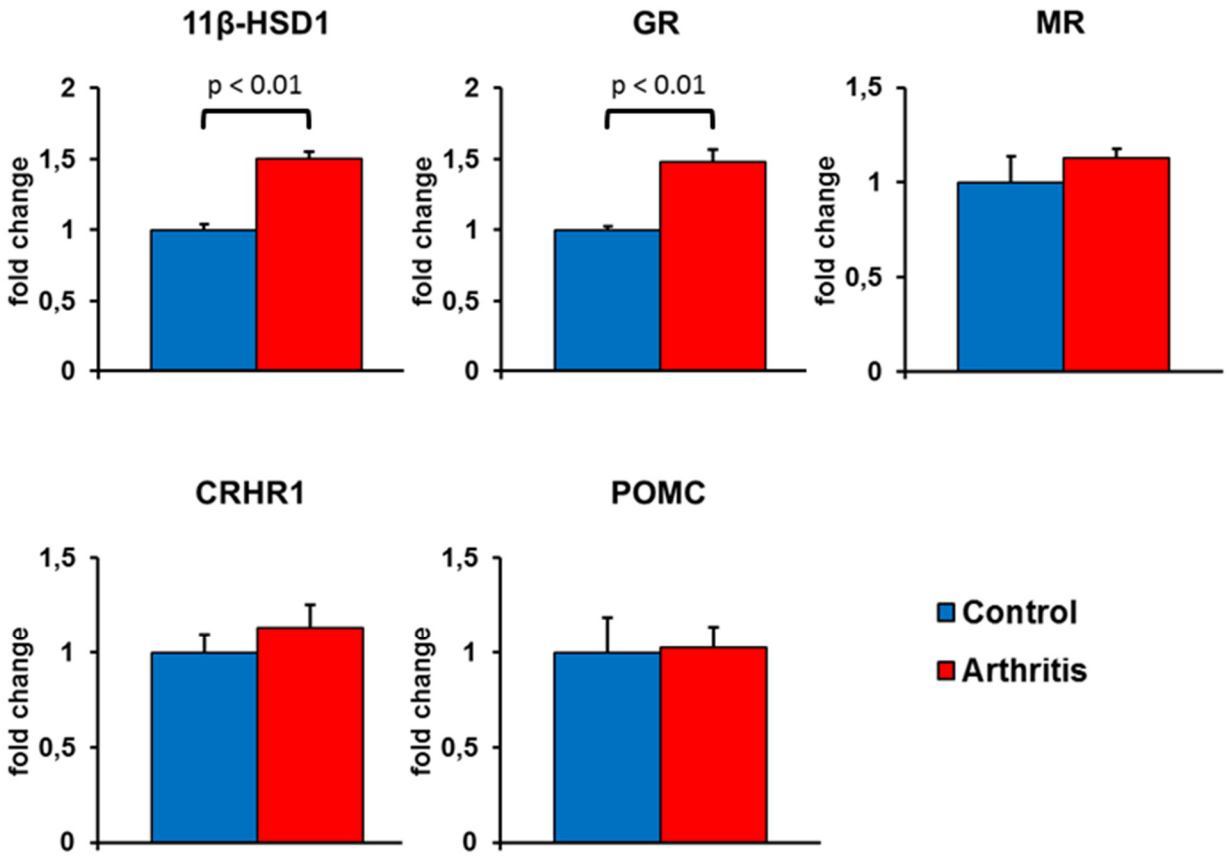

Figure 19: Comparison of mRNA expression levels in the pituitary between acute arthritis mice and their controls.

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and glucocorticoid receptor (GR) were upregulated in the arthritic mice by 1.5-fold ($p = 0.0004$) and 1.48-fold ($p = 0.003$) respectively. Mineralocorticoid receptor (MR), corticotropin-releasing hormone receptor 1 (CRHR1) and proopiomelanocortin (POMC) expression did not change significantly.

5.4.2.3 Hippocampus

Expression levels of 11β-HSD1, CR and MR in the hippocampus were compared between AA mice and their controls (Figure 20). Whereas the receptor expression levels did not change significantly, 11β-HSD1 expression was downregulated in the AA group. This is in contrast to the CA mice, where hippocampal 11β-HSD1 levels did not change in the arthritic animals.

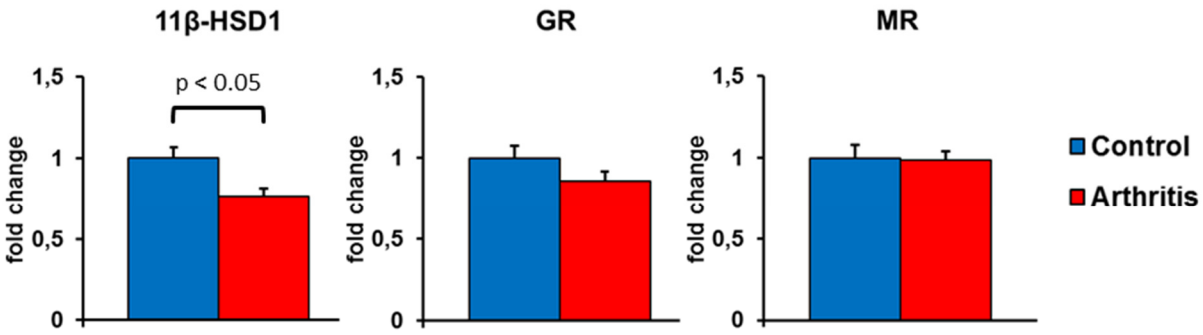

Figure 20: Comparison of mRNA expression levels in the hippocampus between acute arthritis mice and their controls.

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) expression was downregulated by 0.76-fold ($p = 0.016$). Glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) expression did not change significantly.

5.4.3 Hormone assays

As with the CA mice, serum levels of ACTH and corticosterone were determined in the AA mice at the time point of tissue harvest (Figure 21). The control group showed a mean concentration of 8.2 pmol/l ACTH in the serum. The arthritic mice showed average ACTH levels of 10.5 pmol/l and were therefore not significantly different from the controls. This result parallels the finding of unchanged POMC mRNA expression levels in the PG of the AA mice. The AA control group exhibited elevated levels of serum corticosterone compared to physiological corticosterone levels of C57BL/6 mice described before (Garthwaite, Martinson et al. 1980), namely 846.3 nmol/l. In the AA mice, corticosterone blood levels were not raised compared to the control levels, despite acute inflammation. With a mean of 828.7 nmol/l, they stayed on a similar level.

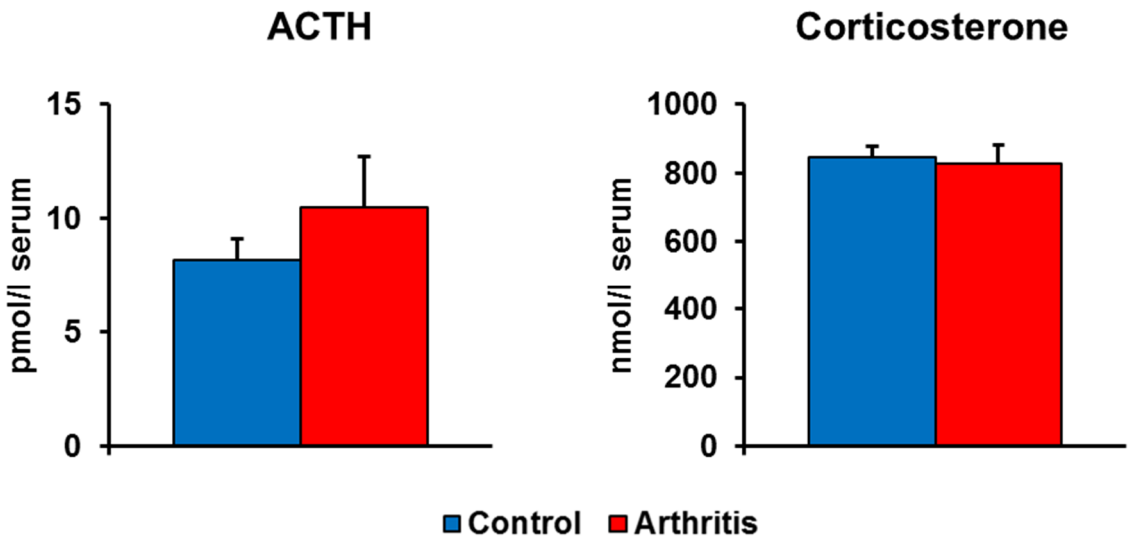

Figure 21: Comparison of serum hormone levels between acute arthritis mice and their controls.

Adrenocorticotropic hormone (ACTH) and corticosterone remained on similar levels in both groups.

5.5 Overview

Table 2 gives an overview of the results of the different experiments conducted in this study.

Measure		AA	CA
mRNA levels	***hippocampus***		
	11β-HSD1	↓	↔
	GR	↔	↔
	MR	↔	↔
	hypothalamus		
	11β-HSD1	↔	↔
	GR	↔	↑
	MR	↔	↔
	CRH	↔	↓
	AVP	↔	↓
	pituitary		
	11β-HSD1	↑	↑
	GR	↑	↔
	MR	↔	↑
	CRHR1	↔	↑
	POMC	↔	↑
serum hormone levels	ACTH	↔	↔
	Corticosterone	↔	↔
immunohistochemistry of the PVN	11β-HSD1	n.a.	↔
	GR	n.a.	↔

Table 2: Overview of findings on arthritic mice compared to their control littermates.

AA = acute arthritis, CA = chronic arthritis, 11β-HSD1 = 11β-hydroxysteroid dehydrogenase type 1, GR = glucocorticoid receptor, MR = mineralocorticoid receptor, CRH = corticotropin-releasing hormone, AVP = arginine vasopressin, CRHR1 = corticotropin-releasing hormone receptor 1, POMC = proopiomelanocortin, ACTH = adrenocorticotropic hormone, PVN = paraventricular nucleus, ↑ = increased, ↓ = decreased, ↔ = no significant change, n.a. = not available.

6 Discussion

This study shows the presence of 11β-HSD1 mRNA expression in the PG and upregulation of the latter in two animal models of immune-mediated arthritis. 11β-HSD1 mRNA expression levels in the other examined parts of the CNS did not change or, in the case of the hippocampus in the AA mice, was decreased in arthritic animals. These findings were not paralleled by any changes in ACTH or corticosterone serum levels. The implications of these findings in the context of the other observations will be discussed here.

6.1 Relative 11β-HSD1 expression in different parts of the CNS

This study reports 11β-HSD1 mRNA expression in murine PG, hypothalamus, hippocampus, cerebral cortex and cerebellum with varying expression levels. This is, to my knowledge, the first time that 11β-HSD1 mRNA expression in the PG of adult mice has been described. Before, its presence has been reported in the PG of rats (Moisan, Seckl et al. 1990, Lakshmi, Sakai et al. 1991), sheep (Yang, Matthews et al. 1995), humans (Korbonits, Bujalska et al. 2001) and mouse embryos (Diaz, Brown et al. 1998). The relative expression of 11β-HSD1 in the PG was by far higher than in the other CNS tissues examined, followed in descending order by hippocampus, cerebral cortex, cerebellum and hypothalamus (Figure 10). This is partly in agreement with studies on the rat brain (Moisan, Seckl et al. 1990), where higher expression levels of 11β-HSD1 mRNA were found in the PG relative to the hypothalamus. But in that study, the expression level of the PG was reported to be lower than in the hippocampus, cortex and cerebellum (Moisan, Seckl et al. 1990). However, later findings of that group showed highest dehydrogenase and reductase levels in the PG compared to other brain parts, indicating a prominent role of 11β-HSD1 there (Lakshmi, Sakai et al. 1991). The same was observed in the sheep CNS, with PG showing the highest dehydrogenase activity amongst the examined CNS tissues (Kim, Wood et al. 1995). In conclusion, the mouse models used in this study showed expression of 11β-HSD1 in all examined CNS parts. Moreover, the remarkably high expression in the PG could indicate a more prominent role of 11β-HSD1 there than assumed previously. As the role of 11β-HSD1 in

the PG has not been in the focus of research so far, these findings have to be evaluated further.

6.2 The pituitary gland

6.2.1 11β-HSD1 upregulation in the pituitary

11β-HSD1 mRNA was upregulated in the PG in both examined mouse models of arthritis. This is, to my knowledge, the first time that PG 11β-HSD1 mRNA expression has been studied under inflammatory conditions.

In many tissues, inflammatory stimuli cause an upregulation of 11β-HSD1 mRNA expression and trigger an increase in the enzyme reductase activity (Chapman, Coutinho et al. 2009). In line with that, an upregulation of 11β-HSD1 mRNA expression in the PG of the arthritic mice could be observed in both models examined in this study. The other CNS regions examined did not show such an upregulation. It is possible that these tissue-specific differences derive from the restricted access of proinflammatory cytokines to the brain. Proinflammatory cytokines are a trigger for the 11β-HSD1 upregulation in other tissues (Cooper, Bujalska et al. 2001). However, cytokines' access to CNS tissues is hampered by the blood-brain barrier. As the PG is not shielded by the blood-brain barrier, it might be concluded that proinflammatory cytokines exert their stimulatory effect on 11β-HSD1 expression mainly in this part of the CNS.

Studies that looked at both the mRNA expression of 11β-HSD1 and 11β-HSD1 enzyme activity, consistently reported a positive correlation (Cooper, Bujalska et al. 2001, Hardy, Filer et al. 2006). In particular, the upregulation of 11β-HSD1 expression in the rat PG has been associated with an increased reductase activity (Hanafusa, Mune et al. 2002). Therefore, the elevated 11β-HSD1 mRNA expression levels in the PG, as observed in this study, strongly suggest increased 11β-HSD1 reductase activity levels and thus elevated corticosterone levels in the PG.

The finding of an upregulated 11β-HSD1 mRNA expression in the PG is paralleled by upregulated GR expression in the AA but not CA mice. Upregulation of GR has been observed before in untreated RA patients in some tissues and proposed to be involved in the development of RA (Eggert, Klüter et al. 2002, Neeck, Klüter et al. 2002). These

observed changes of 11β-HSD1 and GR indicate increased GC action in the PG during arthritis and the implications are discussed below.

6.2.2 Negative glucocorticoid feedback on the PG

There has been some debate whether the GC-negative feedback is mediated only via the hypothalamus or as to what extend there is also a direct inhibitory effect of GCs on the PG (Keller-Wood and Dallman 1984). Dallman et al. showed in their rat model that neuronal disconnection of the hypothalamus and the PG leads to an absence of the response to adrenalectomy and GC treatment (Dallman, Makara et al. 1985). This and similar studies indicated, that negative GC feedback primarily affects the hypothalamus or other brain regions. Other experiments, in contrast, showed a direct effect of GCs on the PG. POMC transcription activity decreases significantly when anterior PG cell cultures were treated with the potent GC dexamethasone (Eberwine, Jonassen et al. 1987) and, to a lesser extent, with cortisol (Nakamura, Nakanishi et al. 1978). Furthermore, ACTH production was attenuated by addition of dexamethasone to the cells (Roberts, Budarf et al. 1979). Apart from cell culture experiments, Miller et al. showed, that besides the hypothalamus and the hippocampus, corticosterone also binds to the GC receptors in the PG (Miller, Spencer et al. 1992). By looking at the expression of transcription factors in the PG of rats during restraint stress and treatment with a GR agonist, another study concluded that the regulation of POMC happens, at least in part, independently of hypothalamic secretagogues (Ginsberg, Frank et al. 2006). And indeed, mice with GR KO in the PG showed excessive GC levels in their postnatal period and impaired HPA axis activity in adulthood (Schmidt, Sterlemann et al. 2009). These studies suggest direct GC feedback on the PG, particularly in adult animals. Moreover, the genetic points of action, by which GC exert negative feedback on POMC expression in the PG, have been delineated subsequently as reviewed before (Jenks 2009).

In conclusion, it is highly likely, that negative feedback also takes place directly at the level of the PG. It can therefore be assumed that an increased level of active GCs in the PG causes attenuation of HPA axis response.

6.2.3 Function of 11β-HSD1 in the PG

Elevated 11β-HSD1 expression at a feedback site of the HPA axis, as seen in this study, could indicate a suppression of HPA axis activity by increased GC feedback. But is there evidence for this mechanism of action? At least a correlation between an increased 11β-HSD1 activity in the anterior PG and a decreased ACTH and corticosterone response to restraint stress in pregnant rats was reported (Johnstone, Wigger et al. 2000).

Further support for this hypothesis comes from animal models with disrupted 11β-HSD1 activity. In 11β-HSD1 KO mice, the overall production of corticosterone and the nadir levels were elevated (Harris, Kotelevtsev et al. 2001). Furthermore, ACTH and corticosterone responses to restraint stress were increased in KO mice and pretreatment with corticosterone before stress exposure was significantly less effective in inhibiting corticosterone response. The observation of low hippocampal tissue cortisol levels in these mice (Yau, Noble et al. 2001) stressed the crucial role of 11β-HSD1 activity in the brain in initiating these changes. Therefore, Harris et al. provided a conclusive line of evidence for a pivotal role of 11β-HSD1 in the regulation of GC-negative feedback on the HPA axis.

There seems to be variability between different animal strains in their HPA axis response to 11β-HSD1 KO. The described increase of ACTH and corticosterone serum levels was not seen in some other strains (Carter, Paterson et al. 2009, Sooy, Webster et al. 2010, Abrahams, Semjonous et al. 2012). Some strains seem to overcome the 11β-HSD1 KO by an increased GR expression in the hippocampus and hypothalamus (Carter, Paterson et al. 2009), thus restoring GC feedback sensitivity. However, these mice still exhibited increased adrenal gland size, as a sign of increased central HPA axis drive by ACTH (Carter, Paterson et al. 2009, Abrahams, Semjonous et al. 2012). This raises the question if the HPA axis abnormalities in 11β-HSD1 KO mice happen due to changes of GC feedback via 11β-HSD1 in central HPA axis parts or due to the lack of peripheral corticosterone rescue by 11β-HSD1 with a decrease of circulating GCs and hence decreased negative GC feedback.

Possibly both factors contribute to the HPA axis dysregulation in 11β-HSD1 KO mice. As GC production via 11β-HSD1 in the splanchnic bed substantially contributes to

serum levels of GCs, at least in humans (Basu, Singh et al. 2004, Andrew, Westerbacka et al. 2005), these peripherally produced GCs most certainly have an effect on the HPA axis regulation. And transgenic 11β-HSD1 KO mice, that have selective overexpression of 11β-HSD1 only in the liver, showed a complete reversal of the changes seen in 11β-HSD1 global KO mice, with all parameters of HPA axis activity back in physiological range (Paterson, Holmes et al. 2007). On the other hand, serum corticosterone levels remained unchanged in liver-specific overexpression (Paterson, Morton et al. 2004) and liver-specific deletion of 11β-HSD1 (Lavery, Zielinska et al. 2012). These two studies again relativize the influence of peripheral corticosterone rescue via 11β-HSD1 on the HPA axis.

It can be concluded that despite the not yet ultimately solved question of the origin of HPA axis alterations in 11β-HSD1 KO mice, there is evidence towards an important regulatory role of central 11β-HSD1 on the HPA axis. Together with the findings from this project, this supports the hypothesis that the failure of HPA axis upregulation originates in increased GC-negative feedback due to 11β-HSD1 overexpression in the PG of arthritic mice.

6.2.4 11β-HSD1 and other pituitary hormones

According to a study of Korbonits et al., 11β-HSD1 is not colocalized with ACTH producing cells in the PG, at least in humans. Instead, it is expressed in PG cells that produce growth hormone (GH) and prolactin (Korbonits, Bujalska et al. 2001). This might lead to the assumption that 11β-HSD1 mRNA expression changes are not related to changes in the HPA axis response, but rather affect the production of other hormones. A study about the circadian rhythm of PG hormones in rats with adjuvant arthritis revealed a downregulation of GH mRNA expression during the course of the disease, whereas prolactin expression remained unchanged (Roman, Seres et al. 2003). Accordingly, a blunted GH response has been observed in patients with RA (Templ, Koeller et al. 1996). Whether these findings are related to changes in PG 11β-HSD1 expression or activity has not been examined in these studies and would be an interesting point to follow up.

On the other hand, it has been hypothesized that the effect of local GC production can influence the surrounding cells in a paracrine manner as well, (Davies and MacKenzie 2003). This is possible because of the lipophilic structure of physiologic GCs, granting them access to adjacent cells. Therefore, despite the observation that 11β-HSD1 might not be expressed in ACTH producing cells, the increased levels of 11β-HSD1 in the PG could still affect HPA axis feedback.

6.2.5 Pituitary POMC

In the CA mouse model, POMC mRNA expression was upregulated in the PG whereas the expression remained unchanged in the AA mice. Even though the present study examined POMC mRNA expression changes in the PG as a whole, previous studies have shown that POMC expression upregulation, that is regularly reported in arthritic rats, only happens in the anterior part of the PG, whereas the intermediate and posterior parts remain unchanged (Höllt, Przewłocki et al. 1986, Millan, Millan et al. 1986, Harbuz, Rees et al. 1992). As POMC is mainly expressed by ACTH-producing cells in the anterior lobe, this increase has been attributed to an increased HPA axis activity (Harbuz, Conde et al. 1997). However, results about the relationship between PG POMC expression and plasma ACTH and corticosterone levels during the course of arthritis remain inconclusive. While in one study the increased POMC mRNA expression in the anterior PG of adjuvant arthritis rats was associated with a decrease of ACTH plasma levels (Harbuz, Rees et al. 1993), another study showed elevated ACTH and corticosterone levels in susceptible streptococcal cell wall arthritis rat strains (Sternberg, Hill et al. 1989), and a third study on adjuvant arthritis rats showed elevated ACTH and corticosterone plasma levels in the morning while remaining unchanged in the evening (Sarlis, Chowdrey et al. 1992). In the study presented here, the increase of PG POMC mRNA expression in CA mice is not associated with any change of serum ACTH or corticosterone levels. These reported discrepancies raise the question how POMC mRNA expression in the PG can be upregulated without being clearly and reproducibly associated with an increase of ACTH and corticosterone levels. The problem of single time point studies on the examination of hormones that are secreted in a pulsatile manner will be discussed in Chapter 6.3. The relationship between POMC mRNA, ACTH and corticosterone levels is further complicated by the fact that PG POMC mRNA

expression is subject to diurnal changes too, and this pattern changes in arthritic animals (Šereš, Herichová et al. 2004). From this perspective, the increased POMC expression in CA mice does not necessarily have to correspond with high ACTH and corticosterone hormone levels.

In line with previous data (Lightman and Harbuz 1993), the observed POMC upregulation in the PG is not associated with an increased expression of CRH and AVP in the hypothalamus. The trigger for this observed upregulation must come from somewhere else. POMC expression is regulated not only by hypothalamic secretagogues, but also directly by proinflammatory cytokines from the blood circulation (Suda, Tozawa et al. 1990). Most of these cytokines exert a stimulating effect on the HPA axis (Turnbull and Rivier 1999). As circulating proinflammatory cytokines are abundant in arthritic subjects (Feldmann, Brennan et al. 1996), it is possible that the POMC mRNA upregulation seen in the CA mice is not a sign of hypothalamic HPA axis drive, but a direct upregulatory effect of proinflammatory cytokines. For the AA mice, the time of proinflammatory cytokine exposure seems to be too short to result in an upregulation of POMC mRNA.

6.2.6 mRNA upregulation in the PG of chronic arthritis mice

Four out of five genes, that were examined for mRNA expression in the PG, were elevated to a similar extend in the CA mice. These findings raised the question of a normalization error in the qPCR experiment, for example because of changes in the reference gene GAPDH during the experiment. However, the reference gene was tested with the GeNorm software for stable expression levels during the experiment (Chapter 4.4.5). Furthermore, by comparing the average qPCR cycle numbers of GAPDH in the arthritic mice with the controls, it was observed that they were even higher in the arthritic mice. As these higher cycle numbers for the reference gene would usually lead to rather lower relative expression values of the examined gene, this indicates that the changes nevertheless seen in the PG mRNA expression levels are likely to be real and not due to a normalization error.

6.3 Serum hormone levels

Serum ACTH and corticosterone levels did not change significantly between arthritic mice and controls in any of the groups. This has been expected, as the hypothesis of this project originates in the well documented failure of GC levels to rise in RA. It is also in agreement with previous animal studies that showed no change in ACTH or GC levels in arthritic K/BxN mice compared to their controls (Buttgereit, Zhou et al. 2009). As discussed before, a possible explanation for this phenomenon is the increased 11β-HSD1 expression in the PG and hence the increased negative feedback on the HPA axis activity, counteracting stimulatory effects of proinflammatory cytokines.

A remarkable finding of this study was that the serum levels of corticosterone were relatively high compared to previously reported data of K/BxN mice and their controls (Buttgereit, Zhou et al. 2009) as well as for K/BxN serum transfer arthritis C57BL/6 mice (AA) (Coutinho, Gray et al. 2012) and their controls (Garthwaite, Martinson et al. 1980). Furthermore, the CA mice showed increased levels of ACTH in the controls as well as the arthritic mice compared to previous studies (Buttgereit, Zhou et al. 2009). These unexpected high levels of serum hormones make the interpretation of any changes between the groups more challenging. They could be related to the anesthetic used before blood taking. Several studies have shown that the administration of Ketamine for anesthesia can alter GC levels, at least in some species (Bentson, Capitanio et al. 2003, Saha, Xia et al. 2005). In rats, usage of pentobarbital as anesthesia resulted in a strong increase of both ACTH and corticosterone plasma levels compared to controls that were decapitated without anesthesia (Vahl, Ulrich-Lai et al. 2005). Stress of the animals at the time point of tissue harvest can further contribute to the increased hormone levels, as mice were moved from the animal unit to the laboratory well before samples were acquired. This left enough time for the mice to produce additional ACTH and corticosterone as a reaction to the new environment and animal handling (Spencer and Deak 2016). Therefore, care should be exercised when drawing conclusions about the absence of changes in the serum levels, as these changes might be concealed by an overshooting GC response to the anesthetic and/or other stress.

Generally, single time point serum levels of ACTH and GCs have to be handled with care as these hormones are secreted in a diurnal and pulsatile manner (Spiga, Walker et al. 2014). Influences from the diurnal changes were minimized by restricting the

sample collection to a certain time frame during the day, representing the late nadir of corticosterone production. Additionally to the circadian rhythm, ultradian secretion pulses were reported in rats with an interval of about three peaks per hour for ACTH (Carnes, Lent et al. 1989) and about one peak per hour for corticosterone (Jasper and Engeland 1991). Furthermore, the pulsatile secretion pattern of corticosterone changes in rats with adjuvant arthritis (Windle, Wood et al. 2001). As within these peaks, the serum hormone levels increase by a multiple of the baseline values, single time point measures can only be an approximation. It has therefore been proposed to use plasma POMC, which does not follow pulsatile secretion and is not responsive to acute stress as a marker for HPA axis activity (Harno and White 2010). As a follow-up to this study, this would be an interesting next step for a better, perhaps more reliable insight into HPA axis activity.

In conclusion, changes in the serum hormone levels seem to be hard to pin down with the limitation of single time point measurements. It is therefore necessary to put the findings into context with observations from the other experiments, like mRNA expression changes of hormones that control the activity of the HPA axis.

6.4 The hypothalamus

6.4.1 11β-HSD1 in the hypothalamus

In the hypothalamus, no significant changes of 11β-HSD1 mRNA were seen between arthritic mice and their controls in either the CA or the AA mouse model. For the CA mice, this was confirmed for 11β-HSD1 protein levels in the PVN by IHC. Compared to other brain parts and particularly the PG, the expression of 11β-HSD1 was quite low in the hypothalamus (Figure 10). There are two possible explanations for this finding: 1) 11β-HSD1 expression is concentrated in specific nuclei that do not contribute substantially to the overall tissue volume. 2) 11β-HSD1 only plays a subordinate role in the regulation of the hypothalamus.

Possibility 1) is supported by a study on the 11β-HSD1 expression in different parts of the human hypothalamus, which showed specific regional differences in the expression levels (Bisschop, Dekker et al. 2013). Furthermore, the distribution of 11β-HSD1 as

seen by IHC in this study showed differences between the PVN and the surrounding tissue. However, these differences did not seem to be big enough to explain the overall very low expression reported here.

The dissection technique used in this study did not allow for selective dissection of the PVN, and even the borders of the hypothalamus are only an approximation by macroscopic landmarks and could not be reliably discerned. This is reflected by rather variable hypothalamic sample weights in this study as compared to quite stable weights of the other CNS tissue samples. A contamination of surrounding brain tissue with low expression levels of 11β-HSD1 could contribute to the low overall expression levels in the hypothalamus.

Possibility 2), the subordinate role of 11β-HSD1 in the regulation of the hypothalamus, is supported by the findings of relatively low hypothalamic 11β-HSD1 levels in this and previous studies (Moisan, Seckl et al. 1990). On the other hand, it is contradicted by a study on rats, that showed that direct injection of glycyrrhetinic acid, a powerful inhibitor of the 11β-HSD enzymes, into the PVN results in downregulation of the HPA axis, as seen by decreased release of CRH into the hypothalamic portal vein system (Seckl, Dow et al. 1993). However, as glycyrrhetinic acid inhibits 11β-HSD2 more strongly than 11β-HSD1, and 11β-HSD2 is expressed in the rat PVN too (Zhang, Kang et al. 2006), it is not clear which enzyme subgroup is responsible for the observed HPA axis downregulation. Therefore, the role of 11β-HSD1 in the hypothalamus regulation is yet to be specified.

No changes could be seen in 11β-HSD1 mRNA expression in the hypothalamus between the CA mice and their controls. It might be questioned if with the rather low expression levels, possible changes could be detected at all with the technique of qPCR. That is why IHC was used to back up the findings from the mRNA expression studies. In the IHC samples, a clear staining could be seen in most of the cells in the PVN. Corresponding with the mRNA expression studies, there were also no changes between arthritic and control mice seen by IHC. These findings are in line with results published in an abstract before, according to which hypothalamic 11β-HSD1 mRNA expression did not significantly change in K/BxN mice compared to their controls (Verma, Zhang et al. 2014).

Taken together the low overall expression of 11β-HSD1 in the hypothalamus and the nonresponsiveness of 11β-HSD1 mRNA expression to arthritis, 11β-HSD1 in the hypothalamus does not seem to contribute crucially to the failure of HPA axis upregulation in arthritis, at least in the animal models examined here. This study rather indicates a role of 11β-HSD1 in the PG as a regulator of HPA axis activity, as has been discussed before.

6.4.2 GR in the hypothalamus

The importance of GR in modulating HPA axis activity has been described in animal studies with GR KO. Global GR KO in mice results in strongly increased plasma levels of ACTH and corticosterone (Cole, Blendy et al. 1995). This seems to be at least in part due to changes in the CNS, as GR KO in neurons and glia cells of mice results in markedly increased CRH mRNA levels in the PVN and plasma corticosterone levels (Tronche, Kellendonk et al. 1999). This presumably happens because of absence of GC-negative feedback. In the present study, GR mRNA expression in the hypothalamus was unchanged in the AA model and increased significantly but only to a small extend in the CA mouse model. On the protein level, we could not detect any changes of GR in the PVN of CA mice. This is in contrast to what Harbuz et al. have reported before, which is a decrease of GR mRNA expression in the PVN under adjuvant arthritis (Harbuz, Conde et al. 1997). It is hard to say where the discrepancies of these two findings originate from. In the study by Harbuz and colleagues, the PVN was examined specifically, while in the present study, samples of the whole hypothalamus were used. Also, with adjuvant-induced arthritis rats, a different animal model was used. In the present study, the findings of substantially unchanged GR mRNA levels in the hypothalamus are backed up by IHC, and are consistent with unchanged levels of MR and 11β-HSD1 mRNA expression, indicating an overall nonresponsiveness of hypothalamic GC metabolism to arthritis in these mouse models.

6.4.3 CRH and AVP in the hypothalamus

Possibly unrelated to the unchanged 11β-HSD1 expression (Chapter 6.4.1), a decrease of CRH and AVP mRNA expression in the hypothalamus was observed In the CA mice.

The paradoxical effect of a decreased CRH mRNA expression in the hypothalamus during arthritis has been reported before for some strains (Harbuz, Rees et al. 1992, Windle, Wood et al. 2001), and was associated with a decrease of CRH release into the portal blood (Harbuz, Rees et al. 1992). Even though the reason for the downregulation of hypothalamic CRH in chronic arthritis has not been definitely identified, an inhibitory effect of substance P has been proposed (Chowdrey, Larsen et al. 1995). The absence of a CRH upregulatory response has been linked to the susceptibility of strains to the induction of arthritis (Sternberg, Young et al. 1989). A later study revealed a biphasic regime of hypothalamic CRH expression changes, with mRNA levels being elevated 7 days after adjuvant arthritis induction and a decrease at day 14 after induction (Aguilera, Jessop et al. 1997). It is therefore possible that the harvesting time of AA mice in this study fell between the two periods, and that is why they did not exhibit CRH mRNA expression changes, whereas the decrease of CRH mRNA expression could be seen in the hypothalamus of the CA mice.

AVP has been proposed to be a main stimulator of the PG in chronic inflammation, taking over the role of CRH in controlling HPA axis activity (Harbuz, Conde et al. 1997). This was a result of the findings that AVP mRNA in the PVN is upregulated in chronic arthritis and systemic and portal plasma AVP levels are increased (Chowdrey, Larsen et al. 1995, Suzuki, Onaka et al. 2009). However, the present study did not show upregulation of AVP mRNA expression in the hypothalamus. Instead, in the AA mice, the expression remained at control levels, whereas in the CA mice, AVP mRNA was downregulated in the hypothalamus along with CRH mRNA. Where do the differences between the findings of these studies come from?

The different species type and strain used in the studies might have contributed to the observed differences. In a recently published paper, differences in the changes of hypothalamic cytokine constellation were described between rat strains in response to adjuvant arthritis (Bodnar, Taves et al. 2017). This was attributed by the authors to the differential HPA axis response of the animals to adjuvant-induced arthritis.

Compared to the studies reported before, the exposure time to arthritis and the arthritis inducing agent of the animals was different. The CA mice in this study spontaneously developed arthritis and showed clinical symptoms for about 30 days, as opposed to rats with adjuvant-induced arthritis that exhibited clinical symptoms for 4 and 12 days

respectively. The exposure time of AA mice to arthritis was similar to that of the adjuvant-induced arthritis rats, but a different induction detergent for arthritis stimulation was used.

Furthermore, the examined area varied between the studies. Whereas Chowdrey et al. looked specifically at the medial parvocellular division of the PVN and Suzuki et al. at the whole PVN, this study was examining the hypothalamus as a whole. Therefore, influences on the AVP mRNA expression by other structures could not be excluded.

Finally, different diurnal timepoints for the tissue harvest were used. While in the preceding studies, tissues were collected at 8:00 to 10:00 o'clock AM and "in the morning" respectively, in the present study the tissues were collected at noon time. This corresponds to the peak of corticosterone production on one side and the nadir of the latter on the other side. As mRNA expression can be subject to diurnal rhythmicity, this might have an impact on the findings, in particular as this diurnal rhythmicity can change in arthritis (Holmes, French et al. 1995).

In the CA mice, PG POMC mRNA is upregulated despite the downregulation of CRH and AVP as hypothalamic triggers. As this upregulation happens despite the absence of an increased hypothalamic drive, this is likely to be driven by other factors, like proinflammatory cytokines. This possibility was discussed in Chapter 6.2.5.

6.5 The hippocampus

11β-HSD1 mRNA expression in the hippocampus decreased significantly in AA mice but remained unchanged in CA mice. This decrease has been reported in K/BxN serum-induced arthritis mice 15 days after induction (Verma, Zhang et al. 2014), which contradicted an older report of minimally increased hippocampal 11β-HSD1 activity in adjuvant arthritis rats (Low, Moisan et al. 1994). Even though the hippocampus is generally protected by the blood-brain barrier, the presence of selective transport system for proinflammatory cytokines has been discussed before (Banks, Kastin et al. 1995). Furthermore, blood-brain barrier integrity can be disrupted in inflammation (Varatharaj and Galea 2017) and a mild effect of adjuvant arthritis on hippocampal cytokine levels has been described (Bodnar, Taves et al. 2017). It is therefore possible,

that proinflammatory cytokines can influence 11β-HSD1 expression in the hippocampus and that they are responsible for the downregulation seen in this study.

The hippocampus is known to participate in the regulation of the HPA axis (Jacobson and Sapolsky 1991). Most of the evidence supports an inhibitory effect of the hippocampus on HPA axis activity. High GR and MR occupancy in the hippocampus is associated with a decrease of ACTH secretagogues in the PVN (Sapolsky, Armanini et al. 1990). The hippocampus itself is under negative feedback control of GCs, and tissue levels of GCs are strongly determined by 11β-HSD1 expression levels in the hippocampus (Yau, Noble et al. 2001). As 11β-HSD1 seems to work mainly as a reductase in the hippocampus (Rajan, Edwards et al. 1996, Ajilore and Sapolsky 1999, Yau, Noble et al. 2001), a decrease of 11β-HSD1 mRNA expression, as seen in this study, would be expected to result in decreased GC levels in the hippocampus, with the consequence of a disinhibition of the HPA axis. In fact, this phenomenon has been observed in a study on obese rats, where downregulation of 11β-HSD1 mRNA in parts of the hippocampus was associated with an increased corticosterone response to stress (Mattsson, Lai et al. 2003). On the other hand, basal corticosterone levels remained unchanged when 11β-HSD1 was overexpressed in the hippocampus of transgenic mice (Holmes, Carter et al. 2010).

Even though the study presented here showed an upregulation of POMC mRNA in the PG in AA mice, hypothalamic CRH and AVP mRNA levels as well as ACTH and corticosterone serum levels remained unchanged. This makes it rather unlikely that the change of 11β-HSD1 expression in the hippocampus is linked to HPA axis activity. Instead, it was proposed before that the downregulation of 11β-HSD1 expression “increases glycolysis and energy substrate (lactate) in brain” as a response to the inflammation challenge (Verma, Zhang et al. 2014). Another possible explanation is that the downregulation of 11β-HSD1 mRNA expression aims to protect the hippocampal neurons from the toxic effect of GC excess (McEwen and Gould 1990) and associated memory impairment (Holmes, Carter et al. 2010). Perhaps related to the downregulation of hippocampal 11β-HSD1 mRNA, a decrease of GR mRNA expression in the CA1 and dentate gyrus region of the hippocampus was reported in rats with adjuvant arthritis, whereas levels remain unchanged in the CA2, CA3 and CA4 subfields (Harbuz, Conde et al. 1997). At the same time, MR expression remained unchanged in all the

hippocampus regions. In the present study, however, with the limitation of examining the hippocampus as a whole, GR and MR expression levels did not change in either model of arthritis.

6.6 Future directions

The methods applied in this project were subject to inherent limitations so that additional experiments are suggested to further follow up the results.

The study design allows for interpretation on a correlation level only. Differences between the arthritic mice and their controls in the mRNA expression levels were described. However, the present study did not include mechanistic insights or cause-effect relationships. More experiments need to be conducted to reveal these relationships and hence ensure that the correlations are causatively linked.

Examination of the tissue samples was restricted to one time point, when the samples were harvested. As for the limited availability of mouse models, the time point for examination was chosen which in other studies showed highest levels of disease activity. Nevertheless, more studies can be conducted in the future examining the mice at different time points in order to depict the dynamic process of the development of immune-mediated arthritis and its effects on a time axis

In this study, no changes between arthritic mice and controls could be seen in HPA axis activity as judged by serum levels of ACTH and corticosterone. It has been described before that changes in the HPA axis during inflammation do not necessarily change baseline HPA axis activity. Moreover, often the HPA axis response to additional stress (on top of the arthritic stress) is changed (Chikanza, Petrou et al. 1992). To examine the animals under these conditions could reveal latent changes of the HPA axis.

The limits of single time point studies of serum hormone levels are discussed in Chapter 6.3. As the examined hormones are subject to diurnal rhythmicity and pulsatile secretion, it would be preferable to obtain blood samples on several time points of the day at several days during the course of the disease. Thus, a more comprehensive profile of the hormone secretion in arthritis could be created and subtle changes could be observed more reliably. However, the challenges of multi time point blood sampling

and the effect on GC secretion have been described before and tremendous efforts will be necessary to yield reliable results (Spencer and Deak 2016).

Findings on mRNA expression levels have been confirmed only for some parts and genes on the protein level by IHC. As the differences between mRNA expression levels are rather small in this study, future experiments should include a confirmation of all mRNA expression results on the protein level, where this has not been done so far, to elucidate the biological significance of the findings. Furthermore, findings on 11β-HSD1 expression and protein levels do not necessarily reflect the reductase activity of 11β-HSD1, even though a close relationship has been reported before (Ergang, Leden et al. 2010). Future examinations could include enzyme activity assays to address that problem. This is particularly important, as the enzyme directionality of 11β-HSD1 can change depending on the availability of various cofactors.

As shown in Chapters 5.3.2.2 and 5.4.2.2, 11β-HSD1 upregulation in the PG was not associated with a decrease of POMC expression in any of the mouse models. To check if there is a function of 11β-HSD1 in the PG other than regulation of the HPA axis, it would be interesting to investigate the expression changes of other hormones produced there. As colocalization of 11β-HSD1 with prolactin and GH has been described in humans before (Korbonits, Bujalska et al. 2001), the examination of these hormones would be of particular interest.

To further investigate whether GC action in the HPA axis components changes in arthritic mice, additional studies could examine the MR and GR occupancy. Even if there are changes in the GR/MR expression levels, it does not necessarily mean an increased GC action there. Further influential factors, apart from 11β-HSD1 and 11β-HSD2 activity in the cells, is the ratio between GCs bound to proteins and free GCs. GCs can only enter the cell and bind to a receptor if they are not bound to proteins. However, more than 90% of the circulating GCs are bound to proteins, mainly corticosteroid-binding globulin (CBG) (Mendel 1989). As there are high levels of CBG in the anterior PG (De Kloet, Burbach et al. 1977), and CBG production is decreased in inflammation (Savu, Zouaghi et al. 1984, Torpy and Ho 2007), it would be interesting to examine how PG MR and GR occupancy changes during arthritis with the aid of a receptor-binding assay, to get a more precise picture of GC action there.

As mentioned in hapter 5.3.3, the macroscopic dissection technique did not allow for individual nucleus dissection, for example the PVN. Indeed, even the borders of the hypothalamus were not clearly discernable. Another approach to get more detailed information about 11β-HSD1 mRNA expression in smaller areas was shown by Vodička et al (Vodicka, Ergang et al. 2014). That group cut coronal sections of rat brains with a cryostat and dissected different areas of the brain with a laser microdissection system. With that technique, mRNA expression levels of different nuclei can be examined individually. This can help to get a better spatial resolution of potential expression changes and these changes can be clearly assigned to different structures, like individual nuclei.

As this is the first study to report the upregulation of PG 11β-HSD1 in arthritic animals, it would be interesting to see if that finding remains consistent across different strains and species. In this study, the effort was already made to compare two arthritis mouse models with a different pathophysiological background. But as each arthritis model has inherent differences in the disease development, further confirmations from other strains or even species would make the findings more robust. This is especially true, as differences in the role of 11β-HSD1 in HPA axis control have been described in different strains, which can influence the susceptibility to arthritis development (Harris, Kotelevtsev et al. 2001, Carter, Paterson et al. 2009). Finally, all models of RA are only an approximation of RA in humans (Kannan, Ortmann et al. 2005). Due to ethical reasons, the studies performed here cannot be conducted with human samples. However, if results from this study are confirmed in other animal models, it will be more likely that these findings can be generalized for the pathogenesis of RA.

7 Conclusion

This is the first study to report the upregulation of PG 11β-HSD1 mRNA in two mouse models of experimental arthritis. At the same time, there were no changes in serum ACTH and corticosterone levels. The question whether the PG 11β-HSD1 upregulation is related to the known failure of HPA axis activation in chronic arthritis or whether it fulfills another function warrants further investigation. With this study, the HPA axis response to immune-mediated arthritis was further characterized. This establishes a basis for further research about the interdependence between RA and the HPA axis and provides new insights into the pathogenesis of RA.

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„Ich, Janko Sattler, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „The role of 11β-hydroxysteroid dehydrogenase type 1 in the regulation of the hypothalamic-pituitary-adrenal axis in immune-mediated arthritis“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Publications

Research articles:

Sattler J, Tu J, Stoner S, Li J, Buttgereit F, Seibel MJ, Zhou H, Cooper MS. Role of 11β-hydroxysteroid dehydrogenase type 1 in Hypothalamic-Pituitary-Adrenal axis activity during immune-mediated arthritis. Under review in Endocrine Connections.

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Oral presentation:

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