PSYCHOSOCIAL STRESS AND ADDISON'S DISEASE

A NEW APPROACH TO EVALUATE THE RELEVANCE OF ENDOCRINE STRESS RESPONSES FOR HEALTH AND DISEASE

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to Nic

to my and Nic's family

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CONTENTS

FIGURE INDEX

TABLE INDEX

1 INTRODUCTION

The field of psychoneuroimmunology (PNI) is dedicated to the investigation of stress-neuroendocrine-immune interactions. Within this field, one area has attracted considerable attention: The effects of stress on the immune system and hence on health and disease. To date, numerous studies have shown that psychosocial stress leads to an activation of the hypothalamus-pituitary-adrenal (HPA) axis resulting in an increased release of its major end product cortisol, which in turn has a wide range of effects on the immune system. Thereby, these mostly immune-suppressive effects are thought of as protecting the organism against an overactive and thus potentially harmful immune response. Thus, contrary to an endocrinological response to acute stress, HPA dysfunctions or a missing cortisol stress response due to chronic stress are associated with negative health outcomes.

Although a large number of studies have investigated HPA axis function and abnormalities in human diseases, clear-cut evidence for the involvement of glucocorticoids in predisposing individuals to the development or exacerbations of specific diseases have so far predominantly been derived from animal studies or have been deduced from *in vitro* findings. This work introduces an alternative approach, namely the investigation of patients with Addison's disease, to measure the effects of context-independently long-term altered glucocorticoid stress responses on the immune system. Studying such effects in patients with Addison's disease may provide valuable insights in the interplay of endocrine stress systems in the human whole organism, the extend to which an organism is able to compensate dysregulations in these systems, and the clinical relevance of psychoneuroendocrinological and -immunological findings for health and disease.

For this purpose, first the theoretical background of the three key components – the endocrinological stress response, Addison's disease, and stress-neuroendocrine-immune interactions – will be presented in the subsequent chapters. In the first section, the reader will find definitions of stress and different stress concepts. Also the endocrinological stress response as well as its effects on different body systems will be discussed. The second section describes in detail the unique endocrine state 'Addison's disease'. In the third section, the basic principles of psychoneuroimmunology will be discussed. The reader will learn about the components and the functions of the immune system as well as their modulation by acute and chronic stress. Also the immune effects of discrete stress mediators (i.e., glucocorticoids and catecholamines) as well as mechanisms and modulators of glucocorticoid-to-immune system signaling will be presented.

While the third chapter outlines the specific problem formulations of the present thesis, the fourth chapter is divided into four sections, each representing a special set of results of two empirical studies. Since so far no data are available on the quality of the

pharmacological substitution in Addison's disease patients, specifically the resulting concentrations of free cortisol, this basic condition was investigated first (section 4.1). Due to the same reason, in a next step the endocrinological stress response in these patients was investigated and compared with the respective responses in healthy controls (section 4.2). Based on these data, immunological consequences of a missing cortisol stress response were analyzed. To distinguish between effects due to the missing stress response and effects due to other responses or compensatory mechanisms, in half of the patients a normal cortisol stress response was mimicked by hydrocortisone injection (section 4.3). Also one intra-cellular mechanism centrally in mediating glucocorticoid effects on the immune system – and thus also potentially important in compensating processes – was investigated. These results will be presented in the fourth section (section 4.4).

In the last chapter of this thesis, the results of all studies are summarized, discussed in a broader context, and an outlook suggests promising directions for future studies employing the investigation of patients with Addison's disease.

2 THEORETICAL BACKGROUND

In this section the theoretical background for studying the effects of an absent cortisol stress response on the immune system will be introduced. This includes a synopsis of the three main topics in this regard: (1) the neuroendocrine stress response, including the development and current state of stress concepts as well as the components and functions of the endocrine stress axes, (2) a detailed description of the exceptional endocrine state 'Addison's disease', (3) the effects of stress on the immune system, introduced by a short overview of the components and functions of the immune system.

2.1 THE NEUROENDOCRINE STRESS RESPONSE

Asking ten people if they sometimes feel stressed out, ten will answer yes. On the contrary, asking ten people for their own definition of stress, one will get ten different answers. But most probably these ten answers will agree in one point, namely that stress is to be blamed for being the direct precursor of mental and physical illness. This is, stress has predominantly a negative connotation and evokes detrimental and maladaptive physiological and behavioral responses.

Certainly this is a very one-sided and narrow perspective. Therefore, the following sections will try to give a more balanced view and also take positive aspects of stress into considerations. In this regard, first a historical overview about the development of stress concepts will be given (2.1.1). This will be followed by a description of the participating stress systems (2.1.2) and their activation (2.1.3). The last two sections will focus on the effects of stress mediators, namely the above mentioned damaging (2.1.4) but also the protective effects (2.1.3).

2.1.1 Stress Concepts: Historical Development

In searching for a definition of stress, closely intertwined psychological and physiological processes have to be taken into account. This is, stress is neither something we are confronted with helplessly because it is simply an automatic reaction of the organism to external stimuli nor is it just an imagination or a train of thought haunting in people's mind.

Accordingly, an overview about the historical development of stress concepts will show, how different researchers focused on different processes and thus made different but for this very reason important contributions.

2.1.1.1 Claude Bernard

The basis for the later development of stress concepts was provided already in the middle of the 19th century by Claude Bernard (1813-1878). He emphasized that an animal's life depends on the *milieu intérieur* that is, "on extracellular fluids, which provide the physico-chemical conditions for the correct functioning of cells". He reasoned that if correct cell functioning depends on optimal physico-chemical conditions, then these must be constant and there have to be mechanisms that allow such conditions to be maintained. This is, he recognized that the stability of the *milieu intérieur* depends on ensembles of compensating mechanisms. For Bernard, "the constancy of the internal environment was the element conditioning free, independent life" (Bernard, 1878). This new concept entailed the overcoming of the concept of physiology as "anatomy in motion" and a radical shift in perspective. The "new" life sciences needed to penetrate this internal environment and investigate its regulation if they were to study living organisms (Conti, 2001). But for this it was necessary to find a broader concept that would allow for linking together the mechanisms affecting the regulation of the body.

2.1.1.2 Walter Bradford Cannon

This was done fifty years later by Walter Bradford Cannon (1871-1945) in introducing and coining the term *homeostasis*. He suggested this term to describe the dynamic, interactive nature of "coordinated physiological processes which maintain most of the steady states of the organism" (Cannon, 1929). Cannon identified the sympathetic nervous system as an important orchestrator of responses to challenges. He emphasized the role of epinephrine secreted from the adrenal glands, "since it is carried everywhere in the body by the blood and has the same effects on the internal organs as the sympathetic impulses" (Cannon, 1935). These processes restoring a disturbed steady state of the "fluid matrix" he also called "fight-or-flight response". Interestingly, in the article published in 1935, he already used the term *stress* not only in the by then conventional psychiatric context (i.e., to describe mental tension) but also as involving physical as well as emotional stimuli. In a physiological context he summarized various threats of homeostasis, like cold, lack of oxygen, low blood sugar, or loss of blood (Cannon, 1935).

2.1.1.3 Hans Selye

However, the cornerstone in the field of stress research was published one year later by Hans Selye (1907-1982) as a letter to *Nature* entitled "A syndrome produced by diverse nocuous agents" (Selye, 1936a). In this brief report he summarized several years of experimentation, primarily initiated by his search for new hormones in the placenta (Selye, 1952). In this regard, he injected rats with crude ovarian extracts and observed adrenal

enlargement and the atrophy of thymus and lymph nodes. Subsequently he tried to purify the responsible hormone for this putative specific reactions. But neither was he able to purify the sought-after hormone nor did the specificity of the symptoms prove true. Rather it occurred to him after further experiments that these symptoms may represent a nonspecific response to noxious agents and he published his findings in the above mentioned letter. Herein he summarized “that if the organism is severely damaged by acute nonspecific nocuous agents (…), a typical syndrome appears, the symptoms of which are independent of the nature of the damaging agent or the pharmacological type of the drug employed, and represent rather a response to damage as such” (Selye, 1936a). He also suggested to term this syndrome, consisting of (1) an enlargement of the adrenal gland, (2) atrophy of the thymus, spleen and other lymphoid tissue, and (3) gastric ulcerations, the “general adaptation syndrome”. The syndrome develops in three stages of adaptation, an initial “general alarm reaction”, followed by a prolonged period of resistance and a terminal stage of exhaustion and death. All these processes he regarded as a generalized effort of the organism to adapt itself to new conditions (Selye, 1936a).

Starting from this report, Selye developed a theory of stress that aroused intense research. However, the term *stress* itself does not appear in his publications until 1946 and Selye’s definition of stress appeared to vary at different periods, indicating stimulus, response, or interaction between stimulus and response (Mason, 1975a). But in 1950, he proposed stress as a condition within the organism in response to evocative agents, and these evocative agents he termed “stressors” (Selye, 1950). In 1974, he restated “stress is the nonspecific response of the body to any demand made upon it” (Selye, 1974). This is, while each input may have a unique effect, they all produce a consistent set of responses.

Over the time, he repeatedly emphasized the key role of the hypothalamus-pituitary-adrenal axis in the stress response. And while Cannon introduced catecholamines as important mediators, Selye shifted attention to glucocorticoids and hence from the adrenal medulla to the adrenal cortex (Selye, 1956).

2.1.1.4 John W. Mason

In contrast to Selye’s noxious or evocative agents, John W. Mason focused on the role of psychological factors in eliciting a physiological stress response. In 1968, he published a review summarizing more than 200 publications on this subject and despite the wide variety of events and psychological stimuli found to elicit a stress response, he identified conditions which tend to elicit responses of *unusual intensity*. These conditions were: novelty, uncertainty or unpredictability, anticipation of something previously experienced as unpleasant, ego-involvement, and situations in which long-established rules are suddenly changed (Mason, 1968). Thus, a stress response is not solely determined by situational criteria. Rather stimuli are processed by the central nervous system and therefore

intervening factors such as previous experiential history or coping styles determine the endocrine response. Mason also pointed out the marked individual differences in the pituitary-adrenal cortical response and regarded the definition of the multiple determinants of these individual differences as a major goal in the future development of psychoendocrine research, with the organization of psychological defenses as an especially important factor to consider (Mason, 1968).

Mason also suggested that corticosteroid secretion may be a specific response to psychological stimuli and not particularly responsive to physiological stimuli such as heat, exercise, and hunger. Scrutinizing Selye's concept of physiological non-specificity of the stress response, Mason proposed that elicitation of an emotion such as anxiety or fear constituted the basis for similar neuroendocrine responses to different stressors observed by Selye (Mason, 1971; Mason, 1975b). In Mason's opinion "emotional stimuli must be ruled out before it can be concluded that a physical stimulus is capable, by itself, of eliciting increased adrenal cortical activity" (Mason, 1968).

2.1.1.5 Richard S. Lazarus

Over a long period, stress research was implicitly based on stimulus-response (S-R) models: Some researchers focused on the stress response, others emphasized characteristics of stimuli. But it was not until the 1970s that the *cognitive revolution* also found its way into the stress area and criticism arose. How does a stimulus approach explain the wide variability in human responses to ostensibly the same stressor? On the contrary, how helpful is an approach that tends to treat all stressors as equivalent if they produce the same response? Are exercise, surprise, and passion equivalent because of their identical effect on heart rate? Are emotions and fatigue stress responses, stressors or maybe both? And how do response-based views of stress explain different responses in different individuals? It became more and more apparent that treating the stress stimulus or the stress response in a mechanical manner is insufficient, since it ignores the processes that intervene between stimulus and response.

Richard S. Lazarus viewed stress as a relational phenomenon, which only exists in the context of a person-environment interaction. In 1986 he stated: "There is simply no way to define an event as a stressor without referring to the properties of persons that make their well-being in some way vulnerable to that event." And: "There are no environmental stressors without vulnerable people whose agendas and resources influence whether or not there will be stress, the form it will take, and its short and long-term outcomes." (Lazarus and Folkman, 1986). Already 20 years earlier Lazarus had proposed a "transactional" model based on expectancies and appraisals: In a primary appraisal the person evaluates whether the stimulus situation is a threat or not. In the case of a threat, the person considers whether this threat can be met with the resources he or she has. If in this secondary appraisal the answer

is “no” or the person believes that these resources are insufficient, stress occurs (Lazarus, 1966). This is, Lazarus considered different concepts to describe the stress process (Lazarus, 1993): (1) a causal external or internal agent with a strong emphasis on the person-environment relationship and (2) an evaluation that distinguishes what is threatening or noxious from what is benign (appraisal). The next two steps are (3) coping processes and (4) a complex pattern of effects on mind and body (stress reaction). Thereby, coping is viewed as a highly contextual process always mediated by appraisal and affecting subsequent stress reactions in two main ways. Problem-focused coping changes a person’s relationship with the environment, emotion-focused coping changes the way a person attends to or interprets what is happening. Both actions aim for changing the conditions of psychological stress for the better.

With introducing processes like person-environment interaction, appraisal and coping, stress research moved away from S-R to stimulus-organism-response (S-O-R) models. Now individual differences in motivational and cognitive variables, which intervene between the stressor and the reaction, had to be taken into account.

2.1.1.6 Seymour Levine and Holger Ursin

Another approach based on a S-O-R model came from Seymour Levine and Holger Ursin (Levine and Ursin, 1991) and at first glance does not differ widely from the approach presented by Lazarus. Levine and Ursin also view stress as a multidimensional concept and identified three main interacting subclasses: (1) the input or stress stimuli, (2) the processing systems, including the subjective experience of stress, and (3) the output or stress responses.

Levine and Ursin considered psychological as well as physiological stimuli as potential stressors, but propose to classify all input as “loads”. This has the advantage that a load may be of physical nature, however it may act as a stressor through eliciting emotional loads. In this regard psychological emotional loads are the most frequently reported stress stimuli.

But no matter what kind of stimuli or load, all-dominant are previous experiences or learning. Every input is being evaluated or filtered before it gains access to any response systems. Thereby, comparison is the basic principle for the stimulus treatment. Levine and Ursin regarded comparisons most simply as expectations and differentiated between the stimulus expectancy and the response outcome expectancy. The former refers to the storing of information that one stimulus precedes another. In the case of a stimulus signaling an aversive event, the stress response occurs. Alternatively, the person may deny the relationship between the stimulus and the aversive event and defense may block threat signals from producing stress responses. The second filter, response outcome expectancies, refers to learning about the consequences of acts. Whether an individual will show a stress

response is determined by three classes of response outcome expectancies: coping, helplessness, and hopelessness. Here, it is important to differentiate between the use of the term coping by Levine and Ursin from Lazarus'. Lazarus used the term coping to refer to strategies used to face a challenge or threat. Levine and Ursin pointed out that in this denotation coping strategies are independent of the level of stress and offer no possibility to predict physiological consequences of the stimuli. Therefore, they defined coping as positive response outcome expectancies resulting from learning processes. If an individual can control or perceive control in a specific situation, the chosen responses (e.g., escaping, avoiding, obtaining reinforcement) result in a positive outcome. This relationship of response and outcome will then be stored as positive outcome expectancy. The expectation of a highly probable positive outcome reduces stress, the inability to cope, i.e. gaining control, results in high levels of stress. In the case of a very low probability that a response will lead to any consequences at all, helplessness arises. Whereas hopelessness arises in situations with very high probability that all available responses will lead to aversive or negative events. Additionally, these learning processes can further be modulated or permanently altered by individual differences. Factors, which change such tolerance to stressful events, can be prenatal stress, nutritional factors, genetic components but also social factors like the availability of social support.

The stress responses itself are defined as all overt behavioral and physiological changes. The existence of physiological stress responses can not necessarily be deduced from observed behavior, since the behavior may just indicate coping and thus absence of physiological responses may result. Therefore, Levine and Ursin proposed subjective verbal reports instead, which seem to follow the physiological state and are reasonably consistent and reproducible. Regarding the physiological stress response, which occurs whenever there is a discrepancy between what the organism is set for and what really exists, two types can be differentiated: The first general and non-specific alarm response occurs even when coping has been established and is characterized by epinephrine release, pulse rate increases and a modest rise in plasma levels of testosterone. The second response is specific to situations and experiences and is seen in noncoping individuals. This later stages of the stress response are characterized by the release of slower-reacting hormones such as cortisol and are seen as processes aiming at re-establishing physiological balance (i.e., homeostasis).

In his later work regarding the cognitive activation theory of stress (CATS), Ursin also emphasized more explicitly than in the above cited paper a fourth aspect of stress, namely the feedback from the stress response (Ursin and Eriksen, 2004). This is a very important aspect, since feedback from the stress response may alter the stimulus situation and these effects will be stored as response outcome expectancies.

Concluding, Levine and Ursin hypothesized that stress-inducing stimuli can be defined by their lack of information concerning the achievement of positive outcomes or avoidance of negative outcomes. Therefore stress is presumed to be the state that is created whenever the brain registers this informational discrepancy (Levine and Ursin, 1991). As Ursin (Ursin, 1998) pointed out, this definition is closely related to one of the principles of Mason, which is that particularly potent stimuli of the stress system are novelty, uncertainty, and unpredictability (Mason, 1968).

2.1.1.7 Summary

This section aimed at finding a definition of stress. In this regard, the development of stress research and stress concepts over almost one century was depicted. Yet, no generally accepted definition can be presented. Levine even concluded in 2005: “After the completion of our last effort to define stress (Levine and Ursin, 1991), I made myself the promise that I would never again engage in what I consider a futile exercise” (Levine, 2005). Despite this rather disenchanting statement, the most important fact became apparent nevertheless: In stress research, one has to deal with a complex system of physiology, behavior, subjective experiences, and cognitive functions, including feedback and control loops between these different domains. And if focusing on only one of these domains, for example the physiological effects of stress, one has still to be aware of the other major processes influencing and being influenced by the domain of interest.

2.1.2 Central and Peripheral Stress Systems

One aspect repeatedly appearing in the above depicted stress concepts is the link between stress and physiological stress responses. Therefore, the following two sections will describe the two systems mainly involved in a physiological stress response: the hypothalamus-pituitary-adrenal (HPA) axis (2.1.2.1) and the sympathetic nervous system (2.1.2.2).

2.1.2.1 HPA Axis

The hypothalamus-pituitary-adrenal axis is an endocrine system not only forming a fundamental part of the endocrine stress response but also contributing to the maintenance of daily energy balance. The activity of the HPA axis is characterized by a pronounced circadian variability (2.1.2.1.2) and a tight modulation by feedback mechanisms (2.1.2.1.3). Additionally, a wide variety of inputs play an important role in the stress-related activation of the HPA axis (2.1.2.1.4). The following sections will illustrate these aspects in greater detail, prefaced by a delineation of the organization of the HPA axis (2.1.2.1.1).

2.1.2.1.1 Organization of the HPA axis

As implicated by its name, the HPA axis is composed of three structures: the hypothalamus and the pituitary centrally and the adrenal glands peripherally. The hormones involved are corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and glucocorticoids (GC). In the following, each of the three structures as well as the corresponding hormone will be described.

Hypothalamus

The hypothalamus is a very small structure which comprises less than 1% of our brain volume. It is located in the middle of the base of the brain, and encapsulates the ventral portion of the third ventricle right above the pituitary (see fig. 2-1).

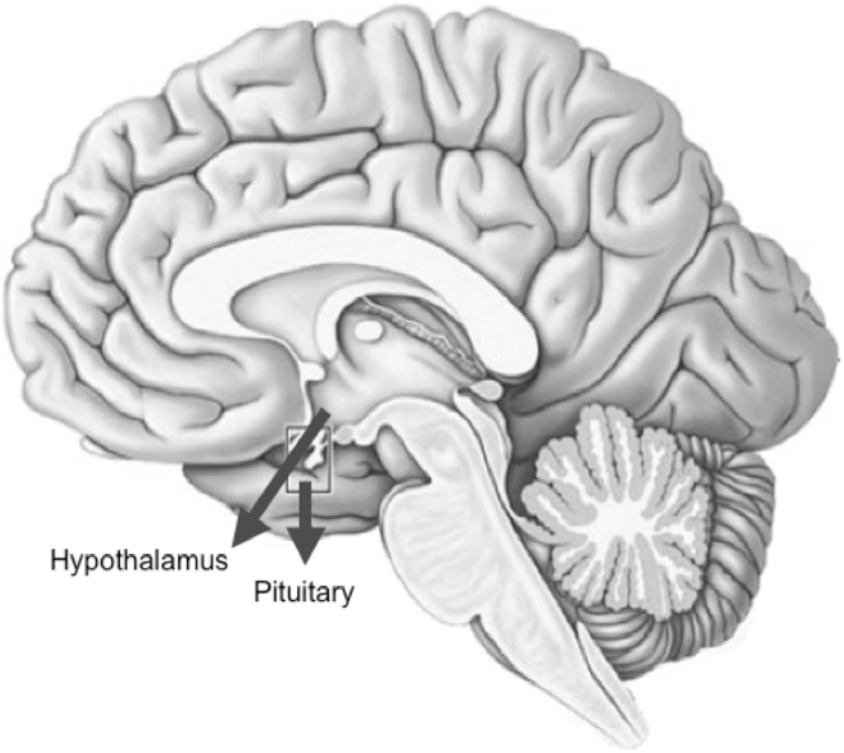

Fig. 2-1: Localization of the hypothalamus and the pituitary gland in the human brain (sagittal section).

The hypothalamus consists of distinct nuclei (see fig. 2-2) and is divided into medial and lateral regions by the fornix, which is a tract of fibers that runs from the hippocampus to the mamillary bodies. At the anterior the hypothalamus is limited by the optic chiasm and anterior commissure, at the posterior by the mamillary bodies.

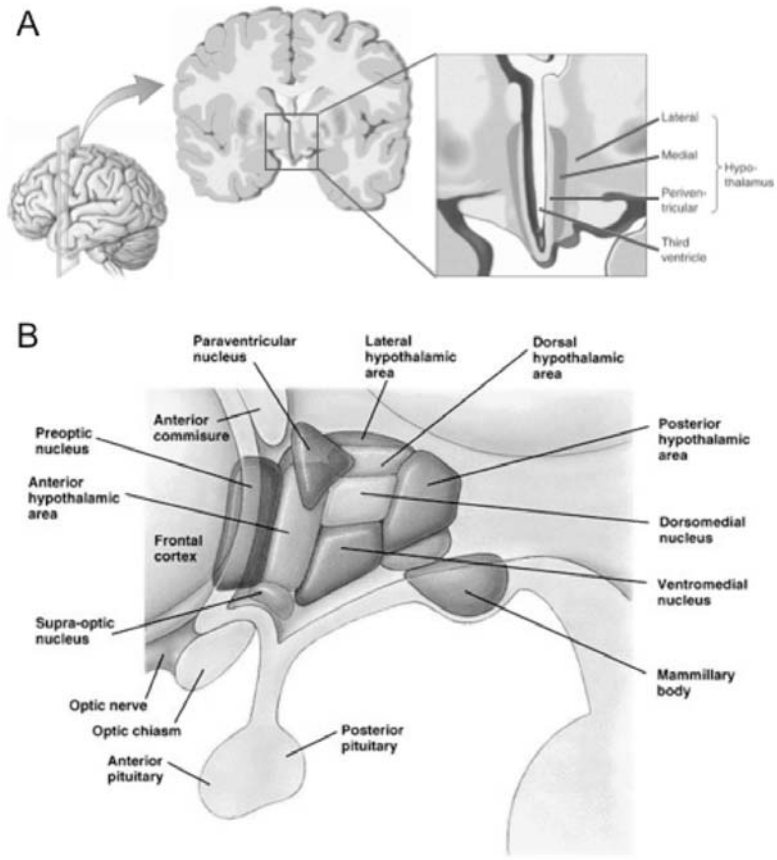

Fig. 2.2: Hypothalamic nuclei (A: frontal section; B: sagittal section).

Regarding the organization of the HPA axis, most important is the hypothalamic paraventricular nucleus (PVN). The PVN is a bilateral structure located either side of the third ventricle and is an elaboration of the periventricular zone of the hypothalamus. It contains two sets of neurons: The dorsal and ventral magnocellular neurons project to the brain stem and spinal cord and regulate behavioral and autonomic motor activity. The second set of neurons is neuroendocrine in nature and can be further divided in magnocellular (larger) cells and parvocellular (smaller) cells. The former secrete either oxytocin or vasopressin, the latter are located more medially than the magnocellular neuroendocrine cells and have terminals in the median eminence. Via the median eminence they secrete hypothalamic releasing hormones into the hypophysial portal vasculature to regulate anterior pituitary function (Kupfermann, 1991; McCann, 1988; Watts, 2000). One of these releasing hormones secreted by the parvocellular neuroendocrine cells of the PVN is the corticotropin-releasing hormone (CRH), the first of three molecules constituting the signal cascade of the HPA axis and discovered in the late 1950s.

CRH is a 41-amino-acid peptide that acts not only as a hormone regulating anterior pituitary function, but also centrally as a neurotransmitter. On this account it is often called corticotropin-releasing factor (CRF). In addition to the CRF-synthesizing neurons in the PVN, other CRF-expressing cell groups are located in the central nucleus of the amygdala, the bed

nucleus of the stria terminalis, the lateral hypothalamic area, parabrachial nuclei, and the dorsal motor nucleus of the vagus. This suggests that the central CRF system regulates not only the neuroendocrine, but also autonomic and behavioral responses to stressors (Dallman et al., 2000).

Pituitary gland

The pituitary gland can be divided into two distinct parts. One part is the posterior pituitary or neurohypophysis, which consists of the median eminence, the infundibular stalk, and the neural lobe of the pituitary gland. Axons from magnocellular cells in the PVN and supraoptic nucleus pass through the infundibular stalk and synapse on the capillaries of the posterior pituitary. When an action potential arrives at a terminal, oxytocin or vasopressin is released from the terminal directly into the bloodstream.

The other part is the anterior pituitary or adenohypophysis. In contrast to the neurohypophysis, the adenohypophysis is non-neural tissue and has three parts: pars distalis, tuberalis, and intermedia. Pars tuberalis makes up approximately 75% of the adenohypophysis and its cells contain corticotropes. Corticotropes synthesize adrenocorticotropin from its precursor proopiomelanocortin (POMC). The decisive signal is CRF. Axons of CRF neurons of the PVN end at the median eminence, and CRF secreted from these diffuses into the hypophyseal-portal blood supply and travels to the anterior pituitary. Through its action on CRF-recepor-1, CRF regulates the synthesis of POMC and eventually the secretion of the 39 amino-acid peptide adrenocorticotropin hormone (ACTH) into the general circulation. ACTH is the second major HPA axis hormone.

Interestingly, vasopressin also has a physiological role in the control of ACTH release as it can potentiate the action of CRF at both hypothalamic and pituitary levels. Therefore, vasopressin and CRH are regarded as hormones closely cooperating in the control of ACTH release (McCann, 1988; Watts, 2000).

Several other tropic hormones are produced in the anterior pituitary and regulated by hypothalamic releasing or inhibiting hormones. In the following these hormones are listed and the releasing or inhibiting hormones are given in parentheses: Thyroid-stimulating hormone (TSH) and prolactin (in both cases thyrotropin-releasing hormone, additionally prolactin-releasing factor in the latter); beta-lipotropin (CRF); follicle-stimulating hormone and luteinizing hormone (gonadotropin-releasing hormone); melanocyte-stimulating hormone (MSH) and beta-endorphin (MSH-releasing factor); and growth hormone (GH; GH-releasing hormone). Furthermore, some of these hormones are regulated by hypothalamic inhibiting hormones. These are: prolactin (prolactin release-inhibiting hormone and dopamine), GH and TSH (GH release-inhibiting hormone), and MSH (MSH release-inhibiting factor) (Kupfermann, 1991). Thereby, the successive processes are similarly to that of CRF-ACTH described above and are depicted in fig. 2-3.

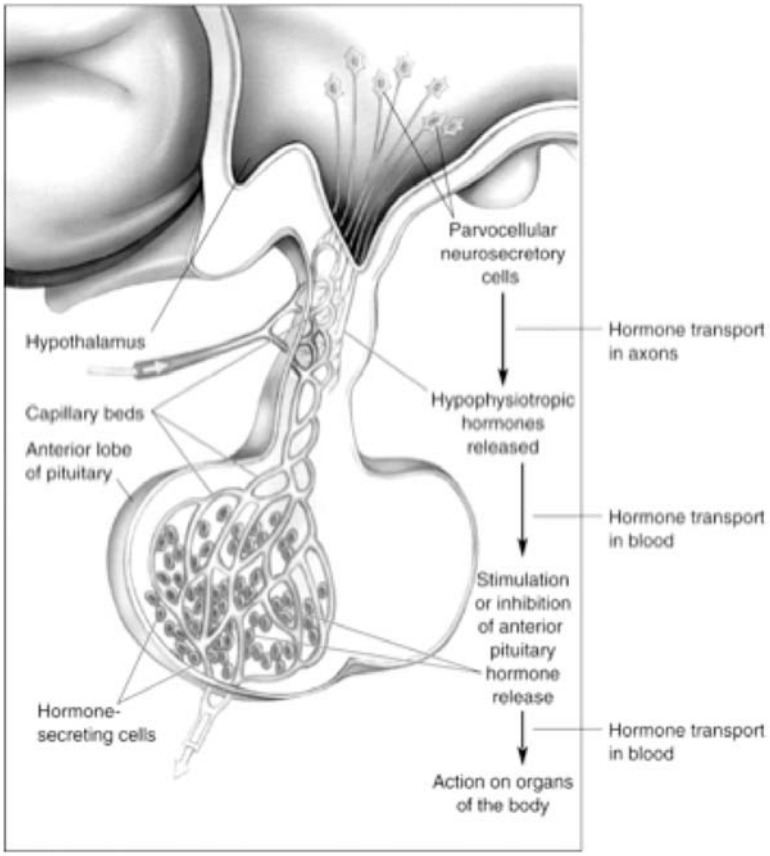

Fig. 2-3: Detailed view of the anterior pituitary gland and its regulation by hypothalamic neurons.

Adrenal glands

The adrenal glands are the third structure constituting the HPA axis and the target tissue for ACTH released by the anterior pituitary. As shown in the left panel of figure 2-4, the adrenal glands are located bilaterally atop the kidneys (ad-renal) and are well supplied with arterial blood. The adrenal cortex comprises 90% of the gland, the medulla the remaining 10%. The medulla synthesizes and secretes catecholamines. Since catecholamines are involved in the second major stress response system, they will be discussed in detail in the respective section (see. 2.1.2.2).

The cortex can be subdivided into three anatomical and functional zones (see right panel of figure 2-4). Below the fibrous capsule, the outer zona glomerulosa is situated. Here, mineralocorticoids, mainly aldosterone, are synthesized. Subsequently follow the middle zona fasciculate and the inner zona reticularis. The former secrets glucocorticoids, the latter androgens, mainly dehydroepiandrosterone (DHEA).

All adrenal steroids are derived from cholesterol by various modifications of its structure (see figure 2-8 in section 2.2.6.2). The rate-limiting step in steroid biosynthesis is the action of the cytochrome P450 side chain cleavage enzyme (scc), which converts cholesterol into pregnenolone. Once pregnenolone is available, sequential modifications by further enzymes rapidly form the various steroids.

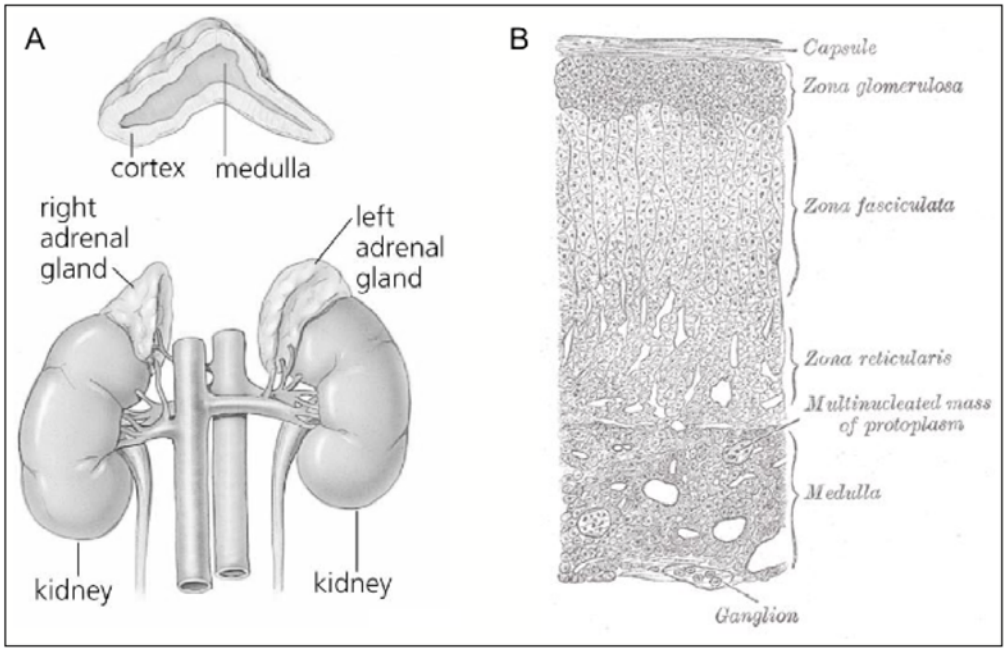

Fig. 2-4: Localization of the adrenal glands (A; source: Carlyn Iverson) and anatomy of the adrenal cortex (B; modified from Gray, 1918).

Since cholesterol is found in storage depots in the absence of stimulation, the very first step has to be its release. One stimulus is ACTH, which binds to its membrane receptor and via the activation of enzymes converts cholesterol esters to free cholesterol. Additionally, as all stimuli of steroidogenesis, ACTH increases the interaction of cholesterol with scc enzyme, thus starting the biosynthetic cascade to cortisol – the third major hormone of the HPA axis (Kaplan, 1988).

Cortisol, sometimes also referred to as hydrocortisone, is the main glucocorticoid (GC) in humans and most mammals, whereas in some rodents corticosterone serves this function. Once Cortisol is secreted and in circulation, it is largely bound to corticosteroid-binding globulin (CBG, synonym: transcortin). Another 15-20% is bound less tightly to albumin, leaving only about 5% of circulating cortisol as unbound (Pearson-Murphy, 2000). These 5% of unbound or free cortisol are thought of as the biologically active fraction, based on the concept known as the “free hormone hypothesis” (Mendel, 1992). Subsequently free cortisol is metabolized in the liver and excreted into the urine, accounting for a half-life of 49 minutes for total cortisol and of 1.8 minutes to 3.5 minutes for free cortisol (Keenan et al., 2004).

Cortisol is essential for life and has a wide variety of effects on different tissues. These effects can be further differentiated into basal and stress functions, which will be discussed in sections 2.1.4 and 2.1.5. Cortisol exerts its effects genomically (for details see also section 2.3.4.1.2): The lipophilic hormone passively diffuses through the cellular membrane, binds to and activates its intracellular receptor; then this complex translocates to the nucleus, where it either interacts with other transcription factors or binds to DNA

response elements (GRE) resulting in up- or down-regulation in the expression of various genes (Pearson-Murphy, 2000).

2.1.2.1.2 Circadian variation

The activity of the HPA axis and thus the secretion of its hormones are subject to a pronounced variation, which allows for adaptive response to environmental demands. Basically, generation of a baseline ultradian pattern of HPA activity depends on a relatively constant oscillation in the activity of parvocellular CRH and vasopressin neurons. Both hormones are secreted episodically at a frequencies of 1 to 3 secretory episodes per hour (Chrousos, 1998b). Regarding ACTH and cortisol, varying numbers of pulses per day are stated, apparently varying with sampling intervals. While van Cauter reports on approximately 15 pulses in a 24-hour span (Van Cauter, 1995), Iranmanesh used various sampling intervals decreasing from 12 to 2 minutes and found 20 to 80 pulses per day (Iranmanesh et al., 1990), which parallels the 1 to 3 secretory episodes per hour given for CRH.

These pulsations are superimposed by several inputs, constituting a circadian rhythm. According to Chrousos, these inputs include: (1) positive circadian input(s) from one or more pacemakers; (2) tonic positive input from locus coeruleus/norepinephrine and dopaminergic mesocorticolimbic systems; (3) tonic negative input from hippocampus and prefrontal cortex; and (4) negative feedback input from the arcuate nucleus-POMC-peptidergic system and the end-hormone of the HPA axis cortisol (Chrousos, 1998b). One pacemaker and master endogenous clock which controls circadian rhythmicity is the paired suprachiasmatic nucleus (SCN) of the hypothalamus, mediating the association with the light-dark cycle (Van Cauter, 1995). All these inputs influence the amplitudes of the baseline pulsation. Thus, the circadian pattern results from increases in the amplitude and not from varying frequencies of pulses (Iranmanesh et al., 1990).

The interplay of episodic pulses and superimposed inputs constitute a typical pattern of ACTH and cortisol secretion throughout the day. Both hormones show an early morning maximum, declining levels throughout daytime, a quiescent period of minimal secretory activity at night, and an abrupt elevation during late sleep (Van Cauter, 1995).

This circadian variation has clear implications for clinical diagnosis and study-design. The time of sample collection has to be chosen adequately, so that for example cortisol levels allow for discrimination between healthy persons and patients. It has also to be taken into consideration that a stimulus response may depend on the timing of stimulus application (Van Cauter, 1995). Additionally, age-related changes in the endocrine circadian rhythm have been well described: Older persons show dampened rhythms and specific phase points of the rhythms occur earlier than in young persons (van Coevorden et al., 1991).

2.1.2.1.3 Feedback regulation

A process is referred to as feedback when the product of an activity in a system modifies the factors that produce that product. Applied to the HPA system, cortisol suppresses the production and release of its precursors CRH and ACTH, which in turn decreases the production and release of cortisol itself. This negative feedback system has to control accurately the activity of the HPA axis under circadian *and* stressful conditions. This covers a range of very low cortisol levels during the trough of basal rhythm to up to the 600-fold at the peak of the circadian rhythm and under stress. It also poses a problem, since receptors characteristically bind their hormones over a 100-fold range (Dallman, 2000). Therefore, two receptors are used to regulate the HPA activity. These two receptors differ in their affinities and their distribution in the central nervous system. The mineralocorticoid receptor (MR) is a high affinity receptor (dissociation constant K_d ~ 0.5nM) for aldosterone, cortisol, and corticosterone. Since glucocorticoids circulate in higher concentrations and enter the brain more easily than the mineralocorticoid aldosterone, brain MRs are predominantly occupied by cortisol and corticosterone. MRs are distributed in the entorhinal cortex, limbic structures, and in motor output neurons, but only a very low density of MRs is found in neurons of the hypothalamus. On the other hand, the glucocorticoid receptor (GR) is a lower affinity receptor (K_d ~ 5-30nM) and preferentially binds glucocorticoids in the order dexamethasone > cortisol > corticosterone > deoxycorticosterone > aldosterone. GRs are virtually ubiquitous in neurons and glia cells and among others found in hippocampus, amygdala, hypothalamus and catecholaminergic cell bodies of the brain stem. Comparing the distribution of the two receptors, MR and GR expression overlap in the hippocampus, more precisely in hippocampal dentate gyrus neurons and CA1 cells (De Kloet et al., 1998). Additionally, the hippocampus has numerous projections to the hypothalamus. Hence it is considered centrally in negative feedback regulation of the HPA axis. Beside the different affinities and largely distinct distribution, also distinct functions are assigned to MRs and GRs. MRs are thought to control basal activity of the HPA axis, whereas GRs (in coordination with MRs) regulate feedback during the circadian peak or after stress.

The regulation of HPA activity by feedback inhibition is the result of a highly complex interplay of various factors. Feedback processes may be rate-sensitive or dose-sensitive and exist in fast, intermediate and slow time domains. Accordingly, they may occur directly or trans-synaptically and result in inhibition of CRF and/or ACTH synthesis and/or release. Again this depends on how MRs and GRs mediate their actions, via activating or repressing gene transcription, or via protein-protein interactions, whereas disproportionate interactions may result in resistance or supersensitivity of the receptors (Dallman, 2000; Dallman et al., 1994; De Kloet et al., 1998; Jacobson and Sapolsky, 1991; Keller-Wood and Dallman, 1984).

2.1.2.1.4 Medial parvocellular PVN-projecting afferents

Like the generation of circadian rhythmicity, generation of stress-related increases in HPA activity depends on relatively constant ultradian oscillations of parvocellular CRH and vasopressin neurons, and the superimposition of stress-related inputs (Chrousos, 1998b). In this regard, limbic afferents play a major role. Specifically, the ventral subiculum, infralimbic and prelimbic region of the medial prefrontal cortex, medial and central amygdaloid nucleus, lateral septum, and paraventricular thalamus are involved in the regulation of the PVN.

In general, the parvocellular PVN receives little or no direct innervations from limbic stress-regulatory regions. Thus, at least one intervening synapse seems to be necessary to relay limbic input to the PVN. These relays are located in the subparaventricular zone, subnuclei of the bed nucleus of the stria terminalis, the medial preoptic area, the lateral hypothalamic area, the ventrolateral region of the dorsomedial nucleus, and the nucleus of the solitary tract. These local neurons contacted by descending limbic projections are mostly GABAergic and to a lesser extent glutamatergic and have inhibitory and excitatory influences on the HPA axis, respectively. Nevertheless, limbic GABA positive neurons (e.g. neurons in the medial amygdala) may excite the PVN through a GABA-GABA disinhibitory process (Herman et al., 2004; Herman et al., 2002).

Other brain regions regulating HPA activity are the SCN and the ventromedial hypothalamic nucleus. Additionally, ascending afferents from the brainstem innervate the PVN (noradrenergic afferents, excitatory) or the peri-PVN region (cholinergic and serotonergic afferents, excitatory) (Herman et al., 2004). CRH and norepinephrine collateral fibers inhibit presynaptic CRH and noradrenergic receptors, respectively, constituting ultra-short negative-feedback loops (Chrousos, 1995). Centrally secreted substance P in turn inhibits hypothalamic CRH neurons but not vasopressin neurons and stimulates the central noradrenergic system (Chrousos, 1998b).

In summary, various interactions of the parvocellular PVN with neuronal systems subserving homeostasis, memory and emotionality exist. Inputs concerned with homeostasis emanate predominantly from a circumscribed set of medial parvocellular PVN-projecting afferents resident in the brainstem, hypothalamus and basal forebrain (Herman et al., 2002). They integrate circulatory stress signals from changes in blood volume or pressure through vagal afferent nerves, osmotic and chemical signals sensed humorally and through vagal afferent signals, as well as inflammatory and pain inputs, also sensed humorally and neurally, through vagal and sensory afferent nerves (Chrousos, 1998b). Furthermore, mnemonic and emotive processes affect parvocellular PVN neurons predominantly via limbic circuits (Herman et al., 2004; Herman et al., 2002).

2.1.2.2 Sympathetic Nervous System

The sympathetic nervous system is the second system involved in physiological stress responses and one of three divisions of the autonomic nervous system (ANS).

2.1.2.2.1 Autonomic nervous system compared to the somatic motor system

In contrast to the somatic motor system that controls voluntary movement, the ANS regulates internal organs and the eyes (Lovallo and Sollers, 2000) and is often referred to as autonomic (or visceral) motor system or involuntary motor system (Dodd and Role, 1991). Beside the extent of voluntary control, the two systems also differ in the anatomical organization. Somatic motor neurons project directly to target skeletal muscles from the central nervous system, i.e., the efferent pathway to skeletal muscle is monosynaptic. Whereas autonomic preganglionic motor neurons project to autonomic postganglionic motor neurons, which in turn synapse on their visceral targets. Thus, in the ANS the efferent pathway to the target is disynaptic. Further, all somatic motor neurons are excitatory and inhibition is exerted indirectly by inhibiting the motor neurons in the spinal cord that excite the muscle. In contrast, the ANS is able to excite and inhibit targets directly (Dodd and Role, 1991).

2.1.2.2.2 Anatomy and neurotransmitters of the autonomic nervous system

The tree divisions of the ANS – sympathetic, parasympathetic and enteric division – in turn differ anatomically in the positions of the preganglionic neurons and in the organization of postganglionic neurons: Preganglionic cells of the (1) *sympathetic nervous system* (SNS) extend from the first thoracic spinal segment to lower lumbar segments (T1 to L3; see left side of fig. 2-5). The cell bodies are found within the spinal cord, primarily within the inter-mediolateral gray matter. The axons of these neurons emerge through the ventral root, enter the spinal nerve, project through the white rami communicantes to the paravertebral chain ganglia, and synapse with postganglionic neurons. The axons may also travel within the ganglionic connective and synapse with postganglionic neurons located in other ganglia than the ganglion corresponding to the segmental level at which their cell bodies are located. This divergence with a ratio of preganglionic to postganglionic fibers of approximately 1:10 permits coordinated activation of sympathetic neurons at several spinal levels. Axons of postganglionic neurons then exit through the gray rami communicantes and travel along branches of the carotid arteries innervating structures in the head or travel in the spinal peripheral nerves innervating autonomic targets like the heart, lungs, or bronchi. Some preganglionic fibers do not synapse in the paravertebral chain ganglion but on neurons of the prevertebral ganglia (celiac ganglion, superior mesenteric ganglion, and inferior mesenteric ganglion) innervating the gastrointestinal system, the kidneys, pancreas, liver, bladder, and

external genitalia. A fourth group of preganglionic fibers runs with the thoracic splanchnic nerve into the abdomen directly innervating cells of the adrenal medulla, which are developmentally and functionally related to postganglionic sympathetic neurons. On the other hand, preganglionic neurons of the (2) *parasympathetic nervous system* (PNS) are located within the brain stem in several nuclei and in segments S2 to S4 of the spinal cord and project to postganglionic neurons in ganglia that are close to visceral targets or actually embedded in them (see right side of fig. 2.5). Neurons of the (3) *enteric nervous system* (ENS) eventually are arranged in interconnected plexuses, situated between the various layers of muscle and endothelium and innervating the gastrointestinal tract, pancreas, and gall bladder. The ENS is composed of local sensory neurons that register alterations, as well as interneurons and motor neurons that control the muscles and the secretory activity of its targets. The enteric nervous system is regulated by an extrinsic innervation that is supplied by the parasympathetic and sympathetic systems, both of which can override intrinsic enteric activity in situations of emergency or stress (Dodd and Role, 1991; Lovallo and Sollers, 2000).

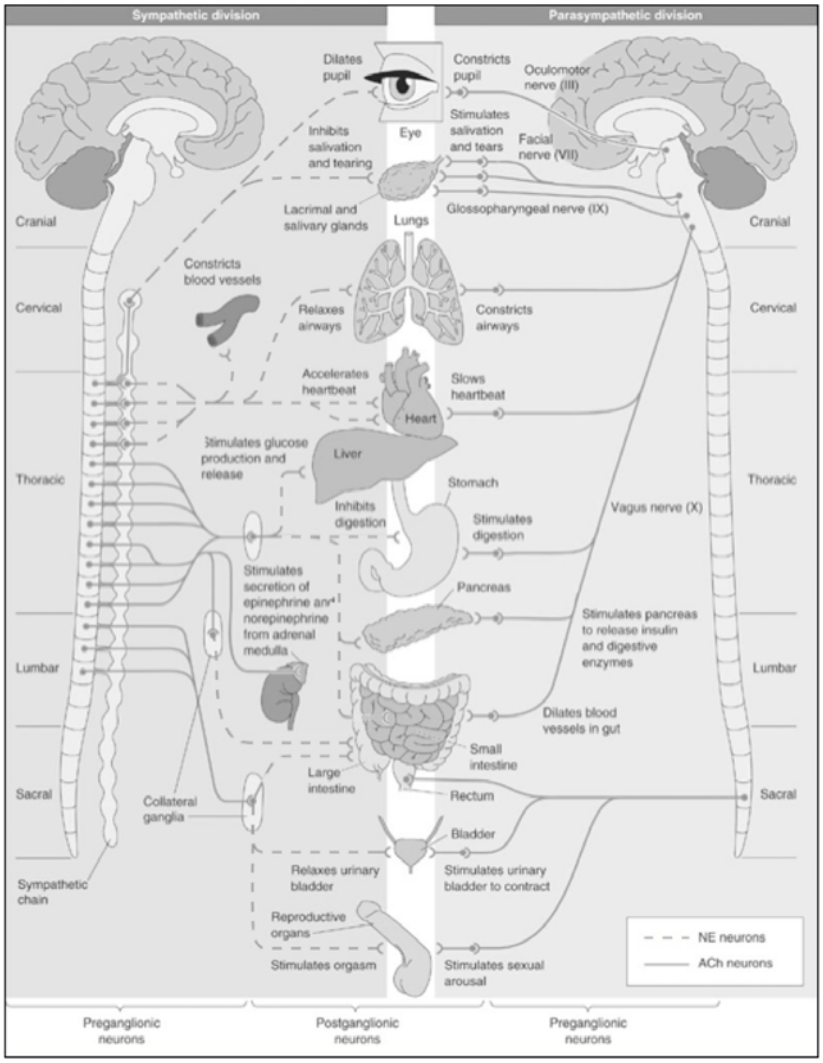

Fig. 2-5: Preganglionic and postganglionic neurons of sympathetic and parasympathetic divisions of the autonomic nervous system and their targets.

Synapses of the ANS use two neurotransmitters, acetylcholine (ACh) and norepinephrine (NE). All preganglionic neurons communicate to their postganglionic neurons using ACh and all postganglionic *para*sympathetic neurons secrete ACh at their neuroeffector junctions. In contrast to the PNS, sympathetic fibers secrete NE at their effectors (see fig. 2-5). However, there are two exceptions: (1) Specialized sweat glands of the palms of the hands and soles of the feet and (2) the medulla of the adrenal gland are activated by ACh. Additionally, small neurons that communicate directly between the larger pre- and postganglionic neurons secrete dopamine, which inhibits the ganglionic neurons (Lovallo and Sollers, 2000).

2.1.2.2.3 Regulation of the autonomic nervous system

The output of the autonomic nervous system is influenced by three control mechanisms. First, local ANS regulation is provided by the action of locally released peptides, neuromodulators, and metabolites. Second, ganglionic mechanisms regulate the output of ANS postganglionic neurons. Third, the brain stem, specifically the nucleus of the solitary tract (NTS), and the hypothalamus exert regulation on the entire ANS. Under psychological stress, the hypothalamus also integrates information of the cerebral cortex, hippocampus, entorhinal cortex, parts of the thalamus, basal ganglia, cerebellum, and the reticular formation. These changes originating in the highest centers of the brain can be seen as a final layer of control over the ANS. To exert control over the ANS, the hypothalamus not only projects to nuclei in the brain stem and the spinal cord that act on preganglionic autonomic neurons, but also acts on the endocrine system to release hormones that influence autonomic function. On the other hand, many autonomic functions do not require continuous monitoring by the hypothalamus. In this regard, sensory fibers project to specific subnuclei within the NTS and these neurons project to lower brain stem nuclei that connect to autonomic motor neurons controlling effectors, thereby constituting a set of reflex circuits by which the NTS controls simple autonomic functions. Elaborate homeostatic adjustments are coordinated by transmitting information from autonomic targets to higher and lower brain regions. In particular, visceral afferents terminate in the commissural nucleus of the NTS, which in turn projects to brain stem and forebrain nuclei, such as amygdala, paraventricular hypothalamic nucleus, and bed nucleus of the stria terminalis. These nuclei then project back to the NTS and lower brain stem nuclei, the latter again projecting directly to the dorsal vagal nucleus and sympathetic preganglionic nuclei (Dodd and Role, 1991; Lovallo and Sollers, 2000).

2.1.2.2.4 Role of the sympathetic nervous system in stress

Sympathetic nervous system activation aims at maintaining homeostasis and is prominent in orthostasis, mild to moderate exercise, thermoregulation, and the postprandial

state. In response to perceived global, metabolic threats like hemorrhage, hypoglycemia, shock, or psychological stress, SNS changes do not suffice and increased neural outflow to the adrenal medulla elicits catecholamine secretion from the adrenal medulla. As mentioned above, the adrenal medulla is one target organ of the sympathetic nervous system, which in addition is innervated directly by preganglionic fibers. The catecholamines secreted are the hormones norepinephrine and epinephrine, which are synthesized in adrenal chromaffin cells from the amino acid precursor tyrosine. Upon entry into adrenal chromaffin cells, tyrosine is converted to L-dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase. DOPA in turn is converted into dopamine by aromatic-L-amino-acid decarboxylase and dopamine is then taken up into storage vesicles and converted into norepinephrine by dopamine-β-hydroxylase. The last step in catecholamine biosynthesis is the conversion of norepinephrine to epinephrine by phenylethanolamine-N-methyltransferase (PNMT). Norepinephrine and epinephrine are stored in granules of separate populations of chromaffin granules and released via exocytosis. In contrast to norepinephrine, the adrenal medulla is the sole source of circulating epinephrine in all mammalian species, including humans, while norepinephrine released from the adrenal medulla makes up to only 35% of levels measured in bloodstream. The remaining 65% are a small proportion of norepinephrine released locally from sympathetic nerve terminals and reaching the bloodstream (Goldstein, 2000; Kvetnansky and McCarty, 2000; Pollard, 2000).

Accumulating evidence supports an independent regulation of sympathetic nervous system and sympathoadrenal system. Each of these two systems can be activated independently from the other by distinct stimuli. While cold exposure and postural changes primarily affect sympathetic nervous system activity, mental effort and caffeine ingestion are considered stimuli for the adrenal medulla. When SNS activity is suppressed by fasting, adrenal medullary responses to various stimuli are enhanced. For certain stimuli the SNS response is biphasic, with an initial suppression followed by subsequent stimulation. During the first phase adrenal medullary secretion is markedly increased (Young et al., 1984). Catecholamines and specifically epinephrine have many of the same actions as direct sympathetic stimulation, but effects last considerably longer. Additionally, a distinction must be made between neural and hormonal effects, since the latter can also reach organs without direct sympathetic innervation. In parallel to the HPA axis, the sympathoadrenal system is often referred to as sympathetic adrenal medullary (SAM) axis and the SAM response with release of predominantly epinephrine “fight or flight” reaction. (Goldstein, 2000; Kvetnansky and McCarty, 2000; Pollard, 2000). As mentioned above, catecholamines further play an important role in the regulation of mpPVN neurons: They stimulate the release of CRH and hence the HPA axis.

2.1.3 Activation of Stress Systems

The former sections described two systems mainly involved in physiological stress response. To identify the stimuli, which are able to activate the HPA and SAM axes and thus to elicit a stress response, numerous studies were conducted and a multitude of situations and events inducing elevated HPA and SAM activity were identified. Therefore, the following descriptions are mainly based on a publication by Mason, reviewing studies published between 1921 and 1968 (Mason, 1968), and on a publication by Biondi and Picardi, reviewing studies published between 1979 and 1999 (Biondi and Picardi, 1999). Both reviews focused on psychological stress, since – as Mason concluded – psychological stimuli are the most potent activators of the HPA axis. Generally, situations and events eliciting a physiological stress response can be subdivided into laboratory stressors and real-life stressors.

2.1.3.1 Laboratory Stressors

Common laboratory stressors are mental arithmetic tasks, mental arithmetic tasks combined with a speech test, the Stroop color-word conflict test, videogame playing, presentation of films and videotapes, interviews, and various multiple task protocols combining several of the aforementioned tasks. These stressors additionally vary in duration and general conditions (e.g., with or without time pressure; attitude of the experimenter; presence of an audience or a video camera; presence of distractors). Significant increases in epinephrine and/or norepinephrine were found in virtually every study, except for studies presenting films and videotapes, and appeared to be related to the amount of effort, feelings of alertness and action proneness, active coping, and sense of control. This does not apply to stress responses in cortisol. Cortisol increases were mainly reported in studies using combinations of mental arithmetic tasks and speech tests, including recording of the speech ostensibly for a subsequent analysis of the verbal and nonverbal performance or for televising to an audience (Biondi and Picardi, 1999). Interestingly, this kind of tasks feature characteristics comparable to the domains proposed by Mason, namely novelty, unpredictability, anticipation, and ego-involvement (Mason, 1968). Applying the stress concept of Levine and Ursin, in these situations positive response outcome expectancies are low and therefore the inability to cope, i.e. gaining control, results in high levels of stress (Levine and Ursin, 1991). A meta-analysis of 80 laboratory studies by Dickerson and Kemeny confirmed these results (Dickerson and Kemeny, 2004): “One important set of determinants of cortisol responses appears to consist of (a) a motivated performance task, (b) relative uncontrollability of task outcome, and (c) presence of social evaluation”. Studies which combined these determinants all used a combination of a mental arithmetic task and a speech task in front of an audience and/or a video camera. The presence of others which

could negatively judge performance can be interpreted as an social-evaluative threat or a threat to the goal of maintaining the social self (i.e. one's social value, esteem, and status, largely based on others' perceptions of one's worth). Notably, social-evaluative threat also is an important element in two domains proposed by Mason. These are the domains ambiguity and involvement, situations in which long-established rules are suddenly changed and in which one must master a difficult task in order to forestall aversive stimuli (Mason, 1968). On the other hand, uncontrollability includes one's inability to avoid negative consequences or succeed despite one's best efforts, corresponding to hopelessness and helplessness, respectively, according to Levine and Ursin (Levine and Ursin, 1991). Dickerson and Kemeny also referred to the interweavement of uncontrollability and novelty, which could certainly be extended to unpredictability: When a situation is novel or unpredictable, the outcomes are less certain and hence less controllable.

2.1.3.2 Real-Life Stressors

Looking at the results of studies investigating potential real-life stressors, very often the same underlying motives and processes can be proposed. Real-life stressors may be loss events (e.g. death of a loved one, loss of employment, or loss of home), academic examinations, anticipation of surgical interventions, work demands, or public speaking, but also acute battle danger, auto racing, or the first parachute jump. A mother perceives hopelessness and helplessness, if the child is dying from leukemia. Academic examinations and public speaking are social-evaluative threats and more or less controllable. This may also apply to a work position with high levels of psychological demands and low level of decision latitude. In situations of acute battle danger, a first parachute jump, or the anticipation of surgical interventions another aspect may come into play. Such situations inhere threats to the goal of physical self-preservation and already Selye (see above) showed the potency of such stimuli to elicit a stress response (Mason, 1968).

2.1.3.3 Physical Exercise

Beside psychological stressors, also physical exercise can activate the HPA axis. Given an adequate work load (i.e., >70% VO_{2max}), cortisol levels will rise and reflect the duration of physical stress (Mason et al., 1973). Similar results are observed e.g. in marathon runners (Cook et al., 1992). But despite the work load, several studies also showed the importance of psychological factors. Pancheri et al. found increases in cortisol levels before the start of a competition only in individual competitors, not in athletes who were members of a team (Pancheri et al., 1982). Beulen et al. investigated ballroom dancers during training sessions and during an tournament and reported sharp cortisol increases only during the tournament. The latter was even more pronounced in pair dancers than in group dancers

(Beulen et al., 2005). These studies not only emphasize the role of social-evaluative threat, but also the role of social support in modulating hormonal reactivity to stress.

Nevertheless, large inter-individual variability remains a stable finding. Therefore, it is important to consider variables repeatedly observed to modulate cortisol stress responses. Among these are genetic predisposition, personality and coping style, age, gender, menstrual cycle, oral contraceptives, smoking, and early experiences (Biondi and Picardi, 1999; Kirschbaum and Hellhammer, 1994; Kirschbaum et al., 1999; Kudielka and Kirschbaum, 2005; Levine, 2000).

2.1.4 Protective Effects of Stress Mediators

While since 1914 (Cannon, 1914) there was scarcely any disagreement regarding effects and biological significance of increases in adrenal medulla hormones (i.e., epinephrine and norepinephrine) during stress, this does not hold true for glucocorticoids.

During the first half of the 20th century, stress-induced secretion of glucocorticoids were thought to increase resistance to stress by stimulating defense mechanisms. This view was proposed also by Selye in an influential review in 1946, in which he additionally proposed the concept of “diseases of adaptation” (Selye, 1946). Such diseases were postulated to be caused by excessive or abnormal adaptive reactions to stress and Selye listed diffuse collagen disease, allergy, and rheumatic diseases as examples. Despite the controversial discussion elicited by his theory that one of the principal causative agents of these diseases was excessive secretion of mineralocorticoids in response to stress, another event would challenge the traditional view and change the history of glucocorticoid endocrinology. In 1949, Phillip S. Hench published – together with Kendall, Slocum, and Polley – a paper with the title: “The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone: Compound E) and of pituitary adrenocorticotropic hormone on rheumatoid arthritis”, in which he described the relief from symptoms of rheumatoid arthritis following treatment with cortisone or ACTH (Hench et al., 1949). Thus, while Selye predicted that over-activity of the adrenal cortex was an etiologic factor in diseases as rheumatoid arthritis, suddenly hormones of the pituitary-adrenocortical axis exerted the contrary, namely suppressive effect. This controversy was resolved without further ado by excluding the anti-inflammatory effects from physiology. Henceforth, attention moved to the pharmacological actions and new clinical applications of these hormones and away from investigating their physiological role.

Notwithstanding this time of change, Dwight J. Ingle investigated the role of adrenal cortex hormones in metabolic processes. In 1952, he emphasized the permissive role of adrenocortical hormones in stress responses, in that their presence at basal levels is necessary but not causative for an adequate metabolic stress response. He reached this

conclusion despite his own observation that “adrenalectomized rats which were on a fixed intake of adrenocortical extract, an amount which represented full replacement under non-stress conditions, had insufficient hormone during stress and the glycosuria fell sharply” (Ingle, 1952). But although Ingle ignored the relevance of this former finding, he uncovered an important and new aspect of glucocorticoid actions in the stress response, i.e. their permissive actions.

About the same time, Marius Tausk published a paper in the periodical of a pharmaceutical firm (Organon, The Netherlands), whose German title roughly translates to: “Does the adrenal gland actually play a role in defense?” (Tausk, 1951). Unfortunately, this paper did not enter the regular literature and hence only quotations by other authors can be given. According to De Kloet, Tausk pointed out that glucocorticoids block primary stress reactions (De Kloet, 2004), respectively restrain defense reaction to stress, which would themselves become damaging if left uncontrolled (De Kloet et al., 1998). According to Sapolsky et al., Tausk compared stress to a fire and the role of GCs to that of preventing water damage rather than putting out the fire (Sapolsky et al., 2000). Eventually Korte et al. wrote about “a somewhat modified version of Tausk’s metaphor of ‘water used by firemen’”, based on which they concluded that “just like the fireman’s water stress responses are ideally beneficial, they can impose a cost to the body, particularly when they are either elicited too often or are inefficiently managed” (Korte et al., 2005). Taken together, already in 1951, Tausk suggested an important role of glucocorticoids in preventing other stress-induced responses from overshooting and subsequently damaging the organism.

Since then, this aspect of glucocorticoid actions has been repeatedly assumed, with the difference that these times it also entered the regular literature. Besedovsky and Sorkin, for example, argued that in the immune system activation of other cells than highly antigen specific cells would lead to a disturbance of the system and hence may be potentially harmful to the host. Additionally, an excessive expansion of lymphoid cell mass would raise the concentration of soluble mediators to an undesirable or possibly even dangerous extent. In this context, “an increase of corticosteroids may well have this very function of suppressing a potentially harmful expansion of lymphoid tissue of low or no affinity for the antigen” (Besedovsky and Sorkin, 1977). Shortly thereafter, Munck, Guyre and Holbrook hypothesized “that (1) the physiological function of stress-induced increases in glucocorticoid levels is to protect not against the source of stress itself, but against the normal defense reactions that are activated by stress; and (2) the glucocorticoids accomplish this function by turning off those defense reactions, thus preventing them from overshooting and themselves threatening homeostasis” (Munck et al., 1984). This new perspective and the discovery that a single, basic molecular mechanism (i.e., via glucocorticoid receptor) initiates most glucocorticoid actions (Munck and Leung, 1977), had an unifying influence on glucocorticoid

endocrinology. In his later works, Munck additionally incorporated the permissive effects of glucocorticoids proposed by Ingle (Ingle, 1952). In his view, under normal conditions, permissive effects prime defenses for action, while after stress the delayed suppressive effects of glucocorticoids prevent stress-activated immediate defenses from overshooting (Munck, 2000; Munck and Naray-Fejes-Toth, 1992).

Chrousos does not draw this distinction but rather regards activation of stress systems as normally adaptive, improving chances of an individual for survival by leading to behavioral and physical changes (Chrousos, 1998a). These changes are listed in table 2-1.

Tab. 2-1: Behavioral and physiological adaptation during acute stress (Chrousos, 1998a).

Behavioral adaptation	**Physical adaptation**
adaptive redirection of behavior	*adaptive redirection of energy*
o increased arousal and alertness	o oxygen and nutrients directed to the CNS and stressed body site(s)
o increased cognition, vigilance, and focused attention	o altered cardiovascular tone, increased blood pressure and heart rate
o euphoria or dysphoria	o increased respiratory rate
o heightened analgesia	o increased gluconeogenesis and lipolysis
o increased temperature	o detoxification from toxic products
o suppression of appetite and feeding behavior	o inhibition of growth and reproductive systems inhibition of digestion-increased colonic motility
o suppression of reproductive behavior	o containment of the inflammatory-immune response
o containment of the stress response	o containment of the stress response

Since Chrousos mainly focused on the clinical implications associated with the stress system, especially HPA axis dysfunction (e.g., Chrousos and Gold, 1998; Chrousos, 2000), the chronology of stress hormone secretion and differential effects of adrenal medulla and adrenal cortex hormones were not considered.

A new framework, which integrated all the above mentioned views and the large array of results on glucocorticoid effects on many different target tissues, was compiled by Sapolsky, Romero, and Munck (Sapolsky et al., 2000). In this regard, they first considered recent findings and, based on them, generated criteria for determining the appropriate glucocorticoid action on target tissues. In doing so, they accounted for the different time-courses of stress mediators as well as the time-courses of their physiological consequences.

Basically, changes in hormone secretory patterns in response to a stressor can be divided into two consecutive but distinct responses, the so-called first wave and the second wave. In the first wave, occurring within seconds, an enhanced secretion of catecholamines from the sympathetic nervous system, the hypothalamic release of CRH, and (approximately 10 seconds later) enhanced secretion of pituitary ACTH, a decreased hypothalamic release of GnRH, and (shortly thereafter) a decreased secretion of pituitary gonadotropins, the

pituitary secretion of prolactin and growth hormone, and the pancreatic secretion of glucagon can be observed. Minutes later, the second wave involves the stimulation of glucocorticoid release and declines in gonadal steroid secretion. The immediate physiological consequences of a stress-response – attributable to catecholamines and CRF – include increased cardiovascular tone, immune activation, energy mobilization, loss of proceptive and receptive sexual behavior, loss of appetite, increased cerebral blood flow and cerebral glucose utilization, and enhanced memory consolidation (Sapolsky et al., 2000).

What are glucocorticoids doing then? To answer this question, Sapolsky et al. distinguished in a first step two classes of glucocorticoid actions. Modulating actions alter an organism's first wave response to stress and preparative actions alter the organism's response to a subsequent stressor or aid in adapting to a chronic stressor. The former are further divided into three subclasses, resulting in four classes of glucocorticoid actions:

1) *Permissive actions*: GCs present before the stressor prime defense mechanisms. In this case, consequences occur whether or not there is a stress-induced increase in GC concentrations (cp. Ingle, 1952).
2) *Suppressive actions*: stress-induced rise in GCs – with an onset of from about 1 hour or more after the onset of the stressor – reins in the stress-activated first wave defense reactions and prevent them from overshooting (cp. Besedovsky and Sorkin, 1977; Munck et al., 1984; Tausk, 1951).
3) *Stimulating actions*: stress-induced rise in GCs enhances the effects of the first wave with an onset of from about 1 hour or more after the onset of the stressor (cp. protection against stress by stress-induced levels of GCs (Selye, 1946).
4) *Preparative actions*: GCs do not affect the immediate response but modulate the response to a subsequent stressor. This action may be permissive, stimulating or suppressive.

Importantly, labeling an action as permissive, suppressive or stimulating depends on the effects of the first wave. If a glucocorticoid action reduces a first wave effect, may that be stimulating or suppressive, the glucocorticoid action is termed suppressive. On the other hand, if a glucocorticoid action enhances a first wave effect, may that be stimulating or suppressive, the glucocorticoid action is labeled stimulating. If the latter effect is due to basal levels present before the first wave, it would be termed permissive.

Beside this so-called criterion of conformity, three further criteria were applied for analyzing the role of GCs in the stress response: the criterion of time course (When does an GC effect occur?), the criteria of hormone subtraction and replacement (Is a first wave effect attenuated or enhanced if there is no stress-induced rise in GC activity, respectively, is it attenuated if basal GC actions are eliminated? Does administering of stress or/and basal levels of GCs restore the stress response?), and the criterion of homeostasis (Which of the

four particular GC actions make more physiological sense in terms of restoring homeostasis?).

In the context of these criteria, Sapolsky et al. reevaluated glucocorticoid actions in selected physiological systems and the following four sections are geared to their framework. These sections are (1) cardiovascular effects, (2) metabolic effects, (3) neurobiological effects, and (4) effects on immunity and inflammation. Since glucocorticoid actions on the immune system are a main focus of this thesis, at this point only a very short synopsis will be given and effects will be discussed in greater detail in the respective section later in this work (2.3.2: brain-to-immune system communication).

2.1.4.1 Cardiovascular Effects

As stated above, catecholamines and CRH mediate the rapid activation of the cardiovascular system, which involves elevated arterial pressure, heart rate, and cardiac output, diversion of blood to muscle via constriction of mesenteric and renal vessels, and dilation of vessels supplying skeletal muscles (Galosy et al., 1981). On the other hand, it is long known that glucocorticoids also increase blood pressure and cardiac output (Sambhi et al., 1965). This narrows the question about GC actions to stimulating or permissive actions. In this regard, most findings point to a permissive action. For example Grünfeld et al. found that in normal unstressed rats increases in systolic blood pressure due to treatment with the GC agonist RU26988 could be prevented by pre- or post-treatment with the GC antagonists RU38486 or progesterone (Grunfeld et al., 1985). Three years later, they observed that while RU486 did not alter basal cardiac output and renal blood flow, pre-treatment significantly attenuated pressor responses to angiotensin II and norepinephrine in Wistar rats (Grunfeld and Eloy, 1987). Additionally, decreased blood pressure in Addisonian and adrenalectomized individuals were observed in numerous studies.

Taken together, these result point to a permissive action of glucocorticoids as proposed by Ingle (Ingle, 1952). However, stimulatory actions are so far untested and hence can not be excluded. Since both permissive and stimulatory actions enhance the first wave response to a stressor, Sapolsky et al. referred to them collectively as helping to mediate the stress response. In this regard, glucocorticoids can clearly identified as mediating the cardiovascular stress response (Sapolsky et al., 2000).

2.1.4.2 Metabolic Effects

During acute stress, blood glucose levels are elevated rapidly by mobilization from existing stores and by inhibition of further storage through a rapid insulin resistance. This is due to catecholamines, glucagon, and GH and makes sure that energy is diverted from storage sites to exercise muscle.

Glucocorticoids are known for also increasing circulating glucose concentrations. This is achieved by several mechanisms: stimulation of glycogenolysis, (hepatic) gluconeogenesis, and glycogen deposition, inhibition of peripheral glucose transport and utilization, as well as induction of lipolysis, mobilization of amino acids, and stimulation of proteolysis (Pearson-Murphy, 2000). Importantly, glucocorticoid actions under basal conditions or after injury (reviewed in Dallman et al., 1993) have to be distinguished from actions taking place under acute stress. Under basal conditions, glucocorticoid actions mostly oppose with those of insulin. Based on these effects and on observations of increases in glucocorticoids after insulin administration (De Feo et al., 1989), glucocorticoids can be termed counter-regulatory or suppressive as they prevent insulin-induced hypoglycemia from overshooting. This corresponds to the view of Munck (Munck et al., 1984).

On the other hand, Ingle termed the role of glucocorticoids in metabolism “permissive” since they permit other hormones to produce normal responses (Ingle, 1952). Since Ingle, the interaction of glucocorticoids, glucagon and epinephrine in the control of glucogenolysis, gluconeogenesis, and lipolysis was repeatedly demonstrated. For example, Exton et al. examined the effects of glucagon and epinephrine on gluconeogenesis and glycogenolysis in the liver and of catecholamines and ACTH on lipolysis in adipose tissue in normal and adrenalectomized rats (Exton et al., 1972). In adrenalectomized rats, they found markedly reduced glucagon or epinephrine stimulation of glucose synthesis from lactate. The gluconeogenic response to glucagon was restored when dexamethason was administered 30 min prior to perfusion. Physiological concentrations of epinephrine and glucagon also did not normally activate glucose release and phosphorylase in livers from adrenalectomized rats. Furthermore, livers from these rats showed diminished activation of gluconeogenesis. Epinephrine stimulation of free fatty acid and glycerol release was impaired, as was the activation of lipolysis by norepinephrine and ACTH reduced.

In integrating results like the above stated into their framework and additionally considering time and dose range required for exerting effects on different processes, Sapolsky et al. summarized glucocorticoid actions in metabolism as follows: Since glucocorticoids synergize with catecholamines and glucagon to stimulate lipolysis and to elevate circulating glucose concentrations by stimulating glycogenolysis and gluconeogenesis, they can be categorized as permissive, whereas the slower stimulation of gluconeogenesis and inhibition of peripheral glucose utilization by stress-induced GCs also can be regarded as stimulatory. Finally, the stimulation of liver glycogen deposition takes hours and this restoring of glycogen levels is best classified as preparative (Sapolsky et al., 2000). Again, this re-evaluation is in concert with Ingle’s view (Ingle, 1952) as well as with Selye’s view of glucocorticoids increasing stress-resistance (Selye, 1946).

2.1.4.3 Neurobiological Effects

In the evaluation of neurobiological glucocorticoid effects, it will be focused on three topics, for there is information as to the effects of the early wave and dose-response information regarding glucocorticoid actions. These topics are (1) cerebral glucose transport and utilization, (2) appetite and feeding, and (3) memory formation.

2.1.4.3.1 Cerebral glucose transport and utilization

Many stressful events are accompanied by increases in cerebral blood flow and energy metabolism and appear to have a common adrenergic mechanism (Bryan, 1990). Since catecholamines do not easily pass the blood-brain barrier, this effect is most probably mediated by the SNS (Sapolsky et al., 2000). On the other hand, glucocorticoids inhibit glucose transport. For example, Kadekaro et al. found increased glucose utilization in adrenalectomy and prevention of this stimulatory effects on cerebral glucose metabolism by additional treatment with dexamethasone (Kadekaro et al., 1988). Horner et al. reported on inhibition of glucose transport in cultures containing both neurons and glia cells. These effects were dose- and time-dependent and preventable by a GR- but not a MR receptor antagonist (Horner et al., 1990). Further, several studies suggested that glucocorticoids may decrease glucose transport by causing translocation of glucose transporters from plasma membrane to an internal location (Carter-Su and Okamoto, 1985; Carter-Su and Okamoto, 1987; Horner et al., 1987). The initial suggestion by these authors that glucocorticoids cause synthesis of some protein which in turn causes the transporter to shift was replaced by findings of Garvey et al., who observed dexamethasone to decrease transporter mRNA (Garvey et al., 1989). All these studies yield a clear picture of inhibitory glucocorticoid actions in cerebral glucose transport and utilization (Sapolsky et al., 2000).

2.1.4.3.2 Appetite and feeding

During stress, appetite is suppressed by CRH acting as neurotransmitter (Arase et al., 1988; Krahn et al., 1986; Morley and Levine, 1982), while on the other hand glucocorticoids at basal levels stimulate appetite (Dallman et al., 1993), but at stress concentrations decrease appetite (Devenport et al., 1989). This latter effect is attributable to inhibitory effects of insulin stimulated by high concentrations of glucocorticoids. When preventing this secondary insulin effect, glucocorticoids stimulate appetite over the entire dose range (Dallman et al., 1993). In this regard and applying the criteria of Sapolsky et al., glucocorticoids can be categorized as suppressive. At first glance this action may not be biologically reasonable. But by glucocorticoids stimulating appetite, energy expenditure during phases of stress are counterbalanced and the organism is prepared for future

demands. Thus, glucocorticoid actions may further be regarded as preparative (Sapolsky, 2000).

2.1.4.3.3 Memory formation

In 1994, Cahill et al. demonstrated nicely the catecholamine-mediated effect on memory (Cahill et al., 1994). In this study, subjects were read one of two stories which only differed in their emotional content. In both stories a boy and his mother are on a trip through town. In the first story they visit the boy's father in the hospital and the staff demonstrates various medical procedures to the boy. In the second story the boy is seriously injured by a car, rushed to the hospital, and various medical procedures are carried out on him. Cahill et al. found marked enhancement of the memory for the emotionally laden details of the accident and subsequent procedures carried out on the boy. This effect was selectively eliminated by beta-adrenergic receptor blockade with propranolol. It is suggested that catecholamines exert their effect on memory by enhancing delivery of oxygen and glucose to the brain, as for example Manning et al. showed that elevation of glucose concentrations enhances episodic memory in the elderly as well as in Alzheimer's patients (Manning et al., 1993).

Glucocorticoids exert more complex effects on memory formation. Diamond et al. demonstrated that stress-induced levels of glucocorticoids disrupt long-term potentiation (LTP) and/or primed burst potentiation (PBP) in hippocampus. However, lower concentrations, such as those seen during the diurnal rise, enhance plasticity (Diamond et al., 1992; Diamond et al., 1994). This biphasic effect of glucocorticoids on LTP and PBP suggests activation of MRs at low levels of glucocorticoids to increase and occupation of GRs by high levels of glucocorticoids to suppress hippocampal excitability (Diamond et al., 1992; Pavlides et al., 1995). This is underlined by findings of adrenalectomy disrupting memory process in animals (Vaher et al., 1994), which is restored by occupancy of MRs, while MR antagonists again disrupt cognition (Oitzl et al., 1994). This is also true in humans. For example, administration of GR agonists to healthy volunteers was shown to disrupt memory within a few days (Wolkowitz et al., 1990) and stress-induced levels of glucocorticoids were repeatedly found to impair performance in declarative memory tasks (Kirschbaum et al., 1996).

Taken together, glucocorticoids at basal levels can be regarded as permissive, since they enhance memory as catecholamines do. However, at stress levels they are suppressive, whereas the biological sense of this remains unclear (Sapolsky et al., 2000). Cumulating evidence indicates that the latter action has to be further differentiated: while glucocorticoids impair processes of memory retrieval and working memory, they dose-dependently enhance consolidation of long-term memory (for review see Roozendaal et al., 2005).

2.1.4.4 Effects on Immunity and Inflammation

Classification of glucocorticoid actions regarding the immune system presents itself as very complex. The main problem arises from the criterion of conformity, since first wave hormones exert both stimulating and inhibitory effects. For example, catecholamines *in vitro* and after lipopolysaccharide injection suppress cytokines like tumor necrosis factor alpha (TNF-α) and interleukin(IL)-6, and stimulate the anti-inflammatory cytokine IL-10 (van der Poll et al., 1994). On the other hand, DeRijk et al. found subcutaneous injection of epinephrine to induce a dose-dependent increase in plasma IL-6 concentration. In parallel, intravenous infusion of epinephrine resulting in concentrations similar to those observed during stress were found to increase IL-6 concentrations (DeRijk et al., 1994). Furthermore, Papanicolaou et al. reports on correlations between peak plasma epinephrine or norepinephrine and IL-6 levels 15 minutes after the onset of a treadmill exercise test run (Papanicolaou et al., 1996). The administration of another first wave hormone, namely CRF, seems to decrease T cell proliferation and natural killer cell cytotoxicity (Jain et al., 1991) but to enhance B cell proliferation *in vitro* (McGillis et al., 1989). Sapolsky et al. conclude that considering the criterion of conformity "offers little information regarding the classification of glucocorticoid actions because there is no consensus as to the effects of that first wave of hormones" (Sapolsky et al., 2000).

However, it is unanimously agreed that various infectious as well as non-infectious (e.g., psychological) stressors rapidly activate the immune system and this immune system activation with the release of various cytokines in turn can stimulate the adrenocortical axis (Besedovsky and Sorkin, 1977). The most general effect of glucocorticoids is in turn to inhibit the synthesis, release, and/or efficacy of cytokines and other mediators of immune and inflammatory reactions (reviewed in (McEwen et al., 1997). Nevertheless, over the past two decades, a large variety of studies have shown that glucocorticoids also exert stimulatory actions on immune functions. Especially striking are effects of glucocorticoids on expression of cytokine receptors. While inhibiting the mediator (e.g. IL-6), glucocorticoids up-regulate the expression of its receptor (Snyers et al., 1990). Wiegers et al. postulated that distinct MR- and GR-mediated effects may underlie stimulatory properties of low physiological concentrations of glucocorticoids and the immunosuppressive action observed after prolonged pre-incubation with high concentrations of corticosteroids (Wiegers et al., 1994). Munck and Náray-Fejes-Tóth combined findings like these and proposed that high mediator levels, but few receptors at low glucocorticoid concentrations and high receptor levels, but little mediator at high glucocorticoid concentrations result in a biphasic or bell-shaped curve of mediator activity. Thus, compared to regulating just the mediator or the receptor, via this dual regulatory actions glucocorticoids can more effectively and tightly regulate mediator activity. By applying this model to basal and stress-induced conditions, Munck and Náray-

Fejes-Tóth conclude that under normal conditions, permissive effects prime defenses for action, while suppressive effects prevent stress-activated defenses from overshooting (Munck and Naray-Fejes-Toth, 1992). Based on the criterion of homeostasis, the same conclusion is also drawn by Sapolsky et al. (Sapolsky et al., 2000): Glucocorticoids restrain defense reactions to stress, which would themselves become damaging if left uncontrolled (Besedovsky and del Rey, 1996). This view of glucocorticoid action on immune system additionally includes that glucocorticoids are no longer thought to unselectively inhibit all immune responses during stress, but rather to "sculpt" the immune response by inhibiting superfluous or autoimmune-prone components (Besedovsky et al., 1991).

Taken together, the new analysis by Sapolsky et al. clearly extended and differentiated conclusions drawn by Munck et al. 16 years earlier (Munck et al., 1984). In applying the criteria of conformity, time course, subtraction, substitution, and homeostasis and in succession distinguishing permissive, stimulatory, suppressive and preparative actions, Sapolsky et al. point out the complex role of glucocorticoids in a complex interplay of different stress mediators. The cardiovascular, immunological, and cognitive effects of glucocorticoids as well as their effects on metabolism permissively help mediating the pending or ongoing stress-response and can be regarded as advantageous for homeostasis. The same is true for the suppressive actions of glucocorticoids on immune parameters, the preparative actions on appetite, and the preparative and stimulatory actions on metabolism. However, the homeostatic role of suppressive glucocorticoid actions on selected neurobiological parameters (i.e., glucose transport and utilization in the brain, appetite, and memory formation) remains to be elucidated.

This analysis perspicuously emphasizes the importance of considering different glucocorticoid actions on different physiological systems at different time points and at different concentrations. This does not only have a bearing on designing studies, but is also of high clinical and therapeutic relevance. Above all, given such a complex interplay, it is conceivable to anticipate serious consequences with the utmost probability if only one of the stress systems shows malfunctions. Or to quote Sapolsky: "Where the trouble begins is when people start excreting those same hormones for reasons of chronic psychological stress" (Sapolsky, 2000). These very consequences, namely the damaging effects of stress mediators will be discussed in the following section.

2.1.5 Damaging Effects of Stress Mediators

Already 70 years ago, Selye recognized that physiologic systems activated by stress can not only protect and restore the organism but also damage the body (Selye, 1936a). Sapolsky et al. likewise differentiated between the beneficial and the damaging actions of

glucocorticoids: “glucocorticoid physiology should be thought of as the salutary responses (be they mediating or suppressive) to noxious stimuli, whereas glucocorticoid pathology occurs when the natural recovery phase to a noxious stimulus is prevented from occurring”. (Sapolsky et al., 2000). With this, Sapolsky already pointed out one potentially harmful condition, namely the failure of shutting down a stress response due to chronic stress. These opposed properties are also emphasized by Chrousos et al. (Chrousos, 1998a). Stress responses and the accompanying responses are normally seen as adaptive and improving chances of an individual for survival. But stress system dysfunctions are associated with various pathophysiologic states, including psychiatric, endocrine, and inflammatory disorders and/or susceptibility to such disorders. In this regard, Chrousos et al. distinguished between HPA axis hyperactivity and hypoactivity and summarized states associated with such altered HPA axis activities (see table 2-2).

Tab. 2-2: States associated with altered HPA axis activity and altered regulation or dysregulation of behavioral and/or peripheral adaptation (Chrousos, 1998a).

Increased HPA axis		Decreased HPA axis
Chronic stress	Diabetes mellitus	Adrenal insufficiency
Melancholic depression	Central obesity	Atypical-seasonal depression
Anorexia nervosa	(metabolic syndrome X)	Chronic fatigue syndrome
Malnutrition	Childhood sexual abuse	Fibromyalgia
Obsessive-compulsive disorder	Psychosocial short stature	Hypothyroidism
Panic disorder	Attachment disorder of infancy	Nicotine withdrawal
Excessive exercise	Gastrointestinal disease	Post-glucocorticoid therapy
(obligate athleticism)	Hyperthyroidism	Post-Cushing syndrome cure
Chronic active alcoholism	Premenstrual tension syndrome	Postpartum period
Alcohol and narcotic withdrawal	Pregnancy (last trimester)	Post-chronic stress
		Rheumatoid arthritis

In summary, all three theories imply that a stress response is basically protective as long as all its elements are well coordinated and the processes are executed in the correct order. They also name examples of failures (HPA axis hyperactivity or hypoactivity) and their consequences. But the main question remains: Under which circumstances and how do these normally adaptive responses exert their maladaptive effects and accelerate pathophysiology?

To answer these questions, in 1993 McEwen and Stellar introduced a new theory which mainly focused on potentially damaging effects of stress mediators (McEwen and Stellar, 1993). And although McEwen et al. repeatedly broadened and further differentiated this theory in the following years, the basic framework persisted (McEwen, 2000a; McEwen, 2000b; McEwen, 2004; McEwen and Seeman, 1999; McEwen and Wingfield, 2003a; McEwen and Wingfield, 2003b). The main advantage of this theory is that it provides a new

terminology, which recognizes the “housekeeping role” of stress mediators, allows for more stress mediators than glucocorticoids, and helps to better understand processes leading to disease (McEwen and Wingfield, 2003b).

The starting point of this theory is the inadequateness of the term homeostasis to describe the observed changes in the physiological systems of interest. Homeostasis in the authors’ opinion applies strictly to a limited number of systems (e.g., pH or body temperature) that are truly essential for life and are therefore maintained within a narrow range (McEwen, 1998a; McEwen and Stellar, 1993). Physiological responses to potentially stressful challenges demand adaptations in broader boundaries. To describe these mechanisms, McEwen et al. adopted the term allostasis introduced by Sterling and Eyer (Sterling and Eyer, 1988). Allostasis is defined as achieving “stability through change”, and is thus still an essential component of maintaining homeostasis. But allostasis applies to systems that maintain systems essentially for life (homeostasis) in balance by fluctuating to meet demands from external forces (McEwen, 2004; McEwen and Wingfield, 2003a; McEwen and Wingfield, 2003b). If and how systems like the HPA axis and/or the SAM axis respond to physical or psychological challenge (i.e., to potentially stressful situations) largely depends on two factors: The way a person perceives a situation and a person’s general state of physical health (McEwen, 1998a; McEwen, 1998b). Thus, the theory emphasizes the important role of cognitive mechanisms and incorporates the stress concepts proposed by Lazarus (Lazarus, 1993) and Levine & Ursin (Levine and Ursin, 1991). And it also makes allowance for influences on physiological responses exerted by personal behaviors like diet, smoking, drinking, or exercise, and individual differences due to factors like genes, development, or experiences (see fig. 2-6).

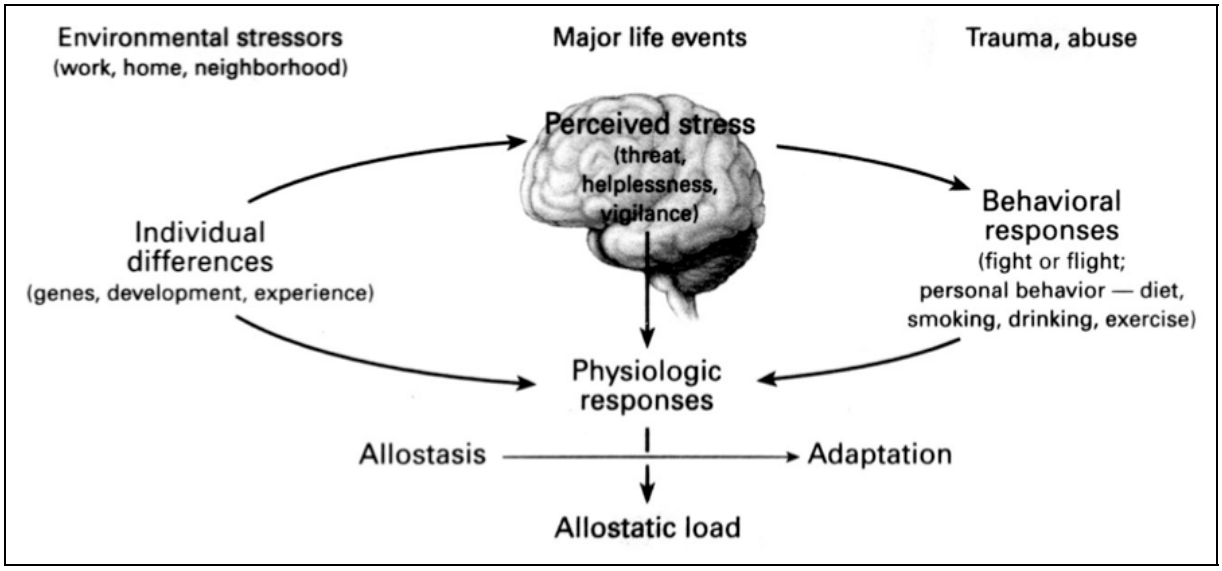

Fig. 2-6: The stress response and development of allostatic load (taken from McEwen and Seeman, 1999).

Shaped by these factors, a physiologic response elicited by physical or psychological challenge is seen principally and in the first place as adaptive. This may even be true if responses are altered or sustained for limited periods[1]. But in the long run, adaptation has a price. These costs of adaptation are termed allostatic load and imply pathophysiologic consequences. Allostatic load is the converged result of stress, perturbations of the diurnal rhythm of stress mediators, genes, early life experiences, living and working environment, interpersonal relationships, influences of diet, exercise, sleep and other lifestyle factors which all affect body chemistry, structure, and function over a life-time (McEwen, 2000a).

Four situations are associated with allostatic load[2] (McEwen, 1998a; McEwen, 2000a; McEwen and Seeman, 1999):

1. Frequent or chronic stress: the stress response is frequently turned on by novel events. For example, in the case of blood pressure, repeated surges can trigger myocardial infarction in susceptible persons (Muller et al., 1989).
2. Lack of adaptation: the stress response fails to habituate to repeated stressors of the same kind. A repeated public speaking challenge usually results in habituation of the glucocorticoid stress response. But some individuals, who seem to lack self-confidence and self-esteem, fail to habituate and subsequently overexpose their bodies to stress hormones (Kirschbaum et al., 1995).
3. Prolonged response: the stress response is not turned of efficiently after the challenge is over. One example of this is individuals with two parents who are hypertensive: they show prolonged elevation of blood pressure after a physiological stressor (Gerin and Pickering, 1995).
4. Inadequate response: inadequate in this regard stands for lack of a response. Such a inadequate response by some allostatic systems in turn triggers compensatory increases in others. This is illustrated by the Lewis rat, which has less corticosterone than the Fischer rat and is therefore missing suppressive GC actions on secretion of inflammatory cytokines. Consequently, Lewis rat are vulnerable to inflammatory and autoimmune disturbances, which are not found in Fischer rats (Sternberg et al., 1989).

[1] In later publications, McEwen et al. termed these altered and sustained activity levels of primary mediators, e.g. of glucocorticoids, ‘allostatic states’ and hypertension is given as an example (McEwen and Wingfield, 2003a).

[2] In later publications, McEwen et al. additionally differentiate between allostatic load and allostatic overload. The former can be considered the result of daily and seasonal routines and is still seen as an adaptive response. The letter results if one superimposes on this additional load of unpredictable events in the environment, disease, human disturbance, and social interactions. Allostatic overload serves no useful purpose and predisposes the individual to disease (McEwen and Wingfield, 2003a; McEwen, 2004).

Importantly, the four given types of allostatic load are not alternative states, since for example frequent stress may lead to prolonged or inadequate responses. And particularly the fourth type of allostatic load demonstrates the advantage of a distinction between homeostatic and allostatic mechanisms as adverse health outcomes may result despite a stable milieu interieur.

Further examples emphasizing the dual role of stress mediators – short-term adaptive actions that are protective (allostasis) and long-term effects that can be damaging (allostatic load) – are given for cardiovascular system, metabolism, brain and immune system. Regarding the cardiovascular system, catecholamines promote adaptation by adjusting heart rate and blood pressure to sleeping, waking, and physical exertion. An example for allostasis is the increase in blood pressure when we get out of bed. Such an increase maintains blood flow, permits an erect posture, and avoids “black-out” (Sterling and Eyer, 1988). Yet, the Whitehall II study showed that lack of control on the job may lead to repeated surges of blood pressure and thus increase the risk of coronary heart disease (Bosma et al., 1997). Closely related to cardiovascular effects is the role of adrenal steroids in metabolism. On the one hand they promote allostasis by activating and maintaining energy reserves, on the other hand HPA over-activity may lead to insulin resistance, accelerate progression towards type II diabetes, abdominal obesity, hypertension, and atherosclerosis (Brindley and Rolland, 1989). Regarding the central nervous system, stress mediators promote retention of memories of emotionally charged events (Cahill et al., 1994), however, HPA over-activity may lead to cognitive dysfunction by mechanisms that involve reduced neuronal excitability, neuronal atrophy, and, in extreme cases, death of brain cells, particularly in the hippocampus (McEwen, 2000b). Such changes, in turn, have clinical implications for disorders such as depression or post-traumatic stress disorder. For the immune system, the importance of balanced physiological responses is especially evident. While chronic over-activity of stress systems result in immuno-suppression associated with an enhanced risk of infectious diseases (McEwen et al., 1997), the absence of glucocorticoids allows other immune mediators to overreact and increase the risk of autoimmune and inflammatory disorders (Sternberg, 1997). Additionally, a study of Fernandez-Real et al. in 228 apparently healthy persons emphasized the interconnectedness of all these systems and the influence of confounding variables (Fernandez-Real et al., 2001): Increased concentrations of proinflammatory cytokines (i.e., IL-6) due to insufficient glucocorticoid signaling were found to be associated with insulin resistance in men (higher in smokers) and hypertension in women, thereby increasing the risk of diabetes and atherosclerosis. But proinflammatory cytokines also induce a syndrome of “sickness behavior”, which overlaps with stress-related neuropsychiatric and physical disorders like major depression and PTSD (Kent et al., 1992). Interestingly, cytokine receptors are found in brain regions, such as the hypothalamus and

hippocampus (Rothwell et al., 1996), and these cytokines are potent stimulators of CRH (Maier and Watkins, 1998).

These latter examples already give an idea of the difficulty to determine the mechanisms leading from allostasis to allostatic load and subsequently to pathology. For example, Raison and Miller summarized just the causes for insufficient glucocorticoid signaling. Insufficient glucocorticoid signaling could thereby result from (1) decreased hormone bioavailability, caused by (1a) decreased production of upstream glucocorticoid secretagogues (ACTH, CRH), (1b) a primary deficit in adrenal hormone production and/or release, alterations in (1c) binding proteins, (1d) enzymes such as 11-beta-hydroxysteroid dehydrogenase, or (1e) the so-called 'multidrug resistance pump', which extrudes cortisol but not corticosterone from the cell, or from (2) decreased receptor-mediated signal transduction/reduced hormone responsiveness, due to alterations in (2a) number of receptors, (2b) binding affinity of receptors, (2c) functional capacity of receptors, or (2d) combination of alterations in all. Additionally, any HPA axis abnormality my also be a primary or a secondary adaptation to alterations elsewhere in the axis (Raison and Miller, 2003). But even if the physiologic origin for an altered concentration of a stress mediator is known, the question remains, what such a measurement represents in the cascade of events that lead from allostasis to allostatic load and finally to disease. For this reason, McEwen and Seeman proposed a new way of classifying such measures, which allows for relating what is measured to a pathophysiological process and also to incorporate new measures (McEwen and Seeman, 1999). For this purpose, the authors introduced a new formulation based on primary mediators leading to primary effects and then to secondary outcomes, which lead, finally, to tertiary outcomes that represent actual diseases. In this regard, primary mediators are chemical messengers, such as cortisol, norepinephrine, or epinephrine that are released as part of allostasis. These mediators cause cellular events (i.e. primary effects), like glucocorticoids regulating gene expression via interaction with DNA. In many cases, these primary effects are organ- and tissue-specific. Additionally, already at this stage pathways of different mediators may converge. Thus, secondary outcomes are mostly the result of more than one primary mediator. They rather reflect the cumulative outcome of primary effects in response to primary mediators (in a tissue- and organ-specific manner). One example for a secondary outcome is elevations in blood pressure. Eventually, tertiary outcomes are actual diseases or disorders such as cardiovascular disease that result of the allostatic load predicted from secondary outcomes.

With this new terminologies, McEwen and coworkers strike a balance between the two extremes of "stress mediators are all protective" and the common sense of "stress makes people ill". The concepts of allostasis and allostatic load overcome the ambiguity of

the term stress and emphasize the inter-individually varying factors constituting a physiological response and hence the resulting susceptibilities. The main advantage of their second framework is that it drives to decide, what a measure, for example elevated blood pressure, ultimately stands for. Such elevations may be part of the pathophysiological pathway of the metabolic syndrome, but they may also be a more primary indication of allostatic load that may lead to accelerated atherosclerosis as well as insulin resistance.

2.1.6 Summary

The sections above gave an overview of stress concept developments, the physiology of central and peripheral stress systems, as well as their activation. Additionally, protective and damaging effects of stress mediators were outlined.

Psychosocial stress was shown to activate the HPA axis as well as the SAM axis. Further, their major end products (i.e., cortisol, epinephrine, and norepinephrine) were shown to have various and complex effects on many important body systems. Most important to the present work, hormones of the endocrine stress response were shown to influence immune functions. These interactions will again be discussed in greater detail in a later section (2.3: psychoneuroimmunology).

However, a closely related question and one of the main questions of this thesis is: What consequences for health and disease are to be expected, if an endocrine stress response is missing permanently and context-independently? In the present work, the exceptional endocrine state ‘Addison’s disease’ was chosen as an approach allowing to investigate this problem formulation specifically in humans. The subsequent section will describe this disease in detail (2.2: Addison’s disease). Given the theoretical background regarding neuroendocrine stress responses outlined in the former sections, the exceptionality of this disease and its potential as an valuable alternative approach for psychoneuroendocrinological and -immunological problem formulations should become apparent.

2.2 ADDISON'S DISEASE

In 1849, Thomas Addison (1793-1860), at this time a 56 year old physician at Guy's Hospital, London, described a remarkable form of progressive pernicious anemia in a paper before the South London Medical Society, published in The London Medical Gazette, n.s: 8: 517-518, 1849:

> *"Its approach is first indicated by a certain amount of languor and restlessness, to which presently succeed a manifest paleness of the countenance, loss of muscular strength, general relaxation or feebleness of the whole frame, and indisposition to, or incapacity for, bodily or mental exertion. (…) the patient experiences a distressing and increasing sense of helplessness and faintness; the heart is excited, or rendered tumultuous in its action, the breathing painfully hurried by the slightest exertion, whilst the whole surface bears some resemblance to a bad wax figure; the patient is no longer able to rise from his bed; slight edema perhaps shows itself about the ankles; the feeling of faintness and weakness becomes extreme, and he dies either from sheer exhaustion, or death is preceded by signs of passive effusion or cerebral oppression." (Addison, 1849)*

Even more interesting than the detailed description of this "idiopathic" anemia is an observation Addison made at the inspections of the bodies of three cases: "In all of them was found a diseased condition of the supra-renal capsules" (Addison, 1849). For the first time this form of anemia was linked to a diseased state of the adrenals.

But it was not until six years later that Thomas Addison was convinced that the concurring facts he observed were not merely casual coincidences. He published a book with the title "On the Constitutional and Local Effects of Disease of the Supra-renal Capsules" (Addison, 1855), in which he described the signs and symptoms of "a morbid state", the leading and characteristic features of which are "anemia, general languor and debility, remarkable feebleness of the heart's action, irritability of the stomach" and – this symptom he did not mention before – "a peculiar change of color of the skin, occurring in connection with a diseased condition of the suprarenal capsules". He additionally reported on the etiopathology and/or the results of post-mortem examinations of 11 cases.

One year later, it is Armand Trousseau (1801-1867), who first proposed to call the suprarenal syndrome "Addison's disease", and in 1928, after reviewing the records of many cases, Brenner narrowed down the cause of Addison's disease and concludes that the symptoms of this disease occur only when most of the adrenal cortex is destroyed and even if the adrenal medulla and the chromaffin tissues are normal (Brenner, 1928).

2.2.1 Definition

Today different forms of adrenocortical insufficiency are distinguished, depending on which level of the HPA-axis is affected and thus the cause of the insufficiency: adrenals, pituitary, or hypothalamus (see fig. 2-7).

Addison's disease or primary adrenal insufficiency (PAI) is caused by bilateral destruction or impaired function of the adrenal cortex, whereas adrenal insufficiency manifests itself as a clinical disease if the functional adrenal mass falls below 10% (Ten et al., 2001). This reduction of adrenocortical cell mass is responsible for the characteristic deficiency of glucocorticoids and mineralocorticoids, or more specifically of cortisol and aldosterone, respectively. (Marzotti and Falorni, 2004). Due to the resulting missing negative feedback, over-secretion of ACTH is another typical symptom of PAI.

Whereas destruction of the adrenal cortex itself is the cause of PAI, secondary adrenal insufficiency (SAI) may occur as a result of pituitary or hypothalamic disease. In both cases the cortisol deficiency is accompanied by an under-secretion of ACTH, and, in the case of hypothalamic diseases, additionally by an under-secretion of CRH. In SAI, only few patients show isolated corticotropin deficiency with adrenal failure (e.g. isolated deficiency of CRH or lymphocytic hypophysitis). Often other hormonal axes are also involved. A more frequent type of isolated SAI is that induced by glucocorticoid therapy (Oelkers, 1996).

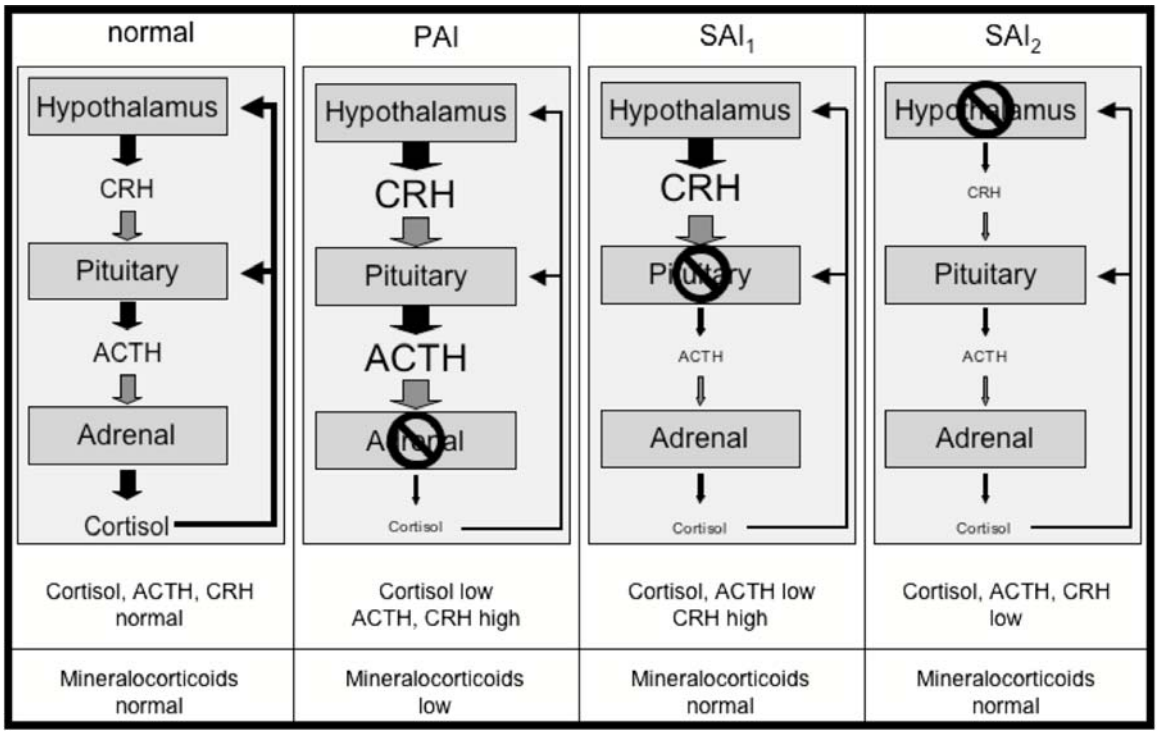

Fig. 2-7: Characteristics of different forms of adrenal insufficiency, compared with a healthy HPA-axis (PAI: primary adrenal insufficiency; SAI: secondary adrenal insufficiency, caused at the level of the pituitary=SAI_1, or at the level of the hypothalamus=SAI_2).

Since this dissertation is focusing on Addison's disease, SAI will not be described in further detail.

2.2.2 Prevalence

Addison's disease is a relatively rare disease with prevalence rates ranging from 39 to 60 per million population according to Oelkers (Oelkers, 1996), 10 to 140 cases per million inhabitants in Western countries according to Marzotti (Marzotti and Falorni, 2004), or near 120 per million according to Ten (Ten et al., 2001). These to some extent incongruent statements can be partially explained by taking into account that prevalence rates for Addison's disease vary between countries: While in New Zealand 4,5 cases per million inhabitants were reported, 50 cases per million are registered in the USA, Northern Europe reports on 40 to 110, and Italy even 117 cases per million inhabitants (Betterle et al., 2002).

Interestingly, Ten et al. point out that a survey of patients with Addison's disease revealed that 60% had sought medical attention from two or more physicians before the correct diagnosis was ever considered. Additionally, no figures are available on the number of undiagnosed patients succumbing to adrenal insufficiency (Ten et al., 2001), leading to the assumption that prevalence rates may be even higher than mentioned above.

The incidence of primary adrenal insufficiency is 4.7-6.2 per million in white populations (Arlt and Allolio, 2003). But while the prevalence is relatively stationary over the years (Betterle et al., 2002), the incidence seems to increase due to variation in rates of causes (Laureti et al., 1999; Lovas and Husebye, 2002). On average, age at diagnosis peaks in the fourth decade of life, with women more frequently affected than men (Arlt and Allolio, 2003). But gender distribution and mean age at diagnosis vary widely depending on the cause of Addison's disease.

2.2.3 Signs, Symptoms, Disease Progression

In Addison's disease, all symptoms and signs can be attributed to glucocorticoid, mineralocorticoid, and/or adrenal androgen deficiency, caused by destroyed adrenals. Unfortunately, most of the symptoms of primary adrenal insufficiency are nonspecific and ill-defined (Betterle et al., 2002). Patients may suffer from fatigue, dizziness, muscular weakness, abdominal pain, vomiting, diarrhea, headache, sweating, muscle and joint pain, and may show behavior changes. Because of this non-specificity of symptoms, the disease may be often misdiagnosed as depression or anorexia nervosa.

Beside these symptoms, signs can be postural hypotension, weight loss, generalized pigmentation, darkened skin creases, pigmented buccal, mucosa, and nail bed, associated vitiligo and/or goiter. Biochemical analyses may reveal high plasma renin levels, hyponatremia, hyperkalemia, hypoglycemia, eosinophilia, and lymphocytosis (Ten et al., 2001). The most specific sign of Addison's disease is the hyperpigmentation of the skin and mucosal surfaces and the most specific symptom is salt craving (Betterle et al., 2002).

An aggravating factor in terms of diagnosis is the time course of the disease. In most cases the destruction of adrenal cells is a rather slow process. A survey, conducted in the Netherlands, comes to the conclusion that the time interval between onset of symptoms and correct diagnosis was, on average, almost 3 years (Zelissen, 1994).

In the case of an acute adrenal insufficiency – i.e., a life-threatening adrenal crisis – typically severe hypotension or hypovolaemic shock, acute abdominal pain, vomiting, and often fever are present. Sometimes these symptoms lead to the wrong diagnosis of an acute abdomen (Arlt and Allolio, 2003). Here the survey reveals that in 50% of patients with Addison's disease, it is an adrenal crisis that leads to the initial diagnosis of adrenal insufficiency (Zelissen, 1994).

2.2.4 Laboratory Assessment of Primary Adrenal Insufficiency

If PAI is suspected, several biochemical test can help to establish the correct diagnosis. If not mentioned otherwise, the following specifications are taken from Oelkers (Oelkers, 1996). The values given are guiding values and can vary according to the assay used.

2.2.4.1 Basal Hormone Measurements

In patients in whom adrenal insufficiency is merely to be ruled out, plasma cortisol levels measured between 8 and 9 a.m. provide a good first assessment of adrenal function. Concentrations of ≥19μg/dl (525nmol/l) rule out adrenal insufficiency, whereas levels ≤3μg/dl (83nmol/l) are indicative of the disorder. For the interpretation of the results it is important to bear in mind that total cortisol levels can be increased as a result of hepatic cortisol-binding globulin production due to e.g. oestrogen. Additionally, cortisol concentrations vary throughout the day. Therefore, the diagnostic usefulness of random samples is limited. Furthermore, hydrocortisone, methylprednisolone, and prednisone cross-react in the cortisol assay and should be avoided within 24 hours of testing (Grinspoon and Biller, 1994).

To localize the cause of insufficiency, ACTH concentrations can be tested. Here ACTH levels are supposed to be higher than normal because cortisol feedback inhibition is absent. Levels ≥100pg/ml (22pmol/l) are indicative of PAI, even if plasma cortisol concentration is in normal range. In PAI, also aldosterone concentrations are low or at the lower end of normal values, whereas the plasma renin activity or concentration is increased because of sodium wasting.

2.2.4.2 Pharmacological Stimulation tests

The most commonly used pharmacological test for the diagnosis of PAI is the short corticotropin stimulation test. 250μg of $ACTH_{1-24}$ (cosyntropin, tetracosatrin, Cortrosyn, or

Synacthen) are given intravenously or intramuscularly before 10a.m., and plasma cortisol levels are measured before and 30 and/or 60 minutes after the injection. In the case of PAI, no increase in plasma cortisol is to be expected, since the adrenal cortex is maximally stimulated by endogenous ACTH. Accordingly, peak cortisol levels of <500nmol/l lead to the diagnosis of PAI.

Since the 250μg ACTH stimulation test induces supraphysiological ACTH concentrations, it has been argued that in the case of acute-onset and mild *secondary* adrenal insufficiency, this dose might over-stimulate partially atrophied adrenals and produce a deceivingly adequate cortisol response (Oelkers, 1996). Therefore, the 1μg synthetic ACTH test has been suggested to be more sensitive (Abdu et al., 1999), especially since the cortisol response to 1μg is equivalent to that obtained with 250μg in normal subjects (Dickstein et al., 1997). Laureti et al. (Laureti et al., 2000) demonstrated that the sensitivity of the low-dose (1μg) ACTH test is similar to that of the high-dose (250μg) ACTH test also for pre-clinical primary adrenal dysfunction and concluded that the low-dose ACTH test has a high diagnostic value also for PAI.

To differentiate between primary and secondary adrenal insufficiency, infusions of $ACTH_{1-24}$ *iv* or *im* over a period of 48-72h with cortisol measurements the day before and repeatedly over the treatment may also be helpful. In PAI, no rises in cortisol levels are to be expected, whereas in secondary adrenal insufficiency cortisol levels starts to rise due to the slow re-activation of adrenal cortex cells (Grinspoon and Biller, 1994).

Another widely used pharmacological test for the assessment of the PHA axis is the insulin tolerance test (ITT). Insulin (0.1U/kg) is administered *iv* to induce hypoglycaemia (<2.2mmol/l). Hypoglycaemia is an indirect stimulus to cortisol release via activation of hypothalamus and pituitary gland. Accordingly, cortisol is measured 15 minutes before, immediately before, and 30, 45, 60, 90, and 120 minutes after injection. An absolute cortisol increase to circulating values >550nmol/l typically together with a relative increase of >200-250nmol/l is considered a normal response (Nylen and Muller, 2004). In PAI, but also in SAI, no increase in cortisol secretion is to be expected. Due to the adverse side effects of this test, it is contraindicated not only in children, elderly, and patients with a history of seizures, ischemic heart disease, or epilepsy (Ammari et al., 1996; Rasmuson et al., 1996), but also unnecessary in patients already known to have low basal plasma cortisol concentrations.

The short metyrapone test was developed specifically to assess the pituitary ACTH reserve, but may also be valuable to diagnose PAI. In this test, a single dose of 2.0g metyrapone is given *po* at 2400 h and ACTH, 11-deoxycortisol, and cortisol is measured the following morning. Metyrapone inhibits the adrenocortical enzyme 11β-OH, leading to a decrease of circulating cortisol, thereby stimulating ACTH production, which in turn causes the cortisol precursor 11-deoxycortisol to accumulate. In PAI, baseline ACTH levels are high

and following metyrapone administration, 11-deoxycortisol levels are still low and ACTH levels do not change (Berneis et al., 2002).

2.2.4.3 Radiologic Evaluation

After an endocrinologic diagnosis is established by hormone tests, abdominal computer tomography scans can be helpful for differential diagnosis. Marked enlargement of the adrenal glands with or without calcifications in patients with tuberculous adrenal insufficiency is usually a sign of active infection and an indication for treatment with anti-tuberculosis drugs. The adrenal glands are also enlarged in patients with adrenal insufficiency caused by fungal infections, metastatic cancer, lymphoma, and AIDS (Oelkers, 1996). In contrary, in patients with autoimmune AD the adrenal glands are small and often weigh only about 1g in end-stage disease (Betterle et al., 2002).

2.2.4.4 Adrenal Autoantibody Tests

Adrenal autoantibodies are an important marker of an ongoing adrenal autoimmune process and hence adrenal autoantibody tests are valuable for early diagnosis but also for differential diagnosis. Therefore, adrenal autoantibodies are discussed in detail in section 2.4.2.1 (Idiopathic Addison's Disease as an Autoimmune Disease) and in section 2.4.2.3 (Serology and Immunogenetics).

2.2.5 Treatment

In the late 1920s and early 1930s, growing interest in the role of the adrenals in diseases led physicians to start working with adrenal cortex extracts. In most cases weak positive effects were observed in adrenalectomized cats and dogs (Stewart, 1929) and patients suffering from Addison's disease (Hartman, 1930). Meanwhile, also the physiologic function of the adrenal cortex came into focus, particularly in regard to sodium and water metabolism. In 1927, Marine and Baumann reported of increased survival of ADX cats by injection of Ringer's solution or sodium chloride (Marine, 1927). One year later, Rogoff and Stewart reported similar results in dogs (Rogoff, 1928), and in 1933, Loeb published the effects of sodium chloride treatment in patients with Addison's disease (Loeb, 1933). In 1937, DeFremery reported on the isolation of corticosterone from the adrenal (DeFremery, 1937) and Steiger and Reichstein on the synthesis of deoxycorticosterone (Steiger, 1937). First reports of treatment of patients with Addison's disease came from Simpson (Simpson, 1938) and Thorn et al. (Thorn, 1939).

Today, treatment of patients with Addison's disease accounts for both missing glucocorticoids and mineralocorticoids. Glucocorticoid replacement is usually given in two or three daily doses, with a half to two-thirds of the daily dose administered in the morning to

mimic the circadian secretion pattern of cortisol (Arlt and Allolio, 2003). The dosage is based on the normal daily cortisol production rate of 5.7±0.3mg/m^2·day (Kerrigan et al., 1993), which translates to about 10-20mg/m^2·day of oral hydrocortisone (Ten et al., 2001), accounting for step-down losses from absorption, hepatic processing, and metabolic bioavailability. Initially, the treatment starts with doses of 25mg hydrocortisone (HC, cortisol, 15 + 10mg) or 37.5mg cortisone acetate (25 + 12.5mg) (Oelkers, 1996). The daily dose may be decreased to 20 or 15mg of HC to prevent over-replacement, which can lead to weight gain, osteoporosis and impaired glucose tolerance. On the other hand, under-replacement bears the risk of incipient crisis and impairment of wellbeing. The goal should be to use the smallest dose that relieves the patient's symptoms (Betterle et al., 2002). Determination of the appropriate dose is mainly based on clinical judgment, taking into account signs and symptoms potentially suggestive of glucocorticoid over-replacement or under-replacement, or relies on urinary cortisol measurements. The latter is suitable as a marker to only a limited extent. Since after HC administration cortisol-binding globulins are rapidly saturated, renal cortisol excretion is transiently but clearly increased. Urinary cortisol should therefore only be used as a marker for HC over-treatment (Peacey et al., 1997).

Mineralocorticoid replacement consists of oral administration of fludrocortisone in a single daily dose of 50-200μg. The dose is guided by measurements of blood pressure, serum potassium, and plasma renin activity (Betterle et al., 2002). Additional markers can be serum sodium concentrations and appearance of peripheral oedema (Arlt and Allolio, 2003).

Optional is the replacement of dehydroepiandrosterone (DHEA) to increase well-being and mood, taken daily as one dose of 25-50mg DHEA in the morning (Arlt and Allolio, 2003). However, Lovas summarizes that so far the evidence for beneficial effects of DHEA replacement is weak and long-term safety requires further consideration (Lovas and Husebye, 2003).

Acute adrenal insufficiency has to be treated immediately with high doses of HC *iv*. Usually, a bolus dose of 100mg is followed by infusion of 100-200mg over a period of 24 hours (Oelkers, 1996). Large volumes of isotonic saline and glucose *iv* are also recommended to obviate hypovolemia and hyponatremia. In the case of febrile illness or injury the dose of HC should be doubled or tripled temporarily and in the case of vomiting glucocorticoid suppositories are recommended (Betterle et al., 2002).

2.2.6 Etiology of Addison's Disease

Addison's disease can have various etiologies. According to Ten et al. (Ten et al., 2001), they can be grouped into three categories: 1) adrenal dysgenesis; 2) adrenal destruction; and 3) impaired steroidogenesis.

Adrenal dysgenesis may be due to congenital adrenal hypoplasia (AHC), mutations of steroidogenic factor-1 (SF-1), or ACTH unresponsiveness. The destruction of the adrenal glands can be caused by autoimmune polyglandular syndrome (APS), adrenoleukodystrophy (ALD), adrenal hemorrhage, adrenal metastases, infections, or amyloidoses. Impaired steroidogenesis may be due to congenital adrenal hyperplasia (CAH), mitochondrial disorders, or the Smith-Lemli-Opitz syndrome (SMOS).

Another classification offers Betterle (Betterle et al., 2002). He distinguishes between autoimmune adrenalitis, infectious adrenalitis, Addison's disease due to neoplastic diseases, adrenal hemorrhage, or adrenal thrombosis, drug-induced adrenal insufficiency, neonatal causes, and genetic causes of Addison's disease (see table 2-3).

Many of the listed causes are very rare. For example, Betterle studied 317 patients with PAI and found 9 cases with adrenoleukodystrophy (2.8%), and 3 cases with neoplastic diseases (0.9%; Betterle et al., 2002). Therefore, the following sections will focus on the main causes of Addison's disease, which are infectious, or more precisely, tuberculous adrenalitis (section 2.4.1), and autoimmune adrenalitis (section 2.4.2).

Tab. 2-3: Etiology of Addison's disease (modified from Betterle et al., 2002).

Autoimmune adrenalitis	
Infectious adrenalitis	Tuberculosis, fungal, viral
Neoplastic diseases	adrenal carcinomas, metastasis
Adrenal hemorrhage	Waterhouse-Friderichsen syndrome, anticoagulation therapy, traumas
Adrenal thrombosis	Systemic lupus erythematosus, panarteritis nodosa, antiphospholipid syndrome, traumas
Drug-induced	adrenolitic therapy, other agents, anticoagulation
Other causes	sarcoidosis, amyloidosis, hemochromatosis, histiocytosis
Neonatal	maternal Cushing's syndrome, traumas at birth
Genetic	adrenoleukodystrophy, congenital adrenal hypoplasia, familial ACTH resistance syndromes (familial glucocorticoid deficiency, Triple A syndrome), Kearns-Sayre syndrome, congenital adrenal hyperplasia, Smith-Lemli-Opitz syndrome

2.2.6.1 Infectious Addison's Disease

On postmortem examination of 11 of his patients, Addison found six cases with adrenal tuberculosis. During this times, tuberculous adrenalitis was by far the most prevalent cause of adrenal insufficiency. Today, tuberculosis is still a major cause of disease in the developing world (Ten et al., 2001). For example, Soule reviewed retrospectively the case notes of patients with Addison's disease admitted to a South African Teaching Hospital from 1980 to 1997. In 34% of all patients, active or previous tuberculosis was diagnosed (Soule,

1999). Moreover, with an incidence of 6%, the adrenal gland is the fifth major organ involved in extra-pulmonary tuberculosis (Lam and Lo, 2001) and the most commonly involved endocrine organ in tuberculosis (Kelestimur, 2004). Tuberculosis is also the most common cause for infectious forms of Addison's disease and with approximately 74% (Betterle et al., 2002) the main non autoimmune cause overall.

In developed countries the prevalence of tuberculous adrenalitis is usually below 10% (Zelissen et al., 1995), but it is conceivable that in future studies relatively more cases of Addison's disease of tuberculous origin will be encountered (Rieder, 1992), since the incidence of tuberculosis in many industrialized countries recently failed to decline and in eastern Europe and the former Soviet Union cases and deaths are even increasing (Raviglione et al., 1995).

Tuberculous Addison's disease may be characterized by enlarged or atrophied adrenal glands. CT scans reveal large glands in a recent and probably active infection, whereas small calcified glands represent remote and probably inactive infection. In the case of recent or active infection it is unclear, whether the adrenal glands are enlarged as a result of direct involvement of the glands by tuberculosis, or as a result of stressful conditions due to tuberculosis which increases cortisol requirement (Kelestimur, 2004).

More uncommon causes in infectious adrenalitis are fungal diseases, such as histoplasmosis and coccidioidomycosis (Ten et al., 2001), or viral infections (i.e., cytomegalovirus and HIV) (Betterle et al., 2002). Regarding viral infections, specifically HIV infections, interdependency between HIV, tuberculosis and Addison's disease has to be considered: in HIV-infected persons the incidence of active tuberculosis is increased (Selwyn et al., 1989), and tuberculosis in turn increases the risk for Addison's disease (Freda et al., 1994).

2.2.6.2 Autoimmune Adrenalitis

In developed countries, autoimmune adrenal destruction is the major cause of Addison's disease, affecting approximately 90% of all patients with primary adrenal insufficiency (Winqvist et al., 1996). The following section addresses findings, which led to the view of idiopathic Addison's disease to be an autoimmune disease. Subsequently, the association of Addison's disease with other autoimmune disorders and its role as part of an autoimmune polyendocrine syndrome (APS) will be discussed. Additionally, serological and immunogenetic findings will be presented.

2.2.6.2.1 Idiopathic Addison's disease as an autoimmune disease

Until 1957, the cause of many cases of Addison's disease were unknown and hence labeled idiopathic. Already Addison reported of one case of adrenal fibrosis of unknown origin, which he described as follows: "the two adrenals together weighted 49 grains, they

appeared exceedingly small and atrophied, so that the diseased condition did not result as usual from a deposit either of a strumous or malignant character, but appears to have been occasioned by an actual inflammation that inflammation having destroyed the integrity of the organs, and finally led to their contraction and atrophy" (Addison, 1855). With this description of adrenals destroyed by inflammation, Addison provided the first indication of the cause of idiopathic adrenal insufficiency.

More than 100 years later, in 1957, Anderson et al. discovered circulating adrenal cortex autoantibodies (Anderson et al., 1957) and subsequent reports indicated that idiopathic Addison's disease might be autoimmune in nature: Blizzard & Kyle demonstrated the organ specificity of the adrenal antibodies and isolated specific antigens in the adrenal cortical microsomes and mitochondria (Blizzard and Kyle, 1963) and Khoury et al. reported the presence of *surface*-reactive autoantibodies to human adrenal cells (Khoury et al., 1981). Studies of Furmaniak et al. suggested that autoantibodies in the serum of patients with Addison's disease interact with a 55 kDa adrenal specific protein (Furmaniak et al., 1988). Four years later, Krohn et al. identified this 55 kDa adrenal protein as steroid 17 alpha-hydroxylase (17α-OH) (Krohn et al., 1992) and Winqvist et al. reported on a protein with an apparent molecular weight of 54 kDa as another major autoantigen in idiopathic Addison's disease, which they identified as 21-hydroxylase (21-OH) (Winqvist et al., 1992). Both enzymes are members of the family of P450 cytochromes and key enzymes in the steroid hormone synthesis of the human adrenal cortex (see fig. 2-8). Bednarek et al. hypothesized that in early onset Addison's disease, which is most commonly associated with hypoparathyroidism and mucocutaneous candidiasis (see below: APS-1), autoantibodies seem to be directed against 17α-OH, whereas in adult onset Addison's disease autoantibodies are directed against 21-hydroxylase (Bednarek et al., 1992). In 1993, Winqvist et al. revised the findings of Krohn et al. regarding 17α-OH and stated that P450 side chain cleavage (P450scc), an enzyme located in the mitochondria, is the major adrenal autoantigen identified by sera of patients with early onset Addison's disease (Winqvist et al., 1993). Yet, as Winqvist et al. pointed out, the role of autoantibodies in the pathogenesis of Addison's disease is still unknown, since it is hard to imagine that autoantibodies can react with their intracellular autoantigens in intact cells, and the antigens in turn has not been convincingly shown to be present on the cell surface (Winqvist et al., 1996).

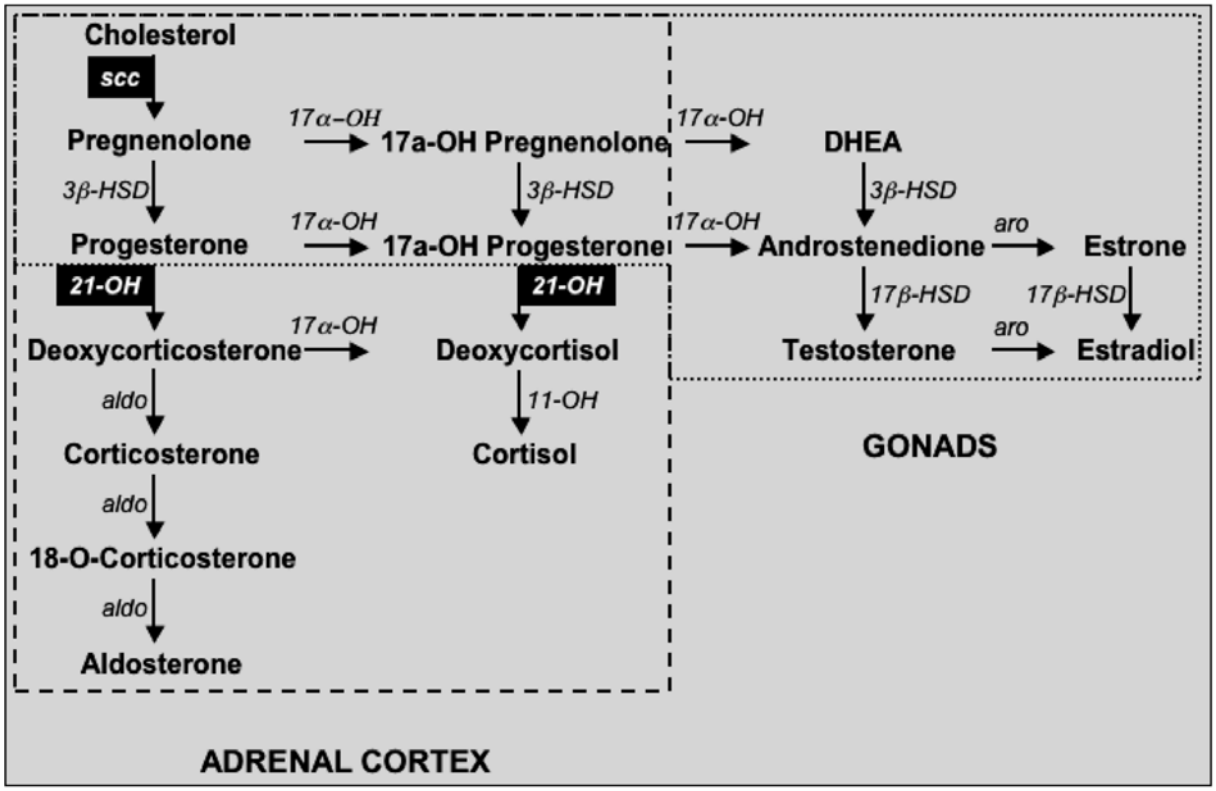

Fig. 2-8: Steroid hormone synthesis pathways (scc: side chain cleavage; 17α-OH: 17-alpha-hydroxylase; 21-OH: 21-hydroxylase; 11-OH: 11-hydroxylase; 17β-HDS: 17-beta-hydroxysteroiddehydrogenase; aldo: aldosterone synthase; aro: aromatase), white on black: steroid synthesis limiting enzymes in Addison's disease.

On the other hand, there is evidence suggesting that also cellular immunity plays a critical role in the pathogenesis of Addison's disease: In 1958, Colover and Glynn observed lesions in the adrenal gland and infiltrating cells with irregular basophilic nuclei after injections of Freund's adjuvant (i.e., homologous guinea pig adrenal gland with heat-inactivated human tubercle bacillus residues) in guinea pigs and hypothesized that this experimental adrenalitis may be of immunological nature (Colover and Glynn, 1958). Post-mortem examinations of adrenals from patients with Addison's disease revealed infiltrating lymphocytes and Fujii et al. observed infiltrations with polymorphonuclear leukocytes in the cortex regions of the adrenal glands in mice after repeated injection of adrenal extract (mixed with KO3 LPS), which were later replaced by mononuclear cells, such as small lymphocytes and macrophages (Fujii et al., 1992). Furthermore, the repeated immunization caused a delayed type hypersensitivity to adrenal antigens and Fujii et al. were able to produce adrenalitis in normal mice by transfer of spleen cells from hyper-immunized mice. Freeman and Weetman showed a proliferative T-cell response to an adrenal-specific protein fraction in the molecular weight range of 18-24 kDa (Freeman and Weetman, 1992). These results point to a possible interaction of B- and T-cell response in Addison's disease, in which peptides derived from the B-cell autoantigen 21-OH may be involved in the T-cell-mediated autoimmunity of Addison's disease (Winqvist et al., 1996). Additionally, Partanen et al. showed that patients with isolated Addison's disease or Addison's disease in association with

autoimmune hypothyroidism, Graves' disease, and/or type 1 diabetes share highly similar major histocompatibility complex (MHC) class II and class III alleles (Partanen et al., 1994). But although the genes encoding 21-OH enzyme are located within the MHC-III region, the presence of autoantibodies against 21-OH was not found to be directly determined by the MHC alleles, as patients with identical markers but different clinical forms of Addison's disease had different reactivity against 21-OH.

Altogether, these studies give proof of the autoimmune nature of idiopathic Addison's disease according to the revisited postulates of Witebsky by Rose & Bona (Rose and Bona, 1993) (see table 2-4) and Betterle et al. summarized these evidences as follows: 1) histopathological findings of a diffuse mononuclear cell infiltration progressing to atrophy of all the three layers of the adrenal cortex; 2) demonstration of a cell-mediated immunity to adrenal cortex antigens; 3) ability to induce the disease in animal models by immunization with adrenal cortex extracts; 4) identification of steroidogenic enzymes expressed in adrenals as self-antigens; 5) association with other organ-specific autoimmune diseases; 6) association with antigens of the major histocompatibility complex (Betterle et al., 2002).

Retrospectively, the case of adrenal fibrosis of unknown origin described by Addison turns out to be the very first description of an autoimmune adrenalitis in the literature.

Tab. 2-4: Revisited postulates of Witebsky by Rose & Bona (Rose and Bona, 1993).

direct proof: based on a) production of the disease by human-to-human transfer of autoantibodies, b) production of the disease by human-to-animal transfer of autoantibodies, c) production of the disease by transfer of autoreactive T cells to SCID mice, or d) in vitro destruction of cells carrying the corresponding antigen by autoantibodies)
indirect evidence: based on e) reproduction of the autoimmune disease in experimental animal models, f) genetically induced disease models, isolation of autoantibodies, or g) isolation of self reactive T cells form organs representing the major target of autoimmune disease
circumstantial evidence: based on distinctive clinical clues a) association with other autoimmune diseases in the same individual / the same family, b) lymphocytic infiltration of target organs, c) statistical association with a particular MHC haplotype or aberrant expression of MHC class II antigens on the affected organ, or

2.2.6.2.2 APS: autoimmune polyendocrine syndrome

Starting in the mid seventies, numbers of records were increasing, which show that patients with autoimmune Addison's disease also have a higher prevalence of other autoimmune disorders (Irvine, 1975). In 1981, Neufeld, Maclaren and Blizzard distinguished two types of autoimmune Addison's disease, which are associated with different polyglandular autoimmune (PGA) syndromes (Neufeld et al., 1981).

The following sections introduce these two types of polyglandular autoimmune syndromes, also called autoimmune polyendocrine syndromes (APS-1 and APS-2) as well as a third and a fourth type, namely APS-3 and APS-4. Isolated autoimmune Addison's disease will be discussed in the last section.

APS-1

The first description of an association between hypoparathyroidism and candidiasis was published in 1929 (Thorpe and Handley, 1929), and the association of these two diseases with idiopathic adrenal insufficiency was reported in 1946 (Leonard, 1946). Since then, the autoimmune polyglandular syndrome type 1 (APS-1) has been described under varying names, such as Whitaker's syndrome (Esselborn et al., 1956), polyglandular autoimmune disease type 1 (Neufeld et al., 1981), or autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED; Ahonen et al., 1990). Independent of the label, its major components are chronic mucocutaneous candidiasis, chronic hypoparathyroidism, and autoimmune adrenal insufficiency. To define this syndrome, at least two of these diseases have to be present in one individual, while the three main components of APS-1 are present together in only about one third to one half of the cases (Betterle et al., 1998).

APS-1 is inherited in an autosomal-recessive mode and a very rare disorder. The highest numbers of patients are found among Finns, Sardinians, and Iranian Jews, with estimated prevalence rates of 1 in 25.000, 14.000 and 9.000 inhabitants, respectively, whereas only limited numbers of cases are found in other parts of the world (Peterson et al., 2000). It is generally thought that due to the autosomal recessive mode of inheritance males and females are equally affected. Nevertheless, the female/male ratio varies widely between 0.8 in Finland (Ahonen et al., 1990) and 2.4 in Italy (Betterle et al., 1998).

Chronic mucocutaneous candidiasis is generally the first disease and the most frequent of the three main diseases of APS-1. It can appear at the first month after birth and up to 21 year of age, but usually does before the age of 5 (Betterle et al., 1998). It is present in 73-100% of all patients and affects nails, dermis, and oral, vaginal, and esophageal mucous membranes (Betterle et al., 1998). The disease is accompanied by a normal B-cell response of serum antibodies to candidal antigens, which may be responsible for preventing the systemic spread of infection (Peterson et al., 1996). However, the cause of this disease is considered to be a defective suppressor T-cell function to the antigen *Candida albicans* (Arulanantham et al., 1979). For this reason, the World Health Organization (WHO) classified APS-1 in 1995 as an acquired immunodeficiency (WHO, 1995).

Hypoparathyroidism usually occurs after chronic mucocutaneous candidiasis and before Addison's disease at an age between 3 months to 44 years. It is present in 73-90% of the cases of APS-1 (Betterle et al., 1998). Due to the autoimmune destruction of the

parathyroid gland, a common symptom in APS-1 is hypocalcaemia, since the parathyroid gland is one of the major organs involved in CA^{2+} homeostasis (Winqvist et al., 1996).

Autoimmune Addison's disease is the third disease to appear, usually between 6 months and 41 year of age, and occurs in 60-100% of cases of APS-1.

Diseases less frequently associated with APS-1 are summarized according to Betterle, Greggio, and Volpato, including details on frequencies, where given (Betterle et al., 1998): other autoimmune endocrinopathies (hypergonadotropic hypogonadism: 17-50%; insulin-dependent diabetes mellitus: 1.2-12%; autoimmune thyroid diseases: 2-13%; and pituitary defects), autoimmune or immuno-mediated gastrointestinal diseases (chronic atrophic gastritis: 13-15%; pernicious anemia: 11-13%; and malabsorption), chronic active hepatitis (8-26%), autoimmune skin diseases (vitiligo: 8-13%; alopecia: 29-32%), ectodermal dystrophy (77-82%), keratoconjunctivitis (8-41%), immunological defects (cellular and humoral), asplenia, and cholelithiasis.

In summary, APS-1 is associated with a broad clinical spectrum with the majority of patients having three to five manifestations, some of which may not appear until the fifth decade. Therefore, Ahonen et al. pointed out that all patients need lifelong follow-up for detection of new components of the disease (Ahonen et al., 1990).

APS-2

In 1926, Schmidt described two patients with nontuberculous Addison's disease and chronic lymphocytic thyroiditis (Schmidt, 1926), a syndrome later called Schmidt's syndrome. Five years later, the first case of Addison's disease with *hyper*thyroidism and diabetes mellitus was described (Rowntree and Snell, 1931), and in 1932 Gowen reported on a patient affected by Addison's disease, *hypo*thyroidism and diabetes mellitus (Gowen, 1932). Since then reported cases increased every decade. Today, the autoimmune polyglandular syndrome type 2 (APS-2) is defined as a combination of autoimmune Addison's disease, which must always be present, and autoimmune thyroid disease and/or type 1 diabetes mellitus (Betterle et al., 2002). APS-2 is also termed Carpenter's syndrome (Betterle et al., 2004). In the vast majority of cases, the thyroid autoimmune disease is Hashimoto's thyroiditis or idiopathic myxedema (i.e., hypothyroidism), and in the remaining cases Graves' disease (i.e., hyperthyroidism) (Carpenter et al., 1964).

However, the complete triad can be observed only in approximately 11% of all cases (Betterle et al., 2002) and approximately 28% of patients with autoimmune Addison's disease are also diagnosed APS-2 (Zelissen et al., 1995). APS-2 is therefore a rare condition with 1.4-4.5 cases per 100.000 inhabitants (Betterle et al., 2004). The clinical onset of APS-2 usually starts in early adulthood with peak age at onset between 30 and 40 years (Zelissen et al., 1995). The majority of patients with APS-2 are females, with a female/male ration of 2-3.7 (Betterle et al., 2002).

The components of APS-2 tend to develop in a specific sequence. Based on data of sixty patients with APS-2, Betterle et al. summarized that type 1 diabetes mellitus develops in general before autoimmune Addison's disease, whereas autoimmune thyroid diseases develop before, contemporary with, or after Addison's disease (Betterle et al., 1996). Eight years later, now based on data of 146 patients with APS-2, Betterle, Lazzarotto, and Presotto reported on mean ages at disease onset of 28.4 (range: 2-63) in type 1 diabetes mellitus, 34.6 (range: 1-85) in Addison's disease, and 40.2 (range: 12-80) in chronic thyroiditis. Among thyroid disorders, Graves' disease usually precedes (mean age at disease onset: 33.4; range: 7-58), whereas Hashimoto's thyroiditis follows Addison's disease (Betterle et al., 2004).

Beside the three main diseases, several minor diseases are reported in APS-2. Among these, the more frequently diagnosed are vitiligo (12% of cases) and hypergonadotropic hypogonadism (10% of cases). Less than 5% of patients with APS-2 additionally suffer from chronic autoimmune hepatitis (3%), alopecia (4%), pernicious anaemia (2%), or seronegative arthritis (2%; Betterle et al., 2004).

Since hardly ever the syndrome emerges with two or three main autoimmune diseases simultaneously, it is of considerable importance to identify among patients with only one main or minor disease those at risk for future development of fully expressed APS-2. For example, Zelissen, Bast and Croughs found in 58% of patients with isolated Addison's disease autoantibodies to the thyroid gland (Zelissen et al., 1995), putting these patients at a ~50% risk for future development of thyroiditis. On the other hand, patients suffering from thyroid autoimmune diseases or type 1 diabetes mellitus, who are also tested positive for autoantibodies to the adrenal cortex or 21-OH have a 30% risk for future development of Addison's disease (Betterle et al., 2004).

In this context, Betterle, Lazzarotto and Presotto consider the clinically overt syndrome (i.e., APS-2) as only the 'tip of an iceberg', since latent or incomplete forms are much more frequent. Therefore, early identification and treatment of other autoimmune endocrine diseases may be critical and even life-saving (Betterle et al., 2004).

APS-3 and APS-4

In addition to the above described two types of autoimmune polyglandular syndromes, Neufeld and Blizzard distinguished in their publication in 1980 two additional types (Neufeld et al., 1980): Type 3 is classified as thyroid autoimmune diseases (TAD) associated with other autoimmune diseases, *excluding* Addison's disease and/or hypoparathyroidism. Type 4 is classified as combination of organ-specific autoimmune diseases not included in the previous types.

Unfortunately, there is no general agreement regarding the syndrome classification, i.e., the number of APS types. Eisenbarth and Gottlieb summarized the ongoing controversy

and contrasted the “splitters” and the “lumpers” (Eisenbarth and Gottlieb, 2004). The former group discriminates the four already mentioned types of syndromes and additionally subdivides APS-3 in the four groups 3A (TAD + type 1 diabetes mellitus), 3B (TAD + chronic atrophic gastritis and pernicious anemia), 3C (TAD + vitiligo, alopecia, miastenia gravis), and 3D (TAD + other unspecified diseases), according to Neufeld (Neufeld et al., 1980). Betterle and Zanchetta suggested even a revision of the original classification by Neufeld and proposed a new classification of the 4 subgroups of APS-3 (Betterle and Zanchetta, 2003). They differentiate between endocrine diseases (3A), diseases affecting the gastrointestinal apparatus (3B), diseases affecting the skin / hemopoietic system / nervous system (3C), and collagen diseases/vasculitis (3D).

On the other hand, the “lumpers” consider all the combinations of APS type 2, 3, and 4 as APS-2. For example, Dittmar and Kahaly argue that no clinical differences between APS-2 and APS-3 have been described (Dittmar and Kahaly, 2003). Accordingly, they do not differentiate between them and also Eisenbarth and Gottlieb annotate that they tend to agree with this group of “lumpers” (Eisenbarth and Gottlieb, 2004).

Since there is no definite time-point at which one specific disease of the various diseases constituting the syndrome has to appear, as well as no definite specific sequence in which the different diseases have to develop, there is always the possibility that a patient diagnosed with APS-3 or APS-4 will later in his life develop the necessary additional disorder (i.e., Addison’s disease) and therefore “qualify” for APS-2. Therefore, in the following sections it will be distinguished only between APS-1 and APS-2.

Isolated autoimmune Addison’s disease

Isolated autoimmune Addison’s disease is characterized by Addison’s disease in *absence* of any other above described diseases. Here, a very similar problem appears: It is not clear what distinguishes a patient with a single disorder, such as isolated Addison’s disease, from a patient with multiple additional autoimmune disorders. Comparing the age at disease onset, isolated Addison’s disease is possibly just the first manifestation of diseases constituting APS-2 (Eisenbarth and Gottlieb, 2004). Especially, since in both cases a HLA-association as well as thyroid, gastric and islet-cell autoantibodies can be found. This points to a common etiology and suggests that isolated Addison’s disease is in fact a variant of APS-2 (Peterson et al., 2000). Winqvist et al. likewise concluded that isolated Addison’s disease and Addison’s disease as part of APS-2 may be regarded as the same entity (Winqvist et al., 1996). The only difference between isolated autoimmune Addison’s disease and APS-2 may simply be time (Eisenbarth and Gottlieb, 2004).

2.2.6.2.3 Serology and immunogenetics of Addison's disease

In all above described forms of autoimmune Addison's disease, whether isolated or as part of an APS, distinctive serological and immunogenetic features can be observed. The first reports in this regard were already described above.

These distinctive features are not only important for differential diagnosis, but also may help to uncover the diverse underlying immunological mechanisms. Hence, in the following sections the serological as well as the genetic background of autoimmune Addison's disease will be discussed in greater detail and current theories regarding the underlying mechanisms will be quoted.

Serology of Addison's disease

In 50-90% of all patients with Addison's disease adrenocortical autoantibodies (ACAs) can be detected (Peterson et al., 2000). The target autoantigens thereby are P450 cytochromes, more precisely the enzymes 17alpha-hydroxylase (17α-OH), 21-hydroxylase (21-OH), and cholesterol side chain-cleaving enzyme (scc; see figure 2.8). Immunofluorescence studies showed that both 21-OH and scc antigens are found in all three layers of the adrenal cortex, while sera reacting with the antigen 17α-OH do not stain the zona glomerulosa (Uibo et al., 1994).

While 21-OH is present in the adrenal glands only, the antigens 17α-OH and scc are found in all steroid-producing organs, such as the Theca interna and corpus luteum of the ovary, Leydig and Sertoli cells of the testis, and in placental trophoblasts (in the latter scc only; Peterson et al., 2000). To distinguish such antibodies from ACAs, they have generally been named steroid cell antibodies (StCA) and the presence of StCAs correlates with a high risk of gonadal failure in Addison's disease patients (Peterson et al., 2000). Here, the major antigen seems to be scc, but not 17-OH, at least in APS-1 (Soderbergh et al., 2004).

Interestingly, in isolated Addison's disease and in APS-2, predominantly antibodies against 21-OH are found with prevalence rates of 25-94% and 78-96%, respectively. Autoantibodies to 17α-OH and scc are rare in isolated Addison's, but in APS-2 they can be detected in up to a third of patients studied (Peterson et al., 2000). In APS-1, the literature is not unanimous as to which of these three types of antibodies is predominant. Martorell et al. summarize that 17α-OH and scc seem to be the antigens most often involved (Martorell et al., 2002). This is in contrast to findings of Söderbergh et al., who performed a multivariate logistic regression analysis for the presence of ten autoantibodies as independent predictors for different disease manifestations in 90 APS-1 patients from Finland, Norway, and Sweden. Here, all three autoantibodies were associated with Addison's disease, but only 21-OH and scc autoantibodies were independent markers and thus Söderbergh et al. concluded that

these enzymes constitute the major adrenal cortex autoantigens in APS-1 (Soderbergh et al., 2004).

Beside P450 enzymes as the major antigens in Addison's disease, additionally autoantibodies to other organs and cells can be found, especially in the context of APS-1. Klemetti et al. as well as Velloso et al. described high autoantibody titers and T-cell-mediated responses to the 65-kDa isoform of glutamic acid decarboxylase (GAD65), the major autoantigen in type 1 diabetes (Klemetti et al., 2000; Velloso et al., 1994). Autoantibodies to GAD65 were also detected in 14% of patients with idiopathic AD (Laureti et al., 1998a). Rorsman et al. demonstrated autoantibodies against another pancreatic β-cell antigen, aromatic l-amino acid decarboxylase (AADC), in APS-1 patients (Rorsman et al., 1995), which have been found recently also in a small subgroup of patients with isolated Addison's disease and APS-2 (Soderbergh et al., 2000). Since APS-1 is often associated with autoimmune hepatitis, Clemente et al. were interested in liver P450 cytochrome autoantibodies. They found autoantibody reactivity with P4501A1, P4501A2, P4502A6, and P4502B6 cytochromes, but none of the APS-1 patients had autoantibodies to P4502D6, the major target in autoimmune hepatitis (Clemente et al., 1998). Hypoparathyroidism has in one study been suggested to result from an autoimmune reaction directed against the extracellular domain of the Ca^{2+}-sensing receptor (CaSR), a parathyroid gland-specific protein (Li et al., 1996). Ekwall et al. and Hedstrand et al. identified the enzymes tryptophan hydroxylase (TPF) and tyrosine hydroxylase (TH) as autoantigens associated with gastrointestinal dysfunction and alopecia, respectively, in APS-1 (Ekwall et al., 1998; Hedstrand et al., 2000). Additionally, an association between vitiligo and autoantibodies against melanocytes was found in APS-1 patients (Song et al., 1994). Regarding mucocutaneous candidiasis as one of the major disease components in APS-1, the glycolytic enzyme enolase seems to be the immunodominant antigen (Peterson et al., 1996).

In isolated Addison's disease and APS-2, thyroperoxidase autoantibodies, thyroglobulin autoantibodies, H+/K(+)-ATPase autoantibodies, and GAD65 autoantibodies are frequently detected (Falorni et al., 2002), demonstrating again the close relationship between isolated Addison's disease and APS-2.

Nevertheless, P450 enzymes and ACAs as well as StCAs are still of major interest in Addison's disease, since high correlations between ACAs and 21-OH autoantibodies exist also in patients with pre-Addison's disease and both are good predictive markers for AD, especially in children (Peterson et al., 2000). ACAs and 21-OH autoantibodies further correlate with the degree of adrenal dysfunction and the presence of high-levels of 21-OH antibodies strongly signals the destructive phase of the autoimmune process (Laureti et al., 1998b). In this regard, the central and the C-terminal regions of 21-OH seem to be the important site for autoantibody binding (Volpato et al., 1998).

Still, a good serological marker not equates with a pathogenic agent. And despite the fact that several authors found 21-OH autoantibodies to inhibit 21-OH activity *in vitro*, studies by Boscaro et al. were not able to confirm these results *in vivo* (Boscaro et al., 1996). Therefore and since there are no data demonstrating that these autoantibodies can enter living cells, they argue that 21-OH autoantibodies cannot be considered pathogenic agents of adrenal failure, but do represent the best serological markers of progressive autoimmune adrenalitis. Again, this raises the question of the direct pathogenic mechanisms and the role of cellular immunity in Addison's disease. Since despite the various autoantibodies and autoantigens detected, the direct pathogenic mechanism by which the adrenal cortex is destroyed is probably cell mediated.

One possible mechanism is delineated by Peterson et al. (Peterson et al., 2000): It is thought that T cells reactive to adrenal cortex antigens escape antigen-presenting cells in the thymus, maybe due to missing presentation of organ-specific, non-circulating antigens. These auto-reactive T cells subsequently recognize and destroy adrenocortical cells and stimulate auto-reactive B cells, which start producing autoantibodies specific to steroidogenic enzymes. Unfortunately, attempts to demonstrate a cell-mediated immune response towards adrenal cortical cells have been unsuccessful, yet.

Another mechanism is proposed by Bøe et al. based on their finding that autoantibodies against 21-OH and scc enzyme in autoimmune Addison's disease are mainly of the IgG1 subclass (Boe et al., 2004): In humans, IgG1 and IgG3 indicate Th1 responses and are primarily produced in response to protein antigens. The antibody isotypes stimulated by Th1 cells have high affinity for Fc receptors and are strong inducers of complement activation and antibody-mediated antigen uptake, i.e. IgG1 and IgG3 support antibody-dependent cellular cytotoxicity and complement-dependent cellular cytotoxicity towards membrane-associated antigens. Therefore, the predominance of autoantibodies of the IgG1 subclass may indicate that the CD4+ T-cell response in Addison's disease is dominated by Th1 cells, a finding that is consistent with the clinical findings of inflammation in the adrenal glands of Addison's patients.

Dittmar and Kahaly summarized two theories of the immunopathogenesis of Addison's disease/APS: the viral infection theory and the suppressor effect theory (Dittmar and Kahaly, 2003). The viral infection theory is based on an immune response to an environmental agent that cross-reacts with a host antigen, resulting in disease. For example, it was shown by Onodera et al. that reovirus type 1 (that produces diabetes in animals) also triggers an autoimmune response and infected mice develop an autoimmune polyendocrinopathy (Onodera et al., 1981). Regarding the suppressor effect theory, Sakaguchi and Sakaguchi demonstrated that administration of the immunosuppressive drug cyclosporine A to newborn BALB/c mice causes a selective defect of the regulatory T

suppressor cells normally responsible for down-regulation of the expansion of self-reactive lymphocyte clones. Additional removal of the thymus immediately after neonatal cyclosporine A treatment permanently sustained the T-cell-deficient state and produced autoimmune diseases with a higher incidence and in a wider spectrum of organs, e.g., thyroiditis, gastritis, insulitis, and adrenalitis (Sakaguchi and Sakaguchi, 1989). These pathological processes led to the preclinical phase of APS, with production of organ-specific antibodies and progressive immune-mediated destruction of endocrine tissues, characterized by chronic inflammatory infiltration of lymphocytes.

Immunogenetics of Addison's disease

Beside serological markers, also distinctive immunogenetic features can be observed in Addison's disease. These immunogenetic features differ in APS-1 and APS-2.

Immunogenetics of APS-1

As mentioned above, APS-1 has an autosomal recessive form of inheritance, suggesting a possible role of immunogenetic mutations in the pathogenesis of Addison's disease. Accordingly, Aaltonen et al. found in a study investigating 14 Finnish families an association between APS-1 and the chromosome 21q22.3 (Aaltonen et al., 1994). Nagamine et al. isolated a novel gene from this region, AIRE (autoimmune regulator), which encodes a protein containing motifs suggestive of a transcription factor (Nagamine et al., 1997). Two mutations were found in Swiss and Finnish APS-1 patients. One of these, a C→T substitution that changes the Arg 257 to a stop codon (R257X, a nonsense mutation) is the predominant mutation in Finnish APS-1 patients (Nagamine et al., 1997). In the same year, Aaltonen et al. also isolated the AIRE gene but reported of already five mutations (Aaltonen et al., 1997). These data demonstrated APS-1 being the first autoimmune disease caused by the mutation of a single gene. However, clinical disease expression depends on the presence of mutations in both alleles of the AIRE gene (Betterle and Zanchetta, 2003).

AIRE encodes a 58-kDa protein having two plant homeodomain (PHD) zinc fingers, a nuclear localization signal, a proline-rich region, four LXXLL motifs, a SAND (after nuclear proteins Sp100, AIRE-1, NucP41/75 and DEAF-1/suppressin) and a HSR (homogenously staining region-locating) domain (Peterson et al., 2000). It is predominantly expressed in thymic epithelial cells but also in some monocyte-derived cells of the thymus, and in a subset of cells in lymph nodes, the spleen and in fetal liver (Pitkanen and Peterson, 2003). AIRE is thought to be involved in negative selection of self-reactive T-cell clones in the thymus, hence in maintaining normal self-tolerance (Ten et al., 2001).

Over the last eight years, the number of AIRE gene mutations steadily increased from more than 20 in 2001 (Ten et al., 2001) to 45 in 2002 (Vogel et al., 2002) and to at least 50 at present (Villasenor et al., 2005), whereas most are frameshift or nonsense mutations

(Peterson et al., 2000). Betterle and Zanchetta summarized the incidences of the four main mutations (see fig. 2-9): The R257X mutation in exon 6 is present in 82% of the alleles of the Finnish with APS-1. The *del13* in exon 8 is the most common mutation in Caucasian American, while the R139X in exon 3 is the most common in patients from Sardinia. Y85C in exon 2 is the only one identified in Jewish Persians (Betterle and Zanchetta, 2003).

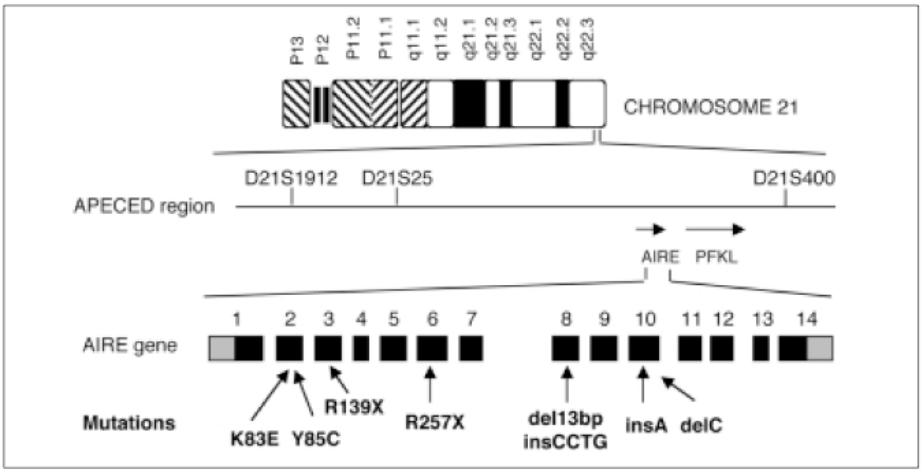

Fig. 2-9: Schematic representation of chromosome 21 showing the map of the APS-1 region (APECED) and the structure of the AIRE gene with the main mutations associated with the presence of APS-1 (Betterle and Zanchetta, 2003).

Despite these interesting associations, the precise molecular mechanism by which AIRE exerts its influence remains unknown. It is also not known at what level AIRE functions. One possibility is that it operates on individual promoters or enhancers, another scenario involves broad regulatory processes at the gene or chromatin level, such as DNA methylation or chromatin remodeling. It is also not unlikely that AIRE complexes with and influences the activity of multiple other transcription factors, acting as a transcriptional regulator. The presence of a SAND domain in AIRE has been interpreted as an important clue to its transcriptional function (Gibson et al., 1998), but evidence for the direct binding of AIRE DNA remains sparse, confined to only one *in vitro* study (Kumar et al., 2001). On the other hand, several studies show that AIRE has the potential to activate transcription. For example, the finding of Pitkanens et al. that AIRE *in vitro* is associated with cyclic adenosine monophosphate-responsive element-binding protein (CREB-binding protein, CBP), a transcriptional co-activator, lend further support towards its involvement in transcriptional regulation (Pitkanen et al., 2000). Furthermore, Anderson et al. demonstrated in AIRE-deficient mice a requirement for AIRE expression in the thymic stroma for the induction of tolerance to tissue-specific antigens and for the prevention of APS-1. DNA-microarray analyses showed that medullary epithelial cells (MEC) of the thymus lacking AIRE exhibited down-regulation of a subset of peripheral tissue antigens, strongly implicating this molecule being involved in regulation of peripheral tissue antigen expression in MECs and in imposition of T-cell tolerance centrally (Anderson et al., 2002).

Taken together, it seems rather unlikely that each of the several hundred genes specifically in MECs has an AIRE-binding site. More likely, AIRE is acting as a transcriptional regulator and/or is involved in broad regulatory processes. There is less uncertainty regarding AIRE's role in the immune system. It is generally agreed that AIRE is involved in negative selection of self-reactive T-cell clones in the thymus, thus playing a central role in maintenance of normal immune tolerance. Hence, in APS-1, mutations in AIRE may be responsible for biased Th2 immune responses to self-antigens and defective protective Th1 responses (Ten et al., 2001).

Beside the role of AIRE mutations in APS-1, also the role of particular human leukocyte antigen (HLA) haplotypes is discussed. Here, some data lend support to the view that there is no such association in APS-1 (Peterson et al., 2000), while others, for example Ahonen et al., found HLA-A28 to be more frequent in patients with APS-1 and HLA-A3 to be more frequent in those with APS-1 and ovarian failure than in those with normal ovarian function (Ahonen et al., 1988). Currently, a general agreement on the role of specific HLA haplotypes in APS-1 has to be awaited.

Immunogenetics of isolated Addison's disease and APS-2

Despite the histological picture of adrenalitis, which favors the view that the primary pathogenetic mechanism – a cell-mediated immune response leading to the destruction of the adrenal cortex – is similar whether AD occurs alone, as part of APS-2 or as a component of APS-1, the mechanisms of tolerance breakdown in these conditions are different. While in APS-1 the failure of AIRE expression in the thymic medulla is the distinctive finding, in isolated Addison's disease and APS-2 it is a strong association with HLA system (Peterson et al., 2000).

The HLA system is the human version of the major histocompatibility complex (MHC). The HLA complex on chromosome 6 contains over 200 genes, more than 40 of which encode leukocyte antigens (see fig. 2-10). The HLA genes that are involved in immune responses fall into two classes, I and II, which are structurally and functionally different. In the HLA region there are some 20 class I genes, three of these, HLA-A, B, and C, are the classic, or class Ia genes. The class II genes are classified according to the class (D), the family (M, O, P, Q, or R), the encoded chain (A or B for α or β chain, respectively), the individual gene (Arabic number), and the allelic variant of the gene (e.g., *0401). For example, HLA-DRB1*0401 stands for allelic variant 0401 of gene 1, which encodes the β chain of a class II molecule belonging to the R family. Class I genes are expressed by most somatic cells, class II genes normally by a subgroup of immune cells that includes B cells, activated T cells, macrophages, dendritic cells, and thymic epithelial cells. The function of both class I and class II molecules is the presentation of short, pathogen-derived peptides to T cells, a process that initiates the adaptive immune response (Klein and Sato, 2000a).

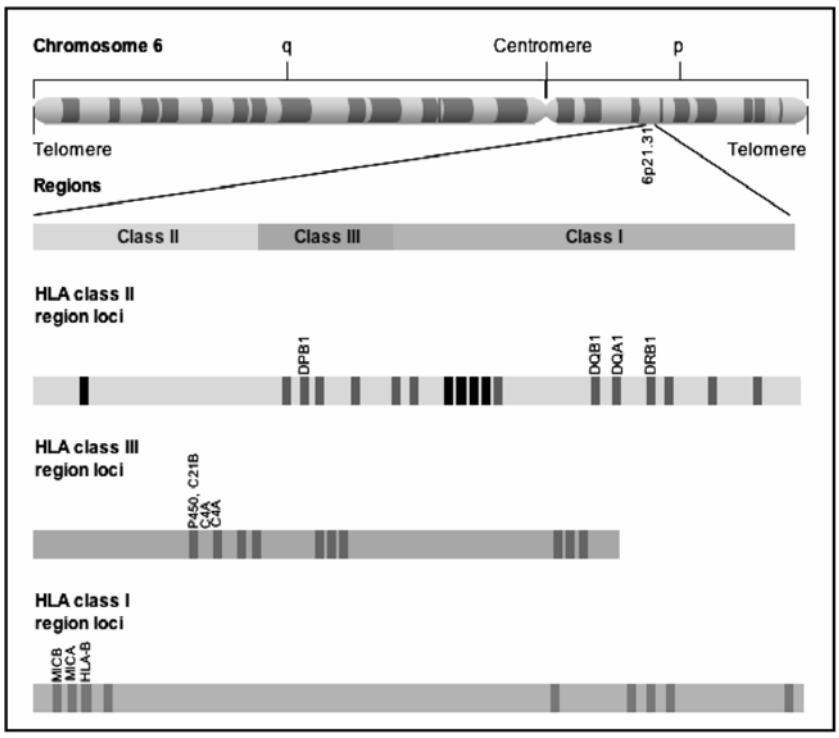

Fig. 2-10: Location and organization of the HLA complex on chromosome 6 (modified from Klein and Sato, 2000a).

Various HLA alleles have been shown to be associate with T cell-mediated autoimmune disorders (Klein and Sato, 2000b). In APS-2 and isolated Addison's disease, strong associations between Addison's disease and alleles within the HLA-DR3 and HLA-DQ2 carrying haplotype are found, including DRB1*0301 and DQA1*0501–DQB1*0201, respectively (Partanen et al., 1994). For example, Partanen et al. found these alleles in 83-92% of the patients, but only in 20-29% of the healthy controls (Partanen et al., 1994). Huang et al. showed that the subtype DQB1*0302 was increased in those patients with Addison's disease and type 1 diabetes mellitus. However, the presence of type 1 diabetes mellitus entirely explained this association (Huang et al., 1996). In Italian patients, in addition to HLA-DR3, also the prevalence of HLA-DR5 was increased in those with both Addison's disease and thyroid autoimmunity (Betterle et al., 1996). In addition, the DRB1*0301 haplotype seems to be associated stronger with APS-2 than with isolated Addison's disease (Partanen et al., 1994). Beside this exception, no difference in HLA class II alleles has been found between the two clinical subgroups, which further suggests that both forms of Addison's disease, isolated and associated with APS-2, have a similar genetic background and etiology.

Patients with Addison's disease also show certain variations of genes in the HLA class III and I region. Regarding genes in the HLA class III region, patients with Addison's disease show deletion of the complement C4 and CYP21A (a pseudogene for cytochrome P450c21) genes, and the tumor necrosis factor β (TNF-β) allele TNFB*1 (Peterson et al., 2000). Interestingly, Partanen et al. found notably similar class II and class III gene markers

in healthy controls and patients, if matched for HLA-DRB1*0301. Therefore, they argue that none of the class III markers are specific to Addison's disease (Partanen et al., 1994).

The same may be true for HLA class I polymorphisms. Here, the HLA-B8 allele and MIC-A (MHC class I chain-related gene A) and MIC-B gene polymorphisms (MHC class I chain-related gene A) are associated with Addison's disease (Gambelunghe et al., 1999). With regard to the HLA-B8 allele, the association with HLA-DR3-DQ2 seems to be stronger, and the B8 allele is only associated with Addison's disease when part of the HLA-B8-DR3 haplotype. Thus suggesting strongly that the HLA-B8 allele is also not a marker specific to Addison's disease.

Other results are observed regarding the MICA and MICB gene polymorphism. The exon 5 microsatellite polymorphism of the MIC-A gene consists of five alleles based on the number of GCT triplet repeat units (alleles A4, A5, A6, and A9) and the presence of an additional nucleotide insertion (allele A5.1; Ota et al., 1997). The intron 1 microsatellite polymorphism of the MIC-B gene consist of 13 alleles based on the number of CA/TG repeat units (Tamiya et al., 1998). In APS-2 and isolated Addison's disease, the polymorphisms MIC-A5.1 and MICB-CA-25 are of special interest. Gambelunghe et al. observed an increased risk for Addison's disease in the presence of MICB-CA-25 in subjects carrying also the MIC-A5.1 allele. Furthermore, they were able to show that both MIC-A5.1 *and* HLA-DR3/DQ2 are necessary to confer increased genetic risk for Addison's disease. Therefore, they conclude that this combination is now to be seen as the most important genetic marker for isolated Addison's disease and APS-2 (Gambelunghe et al., 1999). Nevertheless, to date there is no proof that this association is a primary association, and not also a result of linkage disequilibrium (i.e., a non-random association of alleles at two or more loci on a chromosome) as in the case of the HLA-B8 allele or the HLA class III polymorphisms.

The role of other genomic loci beside HLA genes in Addison's disease is still unclear. One other candidate region is located on chromosome 2q33, where a susceptibility gene has been mapped for type 1 diabetes mellitus (Nistico et al., 1996). This region contains the cytotoxic T lymphocyte antigen 4 (CTLA-4) gene that encodes a receptor on T cells. CTLA-4 is a key regulatory element in the interaction with antigen-presenting cells, since T cell activation requires two signals: the first signal is provided by antigen–T-cell receptor (TCR) engagement and the second co-stimulatory signal is mainly provided by the interaction of the co-stimulatory molecule CD28 with its ligands B7.1/B7.2 (CD80/CD86) on antigen-presenting cells. Importantly, CTLA-4 also binds to B7.1/B7.2 but in contrast to CD28, it provides a *negative* signal to T cell activation and is only expressed on activated T cells (Vaidya and Pearce, 2004).

In the context of Addison's disease, isolated or as part of APS-2, two CTLA-4 gene polymorphisms are of special interest: (1) an A to G transition at position 49, which results in

a threonine to alanine substitution at codon 17 in exon 1 of the CTLA-4 gene (CTLA-4 Ala^{17}) and (2) a microsatellite polymorphism (variant lengths of a dinucleotide $[AT]_n$ repeat) lying in exon 3 of the CTLA-4 gene, specifically a 106 base pair (106-bp) allele. In this regard, Donner et al. found patients with Addison's disease carrying significantly more often at least one CTLA-4 Ala^{17} allele. In their study, increased genetic susceptibility applied only to the subgroup of DQA1*0501 positive patients (Donner et al., 1997). Vaidya et al. reported on similar findings but without the HLA restriction. They suggested that the effect observed by Donner et al. may reflect the presence of an excess of HLA DQA1*0501-associated autoimmune thyroid diseases and type 1 diabetes mellitus in this subgroup of patients. Further, Vaidya et al. found a stronger association of the CTLA-4 Ala17 allele in patients with Addison's disease as a component of APS-2 than in patients with isolated Addison's disease (Vaidya et al., 2000). Regarding the microsatellite polymorphism, results of Kemp et al. indicate an association between the 106-bp allele and Addison's disease. Interestingly, this was true only in the English patients but not in Norwegian, Finnish or Estonian patients (Kemp et al., 1998).

The consistency of allelic associations between the CTLA-4 gene polymorphisms and different autoimmune diseases suggests that at least one of these two polymorphisms may have a functional role in the pathogenesis of autoimmunity. In this regard, Yanagawa et al. hypothesized that the larger size of the 106-bp allele compared to the more common 88-bp allele could adversely affect the stability of the messenger RNA (mRNA) transcript, leading to reduced levels of cell-surface CTLA-4. But they also point out that there is no evidence that a mRNA of 106-bp allele is more stable or less stable than others (Yanagawa et al., 1995). A functional role of the CTLA-4 Ala^{17} allele is generally discounted, and CTLA-4 Ala^{17} is thought simply to be in linkage disequilibrium with the true susceptibility polymorphism. However, it is also possible that this polymorphism determines a subtle alteration in the subcellular localization or a functionally important difference in the folding of the mature CTLA-4 protein (Vaidya et al., 2000). Additional investigations will be necessary to clarify the functional role of CTLA-4 gene polymorphisms and their significance in Addison's disease.

2.2.7 Summary

The outline of Addison's disease given above emphasized the etiological complexity of the disease. However, this rare endocrinological state ultimately and invariably is caused by the destruction of the adrenal cortex. Thus, no glucocorticoids and mineralocorticoids are synthesized and – due to lacking glucocorticoids – also no epinephrine. This lack of vitally important hormones is counterbalanced by pharmacological therapy providing basal cortisol (and aldosterone) concentrations. Nevertheless, in states of stress this disease does not allow for an adequate endocrine response.

The combination of existing basal cortisol concentrations but lacking endocrine stress response predispose ‘Addison’s disease’ towards an valuable approach to gain new insights into the interaction between stress and the immune system. Unfortunately, so far the initial endocrinological conditions are unknown in that no experimental data are available describing how closely the circadian rhythm of cortisol is mimicked by pharmacological therapy. Further, no data are available concerning hormone concentrations during stress. Hence, one aim of the present thesis was to first of all determine experimentally these initial conditions (see section 4.1 and 4.2).

However, the second aim of this work was to investigate the effects of a missing endocrine stress response on the immune system. Thus, first the third main topic in this regard, namely the immune system (2.3.1) will be introduced. Subsequently, studies investigating the effects of stress (2.3.2) and stress mediators (2.3.3) on immune functions as well as mechanisms and modulators of hormone-to-immune system signaling (2.3.4) will be described.

2.3 PSYCHONEUROIMMUNOLOGY

In 1993, Dr. George Freeman Solomon cited in the Normal Cousins Lecture delivered at the conference on research perspectives in psychoneuroimmunology the Transylvanian physician Papai Pariz Ferenc (1980), who in turn reiterated the famous Greek philosopher, natural scientist and influential thinker Aristotle: “When the parts of the body and its humors are not in harmony, then the mind is unbalanced and melancholy ensues, but on the other hand, a quiet and happy mind makes the whole body healthy” (Solomon, 1993).

This demonstrates that on the one hand already the ancient Greeks knew about the mutual mind-body influences (see also the monography by (Sternberg, 2000). On the other hand, a long time this knowledge was opposed by a view of the immune system as autonomous, reactive only to antigen, and self-regulatory (Solomon, 1993) – two positions apparently incompatible. Hence it was not until Metal’nikov and Chorine demonstrated in 1926 that the Pavlovian rules of conditioning also apply to the immune system (Metal'nikov and Chorine, 1926) that this paradox was resolved. In the following 50 years, indications of brain-immune system connectedness accumulated. Five years earlier, Hammar had already observed that emotional factors and hormonal alterations have a major influence on the size of the thymus (Hammar, 1921). Selye then provided the link of shrinkage of the thymus and of lymphoid organs to HPA axis activity (Selye, 1936a; Selye, 1936b). In 1949, Szentivanyi and colleagues discovered that the hypothalamus regulates the anaphylactic response in guinea pigs, demonstrating that the nervous system has a dominant regulatory power over immune reactivity (Filipp and Szentivanyi, 1958; Szentivanyi and Filipp, 1958; Szentivanyi and Szekely, 1958). In 1975, the taste-aversion conditioned immunosuppression experiments by Bob Ader and Nick Cohen eventually put psychoneuroimmunology (PNI) on the map (Ader and Cohen, 1975).

Twenty-four years later, Bruce Rabin defined psychoneuroimmunology (PNI) as “the study of how (1) psychological factors that an individual experiences and that activate neurons in the brain (2) modify the production and release of neuropeptides and endocrine hormones that (3) alter the function of the immune system, which then (4) increases the susceptibility of an individual to diseases that are normally prevented by a healthy functioning immunes system” (Rabin, 1999). But this brain-immune system connection is not one-way. During the eighties of the 20th century, experiments of several laboratories, for example from Besedovsky and colleagues, clearly showed that the immune systems also signals back to the central nervous system (Besedovsky et al., 1981) see also (Blalock, 1989). Therefore, a definition of PNI should emphasize that this field addresses the way in which nervous system and immune system *interact with each other*, and how these interactions influence the state of health of an individual. In their editorial to volume I of the “Neuroimmune Biology Book

Series", Berczi and Szentivanyi pointed out that there is even much more to this interaction than 'bi-directional'. It is a "truly multi-directional, all-inclusive systemic regulatory network formed by the nervous-, endocrine- and immune systems, which controls all bodily functions of higher animals and man" (Berczi and Szentivanyi, 2001).

Since it is not within the scope of this thesis to account for the whole complexity of this multi-directional network, the outline of the following sections is geared to selected questions: How does the immune system protect an organism against disease (2.3.1: the immune response)? What effects have psychological and emotional states on the immune system (2.3.2: brain-immune system communication)? What particular role play glucocorticoids and catecholamines in these interactions? (2.3.3: role of stress mediators)? How and influenced by which modulators do glucocorticoids exert their effects on the immune system (2.3.4: mechanisms and modulation of glucocorticoid-to-immune system signaling)? In the context of the last question, a parameter potentially important in compensating missing glucocorticoid signaling will be introduced: The transcription factor nuclear factor-kappaB.

2.3.1 The Immune Response

Day-to-day an organism is exposed to a wide variety of pathogens. In most cases, this does not cause any major symptoms of disease. Once in a while, an acute infection can cause a temporary state of disease and in very rare cases, this infection can gain the upper hand, leading to death or chronic disease. In the following, the mechanisms accounting for these different outcomes are discussed.

2.3.1.1 First Line of Defense

Generally, the best defense is not allowing a pathogen to enter the organism at all. These external barriers against infections are therefore also called *first line of defense* and include skin, mucous membranes, body fluids, and normal bacterial flora. Normal skin protects an organism by a low pH, which is lethal to most pathogens. Mucous membranes in the respiratory, gastrointestinal, and genitourinary tracts block adherence of microorganisms to epithelial cells by trapping them in the adhesive mucus. Body fluids, such as tears, nasal secretions, saliva, gastric juice, semen, and breast milk include important enzymes and bactericidal elements (e.g., lysozyme in tears, nasal secretions, and saliva; acid in gastric juice; zinc in semen; lactoperoxidase in breast milk). Finally, normal bacterial flora present in the gastrointestinal, respiratory, and genital tracts is protective by competing for principal nutrients and/or by production of substances capable of inhibiting other microorganisms (Uthaisangsook et al., 2002).

2.3.1.2 Second Line of Defense: Innate immunity

What happens, if the first line of defense fails? The second line of defense – or as Parham puts it: “The unsung heroes” – come into play (Parham, 2003). It is also Parham who stated that “the fact that most people are not perpetually sick is testament to innate immunity squelching most of the infections that we contract” (Parham, 2003). This innate immunity is the organism’s second line of defense.

But why ‘unsung heroes’? Contrary to, for example, dendritic cells or complement system, virtually everybody heard of antibodies or immune memory and has an idea what they stand for. From vaccination or from the last flu, it is also known that an immune response is very specific and takes its time to be mounted. As a start, all this does not deal with innate immunity. Innate immunity is rather the direct opposite: It is nonspecific and it has no memory – but it is fast. And exactly these properties are responsible for the fact that we are not perpetually sick.

As the name implies, the innate immune system is present since birth and a phylogenetically ancient defense mechanism found in all multicellular organisms. And although nonspecific, it is capable of differentiating between self and non-self perfectly, i.e., the innate immunity has the ability to recognize invading microorganisms (i.e., antigens) despite their immense molecular heterogeneity and variability.

2.3.1.2.1 The cellular components of the innate immune system

A major component of the innate immune system are white blood cells or leukocytes (for review see Janeway et al., 2001; Rabin, 1999). These cellular components include granulocytes, monocytes, immature dendritic cells, natural killer (NK) cells, γ/δ T cells, and B-1 cells (Uthaisangsook et al., 2002). All these cell types originate from pluripotent hematopoietic stem cells in the bone marrow, which then differentiate into myeloid or lymphoid stem cells. The former are progenitors for granulocytes, monocytes, and immature dendritic cells, the latter for B lymphocytes (B-cells) and T lymphocytes (T-cells) – and regarding innate immunity – specifically for NK cells, γ/δ T-cells, and B-1 cells (Janeway et al., 2001).

The term granulocytes thereby stands for several types of granular leukocytes, also sometimes called polymorphonuclear leukocytes because of their oddly shaped nuclei: They include neutrophils, eosinophils, and basophils, labeled according to their staining capabilities (Rabin, 1999). Granulocytes are all relatively short lived and produced in increased numbers during immune responses. Neutrophils have the capability of binding foreign particles, such as bacteria, to their surface and subsequently ingest (phagocytosis) and kill them. Eosinophils help to protect against infections with parasites, like worms, by releasing toxic proteins. The function of basophils is unknown, but they have granules

containing histamine and surface receptors for the Fc portion of the immune globulin (Ig) E antibody molecule. Primarily, all these different cells circulate in the blood, but, with the exception of basophils, they also enter tissues. Other cells first differentiate in tissues: As monocytes subsequently enter tissue, they enlarge in size and this mature form is then called macrophages. Like neutrophils, macrophages are phagocytes. But they also have another major effector function, namely the presentation of peptides derived from a pathogen on their surface via class I and class II major histocompatibility complex (MHC-I and MHC-II) molecules. Mast cells are cells with a similar morphology like basophils, but they mature in tissues and are mainly found near small blood vessels. Upon activation, they release chemical factors like histamine and mediate many allergic reactions. Likewise, immature dendritic cells migrate from the blood to reside in secondary lymphoid organs, the skin and all tissues of the body. But it is not until they encounter a pathogen that they rapidly mature, i.e., dendritic cells uptake the antigen in peripheral sites, migrate to lymph nodes and there present the antigen via MHC-I and MHC-II molecules to other cells. Therefore, dendritic cells are one of the major so called antigen-presenting cells (APCs). Depending on their location, dendritic cells have a variety of other names. In the skin they are called Langerhans cells, interstitial dendritic cells in the heart, kidney, intestine, or lung, dendritic cells in the blood, and veiled cells in the afferent lymphatics.

NK cells are the third lineage of lymphoid cells beneath T- and B-cells. They are large granular lymphocytes circulating in the blood and have the ability to kill target cells by using perforins and granzymes present in cytoplasmic granules. Located in the paracellular space between epithelial cells, mostly among the epithelial cells of the colon and small intestine, γ/δ T cells directly recognize unprocessed target antigens. They have cytolytic activity and produce cytokine-inducing growth factors. B-1 cells are a lineage of conventional B-cells which express CD5 (CD: cluster of differentiation) on their surface and are therefore also called $CD5^+$ B-cells. Their receptors and the antibodies (predominantly immunoglobulin (Ig) M) produced by them bind many different ligands with low affinity. Contrary to B-cells, B-1 cells recognize antigens without the help from antigen-specific T-cells and therefore mount a response much faster than usual for B-cells (Uthaisangsook et al., 2002).

2.3.1.2.2 Recognition of pathogens: PAMPs and PRRs/PRMs

But how do these cells “know” where to go, what to do, and, most importantly, what is self and what is foreign? To accomplish the latter, the innate immunity uses some kind of “trick”: Innate immune cells do not recognize specific antigens, but nonspecific molecules, called pathogen-associated molecular patterns (PAMPs; Medzhitov and Janeway, 1997a; Medzhitov and Janeway, 1997b; Uthaisangsook et al., 2002). These PAMPs are shared by large groups of pathogens, conserved products of microbial metabolism, essential for the

survival or pathogenicity of the microorganism, and absolutely distinct from self-antigens (Medzhitov and Janeway, 1997a). For example, instead of recognizing an antigen as Escherichia (E.) coli, cells of innate immunity recognize a component of the bacterial cell wall, namely lipopolysaccharide (LPS), shared by most gram-negative bacteria, which E. coli is just one of. Other PAMPs are lipoteichoic acids (LTA) present in numerous gram-positive bacterial cell walls, peptidoglycan (PGN) in the cell-wall of gram-positive, gram-negative and mycobacteria, the unmethylated cytosine phosphate guanine (CpG) motif characteristically for bacterial DNA, double-stranded RNA in RNA virus, or mannan components of yeast cell walls (Medzhitov and Janeway, 1997a; Uthaisangsook et al., 2002). PAMPs in turn are recognized by a set of molecules as well as receptors (cell-surface, transmembrane, intracellular, or soluble receptors) referred to as pattern-recognition molecules (PRMs) and pattern-recognition receptors (PRRs). They represent different functional classes: endocytic receptors like the macrophage mannose receptor, secreted proteins like mannose-binding lectin (MBL) or C-reactive protein (CRP), and signaling receptors like the Toll receptor family (Uthaisangsook et al., 2002). The specificities of PRMs and PRRs are germline encoded, i.e., they arise over evolutionary time due to selection by pathogens at the populational levels (Medzhitov and Janeway, 1997a). Given these prerequisites, the cells of the innate immunity are able to recognize a great variety of molecular structures associated with pathogens with only a limited number of germline-encoded receptors and molecules. And binding of PAMPs to PRRs or PRMs (with subsequent binding of these complexes to PRRs) is the first step in activating these cells to exert their respective above described functions.

2.3.1.2.3 The complement system

Another essential but often underestimated role in immune system activation plays the complement system. The complement system consists of at least 20 different proteins (C1, C2, C3…), which act in a cascade-like fashion. Activation of a complement component (e.g., C3) results in splitting it into fragments, usually designated by the suffixes “a” for the smaller fragment released into body fluid (C3a) and “b” for larger membrane-bound fragment (C3b). Three cascades or pathways are known: The classical, the alternative, and the MBL (mannose-binding lectin) pathway. The latter depends on an above mentioned PRM, namely MBL, which recognizes carbohydrate patterns on cell wall of bacteria, yeast, parasites, mycobacteria, and certain viruses. Binding of MBL to carbohydrates on the pathogen surface is followed by a sequence of reactions resulting in generation of a protease called C3 convertase formed from C2b bound to C4b. The classical pathway initiates complement activation in the same way as the MBL pathway. It just uses a protein very similar to MBL, called C1q, which is part of the C1 complex. C1 can also bind directly to pathogens, but it is primarily activated by antigen-antibody interactions and thus generally provides a later immune response. But again, a C3 convertase is generated. The alternative pathway of

complement activation is initiated by the third component of complement, C3. C3 is constantly activated by spontaneous hydrolysis into the activated C3 protein, C3(H_2O), which interacts with serum complement components to form a C3 convertase. Contrary to pathogens, host cells have complement regulatory proteins on their cell surface, which protect them from the consequences of spontaneous activation of C3 molecules. The alternative pathway can also act as an amplification loop for all three pathways. However, the production of a C3 convertase is the point at which all three pathways converge and the main effector functions of complement are generated. The most important activity of a C3 convertase is to cleave large numbers of C3 molecules to produce C3b and C3a molecules. C3b molecules coat the pathogen surface, a process called opsonization. Simultaneously, C3b binds to complement receptors on phagocytic cells and this induces the phagocytic cell to ingest the bacteria. C3b may also bind to the convertase resulting from the MBL pathway (C4b,C2b) or the alternative pathway (C3b, Bb) to form a C5 convertase complex, which cleaves C5 to form C5b and C5a. C5b then initiates the forming of a membrane-attack complex. For this, C5b triggers the assembly of a complex of one molecule each of C6, C7, and C8. C8 inserts into the cell membrane and up to 16 molecules of C9 are then added to the assembly to generate a channel in the membrane of the pathogen. This channel disrupts the pathogen cell membrane, thereby killing the pathogen. This second function of complement is called lysis. But also the smaller fragments released into body fluid, C3a and C5a, have a biological function. These small peptides attract inflammatory cells to the location at which the complement is being activated, a process called chemotaxis. Additionally, C5a is an important signal to ultimately activate phagocytes to ingest opsonized pathogens, if only C3b binds to the complement receptor CR1 on their surface. Taken together, activation of the complement system may result in lysis, opsonization, and chemotaxis: It not only kills pathogens without any involvement of immune cells, but also provides an alternative mechanism for phagozytic cells, beneath PAMPs, to recognize a pathogen and above all directs them to the side of action (Janeway et al., 2001; Rabin, 1999).

2.3.1.2.4 Communication and "traffic management": cytokines and adhesion molecules

Beneath recognition of pathogens, immune cells also have to communicate to each other to act in concert and eventually mount an effective and coordinated immune response against invading pathogens. The way this communication is accomplished is by mediators called cytokines and chemokines (Janeway et al., 2001; Rabin, 1999; Uthaisangsook et al., 2002). Cytokines are small proteins that are released by various cells, usually in response to an activating stimulus like binding to PAMPs, and induce responses through binding to specific receptors. Cytokines are in most cases designated as IL (for interleukin) followed by a number (e.g., IL-1). Their actions are very complex: Individual cytokines may act on many

different cell types (ambiguity), while different cytokines may have similar actions (redundancy). Several cytokines may also act on one cell synergistically or antagonistically and one cytokine may increase or decrease both the production of another and the expression of receptors for another. For example, activated macrophages may secrete the cytokines IL-1, IL-6, IL-8, IL-12 and TNF-α. IL-1 activates vascular endothelium and lymphocytes and increases adhesiveness of leukocytes. IL-8 is a chemotactic factor recruiting neutrophils, basophils, and T cells to site of infection. TNF-α like IL-1 activates vascular endothelium and increases vascular permeability, leading to accumulation of immune globulin and complement in infected tissues. IL-6 induces B-cell terminal differentiation into immune globulin-producing plasma cells. IL-1, IL-6, and TNF-α all have additionally systemic effects in that they induce an acute-phase response in the liver and induce fever. Finally, IL-12 activates NK cells. But NK cells are also activated by the interferons (IFN) IFN-α and IFN-β, which are produced by viral-infected cells and strongly upregulate the expression of MHC-I molecules on these infected cells.

As already mentioned – and in parallel to LPS and by-products of complement activation – certain mediators (e.g., IL-1, TNF-α) induce the activation of endothelial cells and enhance leukocyte adhesion. These processes are important as they are responsible for the recruitment and migration of leukocytes out of the blood vessels into the infection site. Centrally to this is the induced expression of cell adhesion molecules (CAM) on endothelial cells and leukocytes. CAM are used by cells to grip onto each other, to haul themselves by one another and to travel to injured tissues. Based on their structure, there are three major types of CAM: selectins, integrins and the immunoglobulin superfamily. Selectins are expressed on endothelial cells within a few minutes of exposure to – among others – C5a or histamine (P selectin), or within a few hours after exposure to LPS and TNF-α (E selectin). These selectins interact with carbohydrate epitopes on leukocytes and allow them to roll along and reversibly attach to the vascular endothelial surface. This process is termed rolling. The next step depends on tighter adhesion, i.e. a stronger attachment of leukocytes to endothelium and is supported by IL-8. Adhesion involves integrins on leukocyte (e.g., leukocyte functional antigens-1 (LFA-1) and complement receptor-3) which interact with members of the immunoglobulin superfamily on endothelial cells, mostly intercellular adhesion molecules (ICAMs). As a result, the leukocytes attach and adhere firmly to the endothelium. The subsequent process is known as diapedesis and represents the extravasation of leukocytes through the vessel wall. It additionally involves an Ig-related molecule that is called PECAM or CD31 on both, the leukocyte and the intercellular junctions of endothelial cells. These interactions enable the leukocytes to squeeze between the endothelial cells. Finally in the tissue, the leukocytes just have to be directed to the site of infection, a process called migration. Thereby, chemokines, like IL-8 released by the

macrophages that first encounter a pathogen, form a concentration gradient along which the leukocyte can migrate to the focus of infection (Janeway et al., 2001; Rabin, 1999; Uthaisangsook et al., 2002). This migration of leukocytes to tissue and their accumulation in tissue also constitutes the so-called inflammatory response (Rabin, 1999).

2.3.1.2.5 Inflammation

As already mentioned above, cytokines may have long-range effects that contribute to host defense. In this regard, the pro-inflammatory cytokines TNF-α, IL-1, and IL-6 produced by macrophages communicate an infection to the central nervous system and may cause fever. Additionally, they may initiate an acute phase response (APR) in the liver, thereby amplifying the local immune response by recruiting more phagocytic cells to the site of infection (see above). Several of the acute phase proteins (APPs) produced by the liver upon activation mimic the action of antibodies, but contrary to antibodies, they have a broad specificity for PAMPs. APPs (e.g., SAP: serum amyloid protein, CRP: C-reactive protein, or MBL) bind to bacteria, acting as an opsonin but also activating the classical complement pathway of the complement system, thus facilitating phagocytosis. These processes of cells and soluble substances involved in attracting and activating cells to accumulate at the site of an infectious agent are called inflammatory response (Janeway et al., 2001; Rabin, 1999).

An acute inflammation can be initiated within minutes upon entry of a bacterium into the body. Hence, it is a rapid and efficient response to an infectious agent and will frequently lead to resolution. The characteristics of an inflammation are well known by everybody: (1) redness (erythema) caused by vasodilation, (2) swelling (edema) caused by leakage of fluids from enlarged and more permeable blood vessels, (3) local warmth caused by increased blood flow (fever) and systemic fever caused by the release of cytokines from macrophages acting in the brain, and (4) pain resulting from heat and swelling acting on local nerves. Just in an overwhelming infection or cases of an impaired acute inflammatory response, further steps are necessary resulting in chronic inflammation (Janeway et al., 2001; Rabin, 1999).

In some case, all these efforts may not be sufficient or even be too efficient. For this reason, immune cell of the innate immune system also present peptides derived from a pathogen on their cell surface via MHC-I or MHC-II molecules. Antigen presentation is the fourth major property of innate immunity, beneath recognition of a pathogen, its clearance, and cytokine production. And in combination with specific cytokine profiles, via this fourth property the innate immunity can initiate, direct and control the third line of defense.

2.3.1.3 Third Line of Defense: Adaptive Immunity

In contrary to the innate immunity, the adaptive immunity is very specific and has a memory. However, this comes at the expense of speed: An adaptive or specific immune response may take several days or weeks to develop.

Centrally to the adaptive immunity are its cellular components, T lymphocytes and B lymphocytes, which develop from progenitor cells within the bone marrow. T-cells migrate to the thymus at an early stage as thymocytes, while B cells remain within the marrow for the duration of their development. Early in the development of the cells, antigen-specific receptors occur. The specificity of adaptive immune responses is due to these antigen-specific receptors on both cell types. Instead of being germline-encoded, these receptors are the result of a random rearrangement and splicing together of multiple DNA segments that code for the antigen-binding areas of the receptors. Arstila et al. estimated the repertoire of T-cell receptors and B-cell antibody specificities of over 10^8 and 10^{10}, respectively (Arstila et al., 1999). This diversity is achieved by a mechanism quite similar for both cell types and will be described exemplarily for the B-cell receptor: Despite a constant region, three segments of genes are involved in receptor formation, 25-100 genes for the variable (V) region, approximately 25 genes for the diversity (D) region, and circa 50 genes for the joining (J) region. Any combination of the genes of these three regions may eventually form the final VDJ region of the receptor. Additionally, splicing may be inaccurate and frameshift in basepairs leads to production of a different aminoacid (junctional diversity). Third, the sequence may be altered by the enzyme deoxyribonucleotidyl-transferase inserting nucleotides. The greater repertoire of B-cell receptors is due to further immunoglobulin gene rearrangement occurring during B-cell division after antigen stimulation, called somatic hyper-mutation (Parkin and Cohen, 2001). As a result, every T-cell and every B-cell has its own unique receptor. But this receptor is the product of random processes, hence it may as well bind to a self-antigen, an incident to be prevented in any circumstances. Consequently, several “safety nets” are build in.

The first safety net is the selection of cells taking place in the bone marrow in the case of B-cells and in the thymus in the case of T-cells. Centrally to T-cell selection are APCs, which have MHC-I and MHC-II molecules on their surface occupied with peptides derived from self-antigens. In positive selection, either the CD4 or the CD8 molecule on the T-cell surface has to bind to the appropriate receptor on a MHC-II or MHC-I molecule, respectively. If the T-cell has bound onto a MHC-I molecule, the CD8 molecule will bind to the CD8 receptor on MHC-I and synthesis of CD4 will cease. At the same time, the unique T-cell receptor (TCR) has to fit into the MHC molecule but not bind tightly to the peptide in the MHC groove. Only this combination will positively select the T-cell for survival. This first testing will result in $CD8^+$ or $CD4^+$ cells, which are able to recognize MHC molecules. Without recognizing the body’s own MHC molecules, T-cells would not be able to mount any immune response at all against an antigen. In negative selection, useless or autoreactive cells are sorted out. Useless T-cells are cells which TCRs do not fit into MHC molecules and thus cannot recognize a peptide in a MHC molecule. Without fitting into the MHC molecule, also

the CD4 or CD8 will not bind to the receptor on the MHC molecule. Without any signal provided by binding, these immature T-cells will die by apoptosis. Apoptosis is the death of a cell that occurs when a trigger mechanism instructs the cell to kill itself and is therefore also often termed programmed cell death. On the other hand, autoreactive cells not only bind to a CD4 or CD8 receptor on a MHC molecule, but also the TCR binds tightly to the self-antigen in the MHC groove. A combination which again results in T-cell death by apoptosis. Taken together, via these selection processes it is ensured that only T-cells recognizing MHC molecules but not self-antigens will survive (Rabin, 1999). B-cell undergo negative selection in the bone marrow instead of the thymus. In this case, immature B cells which bind to self cell-surface antigens are removed from the repertoire. The unique receptor of B-cells thereby is an immunoglobulin, more precisely an IgM molecule expressed on the cell surface (Janeway et al., 2001).

But these mechanisms are just the first safety net. Several other precautions are taken with regard to activation of now mature but so far naïve B- and T-cells. These precautions are closely intertwined with innate immune responses and will be discussed with regard to the three phases of an effective adaptive immune response: The (1) induction phase, in which the presence of a pathogen is detected and relevant antigens are presented to T-cells; an (2) activation or expansion phase, which includes the proliferation and mobilization of immune cells important for the eradication of the pathogen; and an (3) effector phase in which the pathogen is neutralized and eliminated by so-called humoral or cellular elements or by an interaction of both (McEwen et al., 1997). Additionally, the (4) formation of immunological memory as one of the most important consequences of the adaptive immune response will be presented.

2.3.1.3.1 Induction phase

One major property of innate immunity is the presentation of antigen in MHC-I or MHC-II molecules on their surface. Which MHC molecule will be presented depends on the antigen. Among others, macrophages digest exogenous pathogens, like bacteria. Once recognized, macrophages engulf these pathogens into cytoplasmic phagosomes (membrane-bounded vacuoles) and the phagosomes fuse with cytoplasmic granules, in which enzymes and antimicrobial peptides inactivate and degrade the pathogen. In the endoplasmatic reticulum, pathogen peptides then are loaded on MHC-II molecules and the antigen-MHC-II complex will be presented on the cell surface. Another cell expressing an antigen-MHC-II complex is the B-cell of the adaptive immunity. B cells internalize soluble antigens, which cannot be taken up efficiently by macrophages. Binding of antigens to a specific surface immunoglobulin receptor results in internalization and processing of the antigen-immunoglobulin complex (Janeway et al., 2001; Medzhitov and Janeway, 1997a; Uthaisangsook et al., 2002). On the other hand, endogenous pathogens like viruses are

already found inside the cell, since they use the cell to replicate. Via proteasomes, the virus is also degraded. But in this case, peptides derived from the virus will be loaded on MHC-I molecules, hence an antigen-MHC-I complex will be presented on the surface (Chaplin, 2003). This is also one important signal to activate NK cells, which then kill the infected cell (Uthaisangsook et al., 2002).

The processing and presentation of a pathogen further results in expression of a co-stimulatory signal. This co-stimulatory signal is another safety net. To activate T-cells of adaptive immunity, they not only have to bind to the antigen-MHC complex, but they also have to receive this co-stimulatory signal (e.g., B7 molecules on the APC binds to CD28 on the T-cell). And since an adaptive immune response is only appropriate when it is specific to pathogen-derived antigens, co-stimulators – which are only expressed in the presence of a pathogen – ensure that an immune response is not directed against a host antigen (Janeway et al., 2001; Medzhitov and Janeway, 1997a; Uthaisangsook et al., 2002).

2.3.1.3.2 Activation phase

So far, innate immunity played the major role and in most cases no involvement of the adaptive immunity is needed. Just when the innate immunity is not able to bring an infection under control, an adaptive immune response is initiated. The link between innate and adaptive immunity is the presentation of antigens by professional APCs (macrophages, dendritic cells, and B-cells). Cells recognizing this signal are T-cells, more precisely $CD8^+$ and $CD4^+$ cells. $CD8^+$ cells only recognize antigens presented with MHC-I and $CD4^+$ cells only antigens presented with MHC-II. Upon activation by binding to the respective antigen-MHC complex and a co-stimulator, $CD8^+$ cells differentiate to cytotoxic T-lymphocytes (CTL) and $CD4^+$ cells to T-helper 1 (Th1) or T-helper 2 (Th2) cells (Janeway et al., 2001).

Since MHC-I molecules are expressed by all nucleated cells, $CD8^+$ cells can be activated by cells infected with a virus, cells infected with other intracellular pathogens, or cells producing abnormal tumor antigen. Upon receiving the two signals (binding to antigen-MHC-I complex and to co-stimulator), $CD8^+$ cells immediately secret the cytokine IL-2 and at the same time express a high affinity receptor for IL-2. Binding of IL-2 to its receptor is the last signal necessary for transition of a naïve $CD8^+$ cell to an armed effector cell. In this regard, activated $CD8^+$ cells first proliferate and after 4-5 days of rapid growth they differentiate to CTLs (Janeway et al., 2001).

$CD4^+$ cells are activated by APCs presenting antigens in the groove of MHC-II molecules and expressing the co-stimulatory signal. Depending on the pathogen, APCs additionally release different cytokines. This cytokine profile determines, whether a $CD4^+$ cell will proliferate and differentiate into a Th1 or a Th2 cell. An APC secreting the pro-inflammatory cytokines IL-12 or TNF-α supports differentiation into a Th1 cell, while the anti-

inflammatory cytokine IL-10 or IL-4 support differentiation into Th2 cells. Via this mechanism, APCs of the innate immunity are able to direct the subsequent adaptive immune response (Fearon and Locksley, 1996).

2.3.1.3.3 Effector phase

As the name implies, T-helper cells assist other cells in exerting their effector function. But the name does not reveal how centrally their function in adaptive immunity actually is. Depending on the type of T-helper cell induced, the following adaptive immune response will be a predominantly cell-mediated (Th1) or a predominantly humoral (Th2) immune response.

Cell-mediated immunity

Cell-mediated or cellular immunity includes processes induced by intracellular infectious agents. Principally, once $CD8^+$ cells are activated and differentiated to CTLs, they are able to kill every cell on which they recognize the specific pathogen initially triggering the differentiation without further co-stimulatory signals. This killing is achieved either by secretion of specific mediators or by inducing apoptosis: Perforin-1 perforates the cell membrane, granzymes have proteolytic properties, and IFN-γ inhibits viral proliferation. Further, binding of the Fas-ligand on CTLs to the Fas receptor of an infected cell induces its self-destruction.

Cells other than APCs do not express the initial co-stimulatory molecule, hence a $CD8^+$ cell, although specific for the presented antigen, still will not become activated. The missing second signal has therefore to be provided by another mechanism. This is done by Th1 cells: The Th1 cell specific for an antigen presented by the infected cell binds to it in parallel to the $CD8^+$ cell specific for an antigen of the same pathogen. Subsequently, the Th1 cell either induces the expression of the required co-stimulatory signal or secretes IL-2, thus activating the $CD8^+$ cell to differentiate into a CTL even in the absence of co-stimulation.

The second major task of Th1 cells deals with phagocytes not able to remove an infectious agent, because it inhibits the fusion of lysosomes to the phagosmones or prevents the acidification of the vesicles, which is required to activate lysosomal proteases. Therefore, such infectious agents may resist and even proliferate inside the phagocytic cell. To activate these phagocytes, Th1 cells recognizing the antigen-MHC-I complex produce IL-2, IFN-γ and TNF-β. Especially IFN-γ is important in that it induces antimicrobial mechanisms in macrophages known as macrophage activation. These cytokines further stimulate the macrophage to produce IL-1, IL-6, and TNF-α, which in turn activate an inflammatory response as described for the innate immune response. The result of Th1 action is a more effective activation and coordination of inflammatory mechanisms, as well as the activation of

macrophages enabling them to suppress the growth of intracellular pathogens. Whereas the latter is regarded as the principal effector action of Th1 cells (Janeway et al., 2001).

Humoral immunity

While the cell-mediated immune response protects intracellular spaces, extracellular spaces are protected by the humoral immune response, in which antibodies produced by B-cells exert the major effects. But before becoming an antibody-secreting plasma cell, once more first the B-cell has to be activated. And B-cell activation is dependent on Th2 cells, i.e. B-cells as well as $CD4^+$ cells have to respond to the same antigen, a process called linked recognition. Just when a $CD4^+$ cell recognized an antigen and differentiated to a Th2 cell and at the same time a B-cell recognized the same pathogen and presents peptides in complex with a MHC-II molecule, the basis for B-cell activation is provided. The Th2 cell will now bind via its specific TCR to the antigen-MHC-II complex expressed on the B-cell. This triggers the Th2 cell to synthesize cell-bound and secreted effector molecules. One particularly important Th2 effector molecule is the CD40 ligand, which binds to the B-cell surface molecule CD40. Additionally, Th2 cells synthesize the cytokine IL-4, which binds to the respective receptor on the B-cell. These two signals along with other signals synergize in driving the proliferation of the B-cell. After several rounds of proliferation and with the help of the cytokines IL-5 and IL-6 secreted by Th2 cells, B-cells further differentiate into antibody-secreting plasma cells. The fact that B-cell activation requires Th2 cells represents another safety net protecting an organism from harmful immune responses to host antigen.

The antibodies secreted by plasma cells, which resemble the specificity of the initial immunoglobulin receptor of the resting B-cell, mediate the humoral immune response in three main ways. First, they may bind to pathogens like viruses and intracellular bacteria. These pathogens have to bind to specific molecules on the target cell surface to enter the cell, a mechanism prevented by antibodies bound to the pathogen. Additionally, antibodies may prevent bacterial toxins from entering cells. These properties of antibodies are labelled neutralization. Second, antibodies may bind to the surface of a pathogen and thus enhance phagocytosis. This property is termed opsonization. Phagocytic cells recognize the constant C region of antibodies bound to a pathogen by their Fc receptors and subsequently engulf and phagocyte the opsonized pathogen. Third, antibodies may bind to the surface of a pathogen and thereby activate the complement proteins, resulting in the above described effects of enhanced phagocytosis, lysis and chemotaxis.

Which mechanisms are engaged in a particular response is determined by the class of antibodies produced. The expression of this class or so-called Ig isotype (i.e., IgG, IgM, IgD, IgE, or IgA) is again regulated by cytokines released by T-helper cells (Janeway et al., 2001).

Taken together, while Th1 cells secrete the cytokines IFN-γ, IL-2 and TNF-β and mediate phagocyte activation and cellular immune responses, Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, which primarily act as growth or differentiation factors for B cells and thus evoke strong antibody responses. Beyond that, Th1 and Th2 responses are mutually inhibitory (see fig. 2-11). This is, Th1 cytokines, such as IL-2 and IFN-γ, inhibit Th2 cells and therefore humoral immune responses and Th2 cytokines, such as IL-4 and IL-10, inhibit Th1 cells, hence macrophage activation and activation of $CD8^+$ cell (Mosmann and Sad, 1996; Romagnani, 2000). Consequently, prevalent Th1 or Th2 responses are linked to conditions like organ-specific autoimmune disorders or atopic diseases, respectively (Romagnani, 2000).

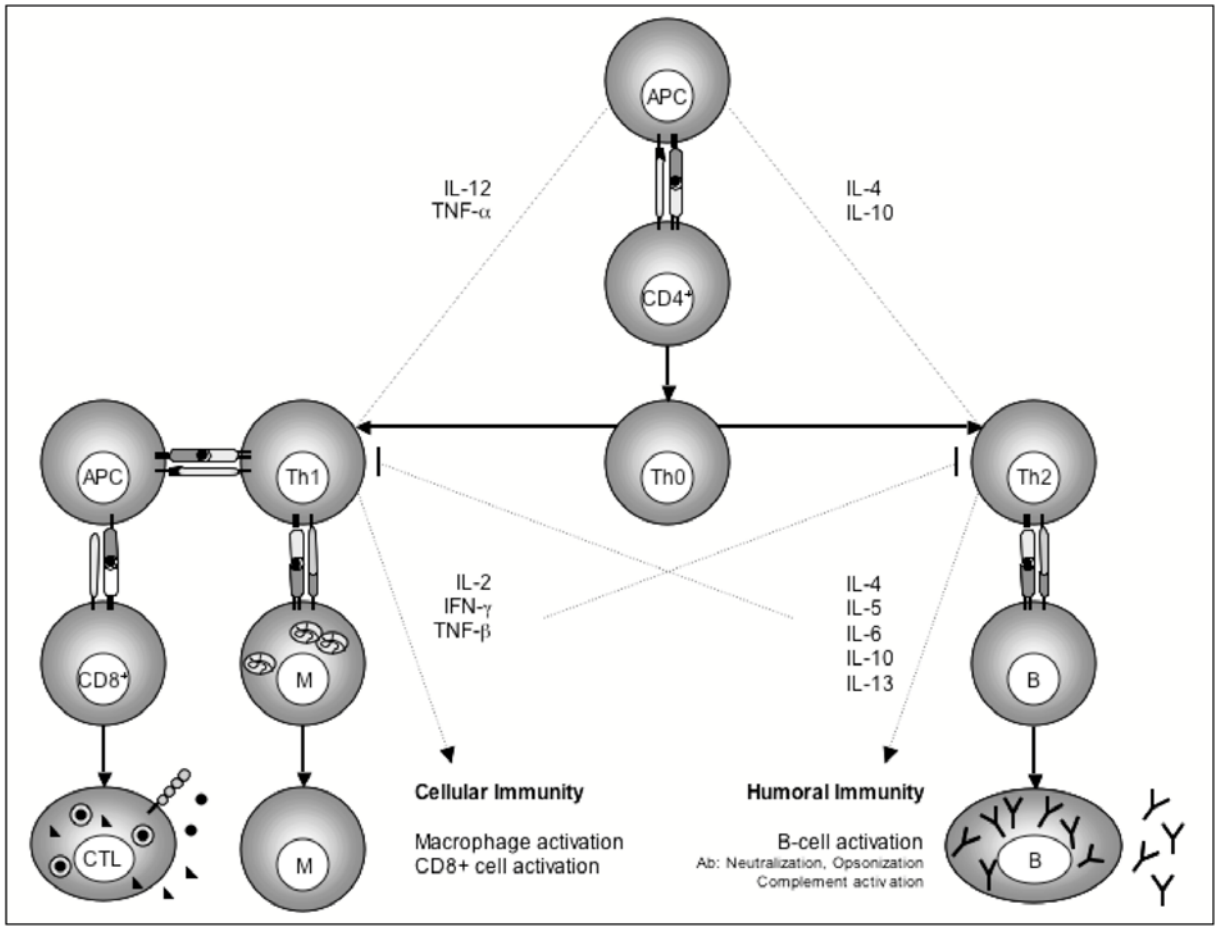

Fig. 2-11: Schematic view of the Th1-Th2 concept (Th: T-helper cell; APC: antigen presenting cell; CTL: cytotoxic T lymphocyte; M: macrophage; B: B-cell; Ab: antibody; modified from Mosmann and Sad, 1996).

However, it should be noted that the classification of activated $CD4^+$ T-cells (Th0 cells) into Th1 and Th2 cells presented in the above sections is based primarily on the cytokines they produce. This circumstance led to some controversy. For example, it was argued that synthesis of any cytokine is a continuously distributed function, hence classification of cells as Th1 or Th2 cells is strongly determined by assay sensitivity (Kelso, 1995). Further, since cytokine genes are independently regulated, T-helper cells may express diverse assortments of cytokine, not just the described two phenotypes (Kelso,

1995). Additionally, at least at present, no specific markers for the two cell types exist. Just some surface molecules – all chemokine receptors – show preferential expression. Of these, more selectively associated with Th1 or Th2 cells are the receptors CCR5 and CRTH2, respectively (Romagnani, 2000). Nevertheless, even if the classification in two types of Th cells may be an oversimplification, the concept has proven helpful in explaining most functions and diseases involving the adaptive immune system.

2.3.1.3.4 Memory formation

After an adaptive immune response is mounted and subsequently the infection effectively repelled, as a next step tissue integrity has to be restored. This includes the removal of most of the effector cells. Since the stimulus, which originally recruited the effector cells is now absent, most cells undergo 'death by neglect', i.e. the cells remove themselves by apoptosis (Janeway et al., 2001). This activation-induced cell death depends on up-regulation of CD95 which interacts with the Fas receptor and eliminates Fas-expressing T-cells. Another mechanism works via gradual up-regulation and stimulation of inhibitory receptors, such as CTL antigen-4, which also down-regulates the immune response (Alam and Gorska, 2003). The dying cells are then rapidly cleared by macrophages (Janeway et al., 2001). As a result, the infection is ended but additionally most of the pathogen-specific effector cells are lost.

However, one key characteristic of adaptive immunity is its memory. This memory is based on some of the effector cells retained and thus providing the "raw material for memory T-cell and B-cell responses" (Janeway et al., 2001). The factors determining which cells die or survive to become memory cells are still unclear. The former hypothesis of lymphocytes being under perpetual stimulation below the threshold for activation by residual antigen has been replaced by the hypothesis of specific memory being maintained by distinct populations of long-lived memory cells that can persist without residual antigen (Janeway et al., 2001). In the case of T-cells, memory T-cell population either can be formed directly from the effector cells themselves or without passing through an effector-cell stage, then referred to as central memory T-cells (Kaech et al., 2002). These memory cells have a lifespan of 10 years or more and react more quickly on subsequent exposure to pathogen (Parkin and Cohen, 2001). B-cell memory on the other hand is strongly associated with isotype switching (see above) and somatic mutations in antigen-binding domains of antibodies (Chaplin, 2003). While encountering a pathogen for the first time, B-cells produce predominantly IgM antibodies. The secondary response is characterized by larger amounts of IgG antibodies, with some IgA and IgE. Additionally, repeated exposure to the pathogen progressively increases the affinity as well as the amount of antibody. It has to be noted that the proliferation of memory B-cells still is driven by antigen-specific T-helper cells (Janeway et al., 2001).

Thus both – memory T-cells as well as memory B-cells – are responsible for mounting a faster and even more effective response to a second or third infection with the same antigen than the primary infection. This so-called protective immunity can also be induced artificially by vaccination. Vaccination results in long-lived immunological protection by “remembering” the first encounter and hence leading to enhanced memory responses that either completely prevent re-infection or greatly reduces the severity of disease (Kaech et al., 2002). The important role in reducing the mortality and morbidity caused by infectious diseases is evident. Therefore, Janeway et al. concluded that vaccination is the most outstanding accomplishment of immunology in the field of medicine (Janeway et al., 2001).

The previous section aimed at giving an overview of the different immune system components and how they function in protecting an organism against invading pathogens. Further, it should have become apparent that a harmonious communication between all these immune system components is imperative for a successful eradication of pathogens. But then, this also implies that at the other hand a dysfunction occurring at one or more levels may impair an effective immune response. Such changes in immune functions are in the focus of PNI research, which is dedicated to stress-neuroendocrine-immune interactions and their consequences for health and disease. In this regard as mentioned before, especially the phenomenon of stress-related immune changes and its clinical significance has attracted considerable attention. This phenomenon is also central to the present work. Therefore, and before focusing on the effects of a missing endocrine stress response, first the effects of acute and chronic psychosocial stress on immune functions will be presented (section 2.3.2). In a next step, this more phenomenological view will be specified by discussing the isolated effects of glucocorticoid and catecholamines on immune parameters (section 2.3.3). The chapter will then be secluded by an overview of mechanisms and modulators of glucocorticoid-to-immune system signaling (section 2.3.4).

2.3.2 Psychoneuroimmunology: Brain – to – Immune System Communication

Since the taste-aversion experiments by Ader and Cohen in 1975 (Ader and Cohen, 1975), numerous studies investigating the effects of psychological stress on immune function have been published. The models deduced from findings regarding the association between stress and immune parameters changed over time. Initially, studies investigating forms of stress like bereavement (Schleifer et al., 1983), marital discord (Kiecolt-Glaser et al., 1987), caregiving for a relative with a chronic disease (Esterling et al., 1994; Kiecolt-Glaser et al., 1991; Kiecolt-Glaser et al., 1996; Pariante et al., 1997), living with a cancer diagnosis (Andersen et al., 1998), or taking school examinations (Dobbin et al., 1991; Glaser et al., 1993) consistently found stress to be associated with suppression of NK-cell cytotoxicity and

lymphocyte proliferation as well as a blunted humoral response to immunization (for review see (Cohen et al., 2001; Herbert and Cohen, 1993). These observations and the broad clinical use of glucocorticoids as an immunosuppressive drug led to the conclusion that stress generally was immunosuppressive (Elenkov and Chrousos, 1999).

However, as Dhabhar and McEwen pointed out, a generalized suppression of immune function under all stress conditions is not an adaptive characteristic (Dhabhar and McEwen, 1997). Evolutionarily, an organism may be injured or infected under conditions of stress, for example when being chased by a predator. Hence, it is quite likely to require an active, instead of a suppressed immune response. Additionally, it seems paradoxical that on the one hand stress is thought to exacerbate inflammatory diseases like psoriasis (Farber et al., 1990), asthma (Joachim et al., 2003), and arthritis (Thomason et al., 1992), while on the other such diseases should be in fact ameliorated by a suppression of immune function. Therefore, Dhabhar and McEwen proposed that the decreases in absolute numbers of peripheral blood T-cells, B-cells, NK-cells and monocytes during acute stress represent a redistribution of these cells from blood to organs, such as bone marrow, lymph nodes and skin (Dhabhar et al., 1996). Such a redistribution appears evolutionarily reasonable, since it may serve to enhance immune function in compartments where it is most likely needed (e.g., when the skin is wounded during fight). But Dhabhar and McEwen also found chronic stress to attenuate this redistribution. Thus, they proposed a biphasic model in which acute stress enhances and chronic stress suppresses cell-mediated immunity (Dhabhar and McEwen, 1997). This biphasic model became modified based on the premise that short-term changes in *all* components of the immune system (i.e., innate and adaptive) would expend too much energy to be adaptive. Instead, stress should shift the balance of the immune response towards activating innate processes and diminishing adaptive processes, since the former can unfold much more rapidly and require less energy rather needed in other bodily systems to support the fight-or-flight response (Dopp et al., 2000; Segerstrom and Miller, 2004).

However, all above mentioned models do not resolve the paradox of stress to be associated with both, inadequate immunity (i.e., infectious disease) and excessive immune activity (i.e., allergic and autoimmune disease). Therefore, a new model had to be found which consolidates such contradictory stress effects. This model is the so-called cytokine or Th1/Th2 shift model. As already described above (see fig. 2.11), Th1-cells and their cytokines activate phagocyte activation and cellular immune responses, therefore providing defense against infection and neoplastic diseases. Contrary, Th2-cells and the respective Th2-cytokines evoke a strong humoral immune response (Mosmann and Sad, 1996; Romagnani, 2000). Since Th1 and Th2 responses are mutually inhibitory, suppression of one response has permissive effects on the other – a shift in the Th1/Th2 balance occurs. Thus, a diminished Th1 response not only increases the vulnerability to infectious and neoplastic

diseases, it also results in an enhanced Th2 response, which in turn increases the vulnerability to autoimmune and allergic diseases. Exactly such a shift has been shown to be associated with stress (Marshall et al., 1998).

Independently of their validity, the different models point out two facts: When studying the effects of psychological stress on immune functions, it is necessary to consider (1) the duration an organism is exposed to stress (acute vs. chronic stress), as well as (2) the type of immunity affected (innate, cellular, or humoral immunity).

Both considerations were accounted for in a meta-analysis including over 300 empirical articles investigating the relationship between psychological stress and immune system parameters recently published by Segerstrom and Miller (Segerstrom and Miller, 2004). With regard to the duration of stress, the authors adopted the taxonomy of Elliot and Eisdorfer (Elliot and Eisdorfer, 1982) to characterize the stressors used in the studies selected for the meta-analysis. They differentiated five types of stressors: Acute time-limited stressors (e.g., laboratory challenges), brief naturalistic stressors (e.g., academic examinations), stressful event sequences (e.g., the loss of a spouse and the series of related challenges), chronic stressors (e.g., caregiving for a relative with a chronic disease), and distant stressors (e.g., having been sexually assaulted as a child). Next, Segerstrom and Miller related these five categories to immune parameters. They accounted for different types of immunity and distinguishing between enumerative and functional measures[3].

Acute laboratory stressors were most commonly utilized, constituting 29% (n=85) of the articles considered. The meta-analysis revealed an up-regulation of innate immunity accompanied by a potential down-regulation of adaptive immunity in response to time-limited laboratory stressors. In detail, increased numbers of NK-cells and neutrophils, as well as an increased production of the pro-inflammatory cytokine IL-6 and the interferon IFN-γ, which stimulates macrophages and NK-cells, were indicators of an up-regulated innate immunity. On the other hand, lymphocyte proliferation was found to be decreased, thus suggesting a down-regulation of adaptive immunity. Taken together, these data are consistent with the modified biphasic model described above, proposing a shift towards activating innate processes and diminishing adaptive processes (Dopp et al., 2000; Segerstrom and Miller, 2004).

[3] Generally, laboratory assays measuring immune parameters can be differentiated in those quantifying cell numbers or concentrations of antibody or cytokine, and those quantifying cell functions. Cell numbers are detected most easily by counting the numbers of specific subsets of peripheral blood mononuclear cells (PBMCs) by fluorescence activated cell sorting (FACS) or by direct counting of cells under the microscope. Cytokine and antibody concentrations can be measured either *in vivo* in plasma or serum, or *in vitro* by stimulating cells to produce these proteins. To quantify immune functions, mostly the ability of NK-cells to lyse target cells (NK cytotoxicity) and the ability of lymphocytes to proliferate if stimulated (lymphocyte proliferation) are assessed *in vitro*. Additionally, immune responses to vaccination or intra-dermal antigen application (delayed type hypersensitivity (DTH) test) may be measured (for review see Vedhara et al., 1999).

Brief naturalistic stressors mostly involved academic examinations and made up 21,5% (n=63) of the articles. Here, data support the Th1/Th2 shift model: Functional measures showed decreases in T-cell proliferation with brief stressors, consistent with a suppression of cellular (Th1) immune responses. Further, the production of the Th1 cytokine IFN-γ was attenuated. On the contrary, the production of the Th2 cytokines IL-6 and IL-10 was increased, as was the antibody production to latent virus.

Regarding the third category – stressful event sequences - Segerstrom and Miller did not find reliable immune changes. Therefore, studies were further divided into three subcategories according to the stressor utilized: loss of a spouse, trauma (disasters), and positive biopsy for breast cancer. Loss of a spouse was associated only with decreases in the cytotoxicity of NK-cells. No changes were found in lymphocyte proliferation and in numbers of Th-cells or CTLs (depending on the parameter, the analysis was based on four to seven studies). In subjects exposed to a natural disaster (n=2-4), on the one hand increases in NK-cell cytotoxicity and lymphocyte proliferation, on the other hand decreases in Th-cell and CTL numbers were found. However, none of these changes were statistically significant. The three studies on women with a positive initial biopsy for breast cancer also did not yield a consistent pattern of immune changes. These inconsistent findings of declines in innate immune responses (loss), increases in innate and adaptive immune responses (trauma) and no immune alterations (breast biopsy) may well be due to the small numbers of studies involved and hence should be considered with caution. But these results also point out that stressors comparable on the fist glance still may be distinct and thus have distinct effects. This is underlined by the different endocrine consequences of loss and trauma. While loss is mostly associated with increases in cortisol (Irwin et al., 1988), trauma is rather associated with decreases in cortisol (Yehuda, 2001).

Chronic stressors also were very heterogeneously and included dementia caregiving, being handicapped, and unemployment (7,8%; n=23). Nevertheless, the effects on immune parameters were quite consistently. While no systematic changes in leukocyte subset numbers were found, NK-cell cytotoxicity, lymphocyte proliferation, and production of IL-2 all were negatively associated with chronic stress. These immuno-suppressive effects of chronic stress on both, innate and adaptive immune parameters, are consistent with the second part of the biphasic model proposed by Dhabhar and McEwen (Dhabhar and McEwen, 1997) or the global immunosuppressive model.

A total of nine studies (3,1%) contributed to the fifth category: distant stressors, such as abuse in childhood or combat exposure. In these studies, only NK-cell cytotoxicity was examined regularly. However, this immune outcome was not reliable associated with stress.

In conclusion, this meta-analysis exemplarily reveals a basic phenomenon: psychological stress not only is reliably associated with immune changes, but these immune

changes are also determined by the characteristics of the stressor. In short, acute time-limited stressors were found to up-regulate innate immunity, brief naturalistic stressors to trigger a Th1/Th2 shift, and chronic stressors to be associated with global immuno-suppression (Segerstrom and Miller, 2004).

2.3.3 Role of Stress Mediators

At this point, the question arises on how these stress effects on immune system are mediated. Since psychological stress is associated with an activation of two stress systems, namely the SAM and the HPA axis (see section 2.1.2), it stands to reason that the immune changes described above are influenced in large part by the hormones epinephrine, norepinephrine, and cortisol. Further, depending on intensity and duration of a psychological stressor, these hormones are secreted in varying concentrations and combinations. Accordingly, they may influence immune parameters differently. Therefore, in the next two sections the effects of the stress mediators themselves on immune functions will be reviewed.

2.3.3.1 Effects of Glucocorticoids on Immune Functions

As described in section 2.1.2.1.3 (feedback regulation), two receptors are used to regulate the HPA activity and mediate cortisol signals: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). These two receptors differ in their affinities and their distribution. The MR is a high affinity receptor for endogenous cortisol and is occupied and activated at lower concentrations than the GR, which is a receptor with 10-fold lower affinity for cortisol but a high affinity for the synthetic glucocorticoid dexamethasone (DEX) (Spencer et al., 1990). As shown above, these two receptors are differentially distributed in the central nervous system (De Kloet et al., 1998). But for glucocorticoids to exert effects on the immune system, these receptors also have to be expressed in immune cells and tissues.

In fact, this was repeatedly described for both MR and GR (e.g., Armanini et al., 1985; Lowy, 1989; McEwen et al., 1997). And as in the central nervous system, the expression of receptor subtypes is considerable heterogeneously. Miller et al. assessed GR protein levels using multiple techniques and found different distributions: The whole thymus and thymus derived T-cells (mostly $CD4^+CD8^+$ double-positive cells) had higher levels of GR than more mature T-cells isolated from the spleen. Neutrophils in turn had fewer GRs than lymphocytes and also than monocytes (Miller et al., 1998). While MRs are not detected in the thymus, both receptors are expressed in the spleen, although MR protein in the spleen is low (Miller et al., 1990).

These data show that basically glucocorticoids effects upon the immune system can be mediated classically by binding to their respective receptors. But they also suggest that

different immune compartments and cell types may have different responses or sensitivity to glucocorticoid hormones. Therefore, in the following glucocorticoid actions already summarized in section 2.1.4.4 according to Sapolsky et al. (Sapolsky et al., 2000) will be reviewed in more detail. Since at least GRs are found in all immune components, glucocorticoids affect virtually every immune function and all immune processes. This includes the development, maturation and death of immune cells, immune cell trafficking, antigen presentation by APCs, cytokine production, secretion and signaling, regulation of the class of immune response and effector functions.

2.3.3.1.1 Development, maturation and death of immune cells

Glucocorticoids modulate the immune system by intervening already at early stages of precursor cell differentiation. Shezen et al. found that glucocorticoids *in vitro* on the one hand enhance the formation of granulocyte colonies, on the other hand they suppress the formation of macrophage colonies, thus causing a shift in the balance of granulocyte vs. macrophage formation (Shezen et al., 1985). Furthermore, glucocorticoids attenuate the differentiation of monocytes (Baybutt and Holsboer, 1990). Chronically elevated levels of glucocorticoids were also shown to cause a rapid depletion of developing B-lineage cells in the bone marrow of mice by reducing the number of cycling precursor B-cells and inducing apoptosis (Garvy et al., 1993). However, glucocorticoids are not simply suppressive in all circumstances. For thymocyte survival and differentiation they are rather necessary, in that T-cell hybridomas and thymocytes are rescued only by the simultaneous stimulation through both the T-cell receptor (TCR) and the GR (Iwata et al., 1991; Zacharchuk et al., 1990). Vacchio et al. showed that absence of stimulation through the GR not only inhibits the progression from double negative $CD4^-CD8^-$ to double positive $CD4^+CD8^+$ cells and augments negative selection, but also results in death of thymocytes that would have otherwise undergone positive selection (Vacchio et al., 1998). Therefore, in antagonizing TCR-mediated death signals and allowing positive selection to occur, glucocorticoids at physiological levels play a critical supportive role in thymocyte development. None the less, Vacchio et al. also showed that beside having the highest numbers of GRs, the thymus also is able to make its own steroids (Vacchio et al., 1994). It is therefore unclear, if stress-induced changes in glucocorticoid concentrations play a role in thymocyte development or if these effects are due to locally produced glucocorticoids only. Furthermore, Weiss demonstrated that glucocorticoids at concentrations found at the diurnal peak are capable of inducing cell death in thymocytes *in vitro* (Weiss, 1971). These conflicting results of glucocorticoids mediating positive and negative effects in the thymus are summarized in an overview by Jondal et al. (Jondal et al., 2004). They suggested that the effects depend on the local hormonal concentration, with basal glucocorticoid levels promoting growth of early thymocytes and increased levels due to stress inducing apoptosis in these cells. Principally,

it has been known for the last decade that glucocorticoids are capable of inducing apoptosis in most nucleated cells. But again, the associations are quite complex. First, the effect of glucocorticoids on apoptosis depends on the cell lineage. Cells of hematopoietic origin (e.g., monocytes, macrophages, lymphocytes) are very sensitive to glucocorticoid stimulation of apoptosis. In contrast, cells of epithelial origin (e.g., mammary gland, ovarian follicular cells, hepatocytes) are protected by glucocorticoids against various stimuli for apoptosis (Amsterdam and Sasson, 2002). Second, as Evans-Storms and Cidlowski pointed out, anti-proliferative and apoptotic effects of glucocorticoids may have the same net result, but these effects are mediated by different pathways. Hence, it is important to separate them (Evans-Storms and Cidlowski, 1995).

2.3.3.1.2 Immune cell trafficking

Glucocorticoids also play an important role in regulation of immune cell distribution or trafficking. Numerous investigators demonstrated that pharmacological glucocorticoid application as well as stress-induced changes in glucocorticoids induce a pronounced decrease in lymphocyte, monocyte, and eosinophil numbers in the blood, accompanied by an increase in numbers of neutrophils (e.g., Fauci and Dale, 1974). These effects are mediated via the GR (Dhabhar et al., 1996) and interpreted as a redistribution of the cells out of circulation into other body compartment (Fauci and Dale, 1974) or to “battle-stations” (Dhabhar et al., 1996), rather than a glucocorticoid-induced cell death. This redistribution of cells is probably due largely to alterations in cell adhesion molecules (Sapolsky et al., 2000). Glucocorticoids can induce down-regulation of cell adhesion molecules in endothelial and polymorphonuclear cells and regulate the expression of cell adhesion molecules in lymphocytes and monocytes, thus alter cell trafficking and hence cell infiltration at sites of inflammation (Cronstein et al., 1992; for review see Pitzalis et al., 2002).

2.3.3.1.3 Antigen presentation by APCs

The induction of an immune response largely depends on the ability and effectiveness of MHC molecules to bind and present antigens. Glucocorticoids interfere with this response not only by altering the effectiveness of antigen presentation, but also at very early stages of the adaptive immune response by modulating differentiation, maturation and function of antigen-presenting cells, specifically of dendritic cells (Kitajima et al., 1996; Tuckermann et al., 2005). For example, glucocorticoids have been shown to down-regulate MHC-II antigens on macrophages and to reduce IFN-γ-induced increases in MHC-II expression in rats (Leszczynski et al., 1986). With regard to APCs, glucocorticoids can suppress the accumulation of dendritic cells in the spleen and bronchial mucosae, where immature dendritic cells reside that have not yet taken up antigen (Moser et al., 1995). In this regard, the finding that glucocorticoids suppress dendritic cell maturation is interpreted as a

positive mechanism, since it promotes the tolerance of dendritic cells to self antigens (Tuckermann et al., 2005). Once activated, APCs migrate into the draining lymph nodes, a process which for Langerhans cells has been shown to be inhibited by glucocorticoids (Cumberbatch et al., 1999). Cumberbatch et al. further found that this effect results from glucocorticoid regulation of synthesis and/or release of TNF-α, which provides one important signal for Langerhans cells to traffic from skin into lymph nodes (Cumberbatch et al., 1999).

2.3.3.1.4 Cytokine production, secretion and signaling

The latter finding emphasized an important mechanism by which glucocorticoids influence the immune system, namely by interfering with the production, secretion and signaling of cytokines. One of the most well-established and important effects of glucocorticoids is the inhibition of cytokine production. This includes IL-1, IL-2, IL-3, IL-5, IL-6, IL-12, granulocyte monocyte colony-stimulating factor (GM-CSF), IFN-γ, TNF-α, and chemokines like IL-8 (reviewed in Wiegers and Reul, 1998). But again, these glucocorticoid effects have to be regarded differentially. As mentioned before, glucocorticoids not only influence cytokine expression, but they may also (up-)regulate the expression of receptors for these cytokines (see section 2.1.4.4). Via this dual regulatory actions, glucocorticoids effectively and tightly regulate mediator activity (Munck and Naray-Fejes-Toth, 1992). For example, Wiegers et al. found in *in vitro* studies that glucocorticoids as expected suppress IL-2 production in anti-TCR-stimulated rat splenic lymphocytes, but by increasing the expression of IL-2 receptors, glucocorticoids also increase the sensitivity of these cells to IL-2 during the phase of enhanced proliferation (Wiegers et al., 1995); for glucocorticoid-dependent stimulation of IL-6 receptor synthesis see (Snyers et al., 1990). These findings show that rather than simply suppressing cytokine production, glucocorticoids exert a regulatory role in cellular immunity by combining stimulatory components and inhibitory properties. It also shows that it is necessary to include the assessment of all primary parameters and full-time course analyses to conceptually define the role of glucocorticoids (Wiegers and Reul, 1998).

2.3.3.1.5 Regulation of the class of immune response

The fact that glucocorticoids influence cytokine synthesis may also have further implications. By suppressing cytokines derived from Th1-cells (IL-2, IFN-γ), dendritic cells, or macrophages (IL-12) on the one hand and enhancing the production of IL-4 and IL-10 derived from Th2-cells, macrophages, dendritic cells and mast cells on the other hand, glucocorticoids cause a shift from cellular to humoral immune processes, hence regulate the class of immune response (Daynes and Araneo, 1989; Elenkov, 2004; Tuckermann et al., 2005). In contrast to these findings, glucocorticoids in pharmacological concentrations

strongly inhibit the secretion of the Th2 cytokines IL-4, IL-5, and IL-13 by T-cells, thereby exerting the immunosuppressive effects being availed clinically in allergic diseases (Barnes, 2001).

2.3.3.1.6 Effector functions

Beside effects on induction and proliferation of immune responses, glucocorticoids have been found to directly inhibit a variety of immune effector functions relating to NK-cells, CTLs, and B-cells. In the case of NK-cells, it has been reported that glucocorticoids *in vitro* inhibit the cytotoxic activity in a dose-dependent manner and also minimize the enhancement of NK cytotoxicity obtainable in the presence of IFN-γ (Gatti et al., 1987). Furthermore, these effects were shown to be due to direct inactivation of cytotoxic function of NK-cells and not due to effects on cellular proliferation (Callewaert et al., 1991). Interestingly, *in vivo* administration of glucocorticoids has often been found to facilitate instead of inhibit NK-cell responses. This facilitation appears to reflect glucocorticoid effects on cell distribution rather than altered cell function (Katz et al., 1984). In the case of cytotoxic T-lymphocytes (CTLs), glucocorticoids were also found to be mostly inhibiting. Here, glucocorticoids may act both indirectly via effects on IL-2 production and directly on CTLs. For example, Schleimer et al. found the inhibitory effects of low but not higher concentrations of dexamethasone to be reversible by IL-2 (Schleimer et al., 1984). But glucocorticoids may affect not only CTL proliferation, but also CTL apoptosis. The former was shown to be the result of glucocorticoids inhibiting IL-2 activity (Redondo et al., 1988), the latter was inhibited by IL-2 and IL-4 (Migliorati et al., 1994). Furthermore, studies by DeKrey and Kerkvliet suggest glucocorticoid effects on CTLs to be dose-dependent (DeKrey and Kerkvliet, 1995): the generation of CTL activity in mice was sensitive to high doses of glucocorticoids or dexamethasone, but was refractory to stress-like glucocorticoid elevations. In the case of B-cells, glucocorticoids have been shown to decrease the expression of MHC class II molecules on B-cells (Dennis and Mond, 1986; Weiss et al., 1996b) and the number of mature B-cells in the spleen (del Rey et al., 1984; Weiss et al., 1996b). Weiss et al. emphasized that for the former effect glucocorticoids are necessary and sufficient, but for the latter, they are just necessary, i.e., other mediators in addition are required (Weiss et al., 1996b). Contrary to early (antigen-stimulated activation and proliferation) or later (proliferative response to B-cell growth factor) events in the B-cell cycle, glucocorticoids were consistently observed to stimulate the immunoglobulin synthesis by cultured B-cells, when added early (Cupps et al., 1985; Grayson et al., 1981; Wu et al., 1991). However, as described above, some of these effects could be secondary to glucocorticoid modulation of cytokine production or activity, such as the shift from Th1 to Th2 cells or the induction of cytokine receptors (Sapolsky et al., 2000; Wu et al., 1991). Furthermore, glucocorticoids *in*

vivo were also found to suppress immunoglobulin production (Goldstein et al., 1992). Thus, it is difficult to evaluate the physiological role of glucocorticoid influences on B-cell functions. Nevertheless, these data point out that endogenous glucocorticoids at least contribute to the regulation of B-cell response to antigen (del Rey et al., 1984; McEwen et al., 1997).

2.3.3.1.7 Summary of glucocorticoid effects on immune function

In summary, glucocorticoids have been shown to influence virtually every immune function, mostly in a suppressive way. However, the outline above also showed that these effects have to be interpreted cautiously when it comes to drawing conclusions about the physiological role of glucocorticoids in immune function, especially the role of stress-induced increases in glucocorticoid concentrations. First of all, many results were obtained using the synthetic glucocorticoid dexamethasone, and furthermore, using rather high concentrations of it. Therefore, it is subject to controversial discussion, if observed effects may be limited to synthetic glucocorticoids or supra-physiological concentration of endogenous glucocorticoids. For example, Dhabhar and McEwen pointed out that acute stress and low doses of corticosterone in laboratory animals increased, instead of decreased specific components of immune function, e.g. cellular immunity in the skin (Dhabhar and McEwen, 2001). Hence, questions arise not only regarding the transferability of studies using dexamethasone, but also regarding the dependency of glucocorticoid effects on the applied dose. Another point concerns the transferability of *in vitro* data to *in vivo* situations. As mentioned above, in some immune parameter, e.g. in immunoglobulin production, contrary effects of glucocorticoids are observable. Additionally, some effects may require other mediators in addition (Weiss et al., 1996b). Furthermore, the studies by Wiegers et al. showed that glucocorticoids may act in a complex manner and exert dual regulatory actions (suppression of IL-2 and stimulation of IL-2 receptor expression; (Wiegers et al., 1995). Therefore, it is necessary to consider various primary parameters and full-time course analyses to reveal such complex interplays. Beside these points for further discussion, also species-differences have to be taken into account. Some effects seem comparable in rodents and humans, while others, like the greater sensitivity of rodent lymphocytes to glucocorticoid-induced apoptosis, differ between species (Weiss et al., 1996b).

Taken together, although many glucocorticoid effects on immune functions seem to be suppressive when considered isolated, this may nevertheless serve a positive mechanism when related to the whole organism. Therefore, glucocorticoids are not thought of as being mostly suppressive and sometimes enhancing, but rather as directing immune reactions in a specific and coordinated manner. This position is also held by Besedovsky et al., who stated that glucocorticoids are *sculpting* the immune response by selectively inhibiting specific immune processes, while stimulating or not affecting others (Besedovsky et al., 1991).

2.3.3.2 Effects of Catecholamines on Immune Functions

As for glucocorticoids, the immunological effects of catecholamines secreted as a stress response is an area of considerable complexity, since epinephrine and norepinephrine also have both stimulating and inhibitory effects (Sapolsky et al., 2000). Generally, catecholamines exert their effects by binding to adrenoceptors, which are transmembrane spanning G-protein coupled cell surface receptors. Adrenoceptors can be classified into three major groups, α1-, α2-, and β-adrenoceptor types, which can be further subdivided into at least three subtypes: α1A, α1B, and α1C, α2A, α2B, and α2C, and β1, β2, and β3 (Hasko and Szabo, 1998). Norepinephrine predominantly activates α- and β1-adrenoceptors and is a weak stimulator of β2-adrenoceptors, whereas epinephrine is a strong stimulator of β-adrenoceptors (Motulsky and Insel, 1982).

For catecholamines to modulate immune functions, the major requirement is the presence of adrenoceptors on immune cells and immune tissues. Until recently, adrenoceptor expression on peripheral blood leukocytes (i.e., lymphocytes, macrophages, neutrophils, eosinophils, and basophils) was thought to be restricted to α2- and β-adrenoceptors (Bishopric et al., 1980; Hadden et al., 1970; Khan et al., 1986; Pochet et al., 1979). This was due to the demonstration of Casale and Kaliner that circulating human blood cells have no detectable α1-adrenoceptors (Casale and Kaliner, 1984). But Rouppe van der Voort et al. showed in 1999 that the expression of α1b- and α1d-adrenoceptors can be *induced* in human monocytes by dexamethasone and the β2-adrenoceptor agonist terbutaline (Rouppe van der Voort et al., 1999); see also (Kavelaars, 2002). Beside differing in the type of adrenoceptor predominantly expressed, lymphocyte subsets differ also in their receptor density, such as NK-cells having the greatest and Th-cells having the lowest number of β2-adrenoceptor, while CTLs, B-cells, and monocytes have an intermediate number of β2-adrenoceptors (Khan et al., 1986; Maisel et al., 1989). Importantly, β2-adrenoceptors seem to be selectively expressed on Th1 cells, while Th2 cells do not express any detectable β2-receptors (Sanders et al., 1997). However, high β2-adrenoceptor density does not necessarily mean high response after stimulation of these receptors. It was demonstrated that NK-cells, CTLs, and monocytes are very responsive to β-adrenoceptor stimulation, while Th-cells and B-cells show only a modest response (Knudsen et al., 1995; Maisel et al., 1989).

Beside type, density and responsiveness of receptors, also the way of communication can be different. Norepinephrine may reach its target cells by (1) neural communication, when released at synaptic junctions and acting across a narrow synaptic cleft on the postsynaptic cell, by (2) non-synaptic communication, when diffusing in the vicinity of the nerve terminal and reaching more distant target cells, and by (3) endocrine communication, when released from the adrenal medulla and reaching cells via the circulating blood.

However, epinephrine reaches its target cells just by endocrine communication (Hasko and Szabo, 1998).

Despite this complex interplay of receptor types, numbers, and sensitivities and ways of communication, the reports of catecholamine effects on immune function rather consistently found most effects to be mediated via α2- and β-adrenoceptors. As glucocorticoids, catecholamines – directly or indirectly – influence virtually every immune function and immune process. In this regard, a clear distinction should be made between the role of sympathetic nervous system control of immune processes and effects of catecholamines on immune functions. For example, specific compartments of the thymus are innervated noradrenergically and hence, norepinephrine but not epinephrine effects on thymocyte development has been described. However, in the following, an overview of catecholamine effects on immune functions will be given. This includes thymocyte development; immune cell trafficking; activity and function of cells of the innate immunity; production of cytokines and regulation of the class of immune response; and effector functions (i.e., lymphocyte proliferation, CTL activity, and antibody production).

2.3.3.2.1 Thymocyte development

To differentiate into T-cells, thymocytes have to be under the influence of a specific microenvironment, which is created by a number of supporting cells. The major cell type involved are supposed to be thymic epithelial cells (TEC) and these TECs synthesize the key factors of the thymic microenvironment, e.g., cytokines like IL-1 or IL-6 (Kendall, 1991). Furthermore, especially subcapsular/perivascular TECs are in close connection to catecholaminergic nerve fibers innervating the thymus and thus exposed to norepinephrine, the main catecholamine in the thymus (Kendall et al., 1988). Additionally, TECs have been shown to express functionally active β1- and β2-adrenoceptors (Kurz et al., 1997). However, von Patay et al. demonstrated that the release of IL-6 from TECs *in vitro* was stimulated only scarcely by catecholamines and moderately by LPS alone, but increased dose-dependently when both were added and thus acted synergistically (von Patay et al., 1998). The regulation of IL-6 expression in TECs by catecholamines is of particular interest, because IL-6 has repeatedly been implicated in T-cell development: In combination with IFN-γ and IL-2, IL-6 has been shown to induce the differentiation of CTLs from immature thymocytes (Takai et al., 1988) and together with IL-2 or IL-4, IL-6 enhances the proliferation of single positive thymocytes (Chen et al., 1989; Suda et al., 1990). Additionally, IL-6 acts as an autocrine growth factor for TECs themselves (Colic et al., 1992). Therefore, it may be speculated that catecholamines play a role in augmenting the production of T-cells.

2.3.3.2.2 Lymphocyte trafficking and circulation

Increases in SNS activity or administration of catecholamines have been shown to affect lymphocyte distribution in a time-dependent manner. In the short term, catecholamines acutely mobilize NK-cells and granulocytes from depots, whereas in the long term, chronically, catecholamines decrease the number of lymphocytes, and particularly of NK-cells in the peripheral blood (Benschop et al., 1996; Schedlowski et al., 1996), whereas T- and B-cell numbers remain relatively unaffected (Benschop et al., 1996). These changes in NK-cell numbers were shown to be accompanied neither by alterations in expression of adhesion molecules on NK-cells nor by changes in plasma concentration of soluble adhesion molecules (Schedlowski et al., 1996). This modulation of NK-cell circulation rather appears to be mediated via β2-adrenoceptor mechanisms, whereas the transient increases of granulocytes after short term catecholamine administration involve α-adrenoceptor stimulation (Benschop et al., 1996). In this regard, several mechanisms by which catecholamines modulate lymphocyte distribution are discussed. The finding that T- and B-cells remain relatively unaffected suggests a role of the varying sensitivities of lymphocyte subpopulations to catecholamine effects. Alternatively, the SNS, which directly innervates the vascular smooth muscle and regulates the regional blood flow, may thereby change the delivery of lymphocyte to post-capillary venules of tissues and thus the opportunity for lymphocytes to enter tissues (Elenkov et al., 2000). Independently of the mechanisms, catecholamines increase the number of circulating NK-cells and granulocytes, i.e., immune cells of innate immunity. And because higher numbers of circulating cells also means more cells can be attracted in cases of tissue damage, catecholamines may help reducing the risk for infections (Benschop et al., 1996).

2.3.3.2.3 Macrophage activity and neutrophil functions

The effects of catecholamines on macrophage functions appear to be rather complex. Catecholamines are repeatedly reported to stimulate (Spengler et al., 1990) as well as suppress (Hasko et al., 1998) the production of TNF-α by macrophages. This apparent discrepancy may be attributed to the state of activation of macrophage populations. It is thought that there is a transient stage of differentiation when monocytes during maturation to macrophages lose their β-adrenoceptor responsiveness. Naïve cells may thus preferentially express α-adrenoceptors, which will result in stimulation of macrophage activity (Spengler et al., 1990). On the other hand, antigen challenge and activation of macrophages may result in an increase in β-adrenoceptors and hence a suppression of macrophage activity (Chou et al., 1996; Hasko et al., 1998). Despite the state of activation or differentiation of macrophages, which may determine the responsiveness and expression of adrenoceptor subtypes, also various other factors may modulate the effect of catecholamines on

macrophage activity. These are: the presence of mediators, such as substance P, which potentiates TNF-α production by macrophages (Ho et al., 1998), peripheral CRH as a potent mast cell secretagogues (Theoharides et al., 1995), and histamine, which suppresses the production of TNF-α and is stimulated via α- or inhibited by β2-adrenoceptors by catecholamines, and the effect of catecholamines on the expression of important co-stimulatory molecules like B7.2.

Contrary to the complex effects of catecholamines on macrophage functions, functions of neutrophils seem to be generally inhibited by catecholamines. Neutrophil phagocytosis, release of lysosomal enzymes (Zurier et al., 1974), respiratory burst of neutrophils associated with degranulation (Nielson, 1987), superoxide generation and formation of oxygen radicals that play an important microbicidal role (Barnett et al., 1997; Weiss et al., 1996a), and chemotaxis of human neutrophils (Harvath et al., 1991) all have been shown to be suppressed by catecholamines. Here, stimulation of β-adrenoceptors seems to play an important role, as for example suppression of superoxide generation and formation of oxygen radicals at nanomolar concentrations of epinephrine could be prevented by β2-adrenorceptor blockade (Barnett et al., 1997; Weiss et al., 1996a).

2.3.3.2.4 Cytokine production and regulation of the class of immune response

In cytokine production, catecholamines appear to have a substantial modulatory role, affecting antigen-presenting cells and Th1-cells, mostly by stimulation of β-adrenoceptors. As for glucocorticoids, most of these effects result in driving a Th2 shift (Agarwal and Marshall, 2000; Chrousos, 2000; Elenkov and Chrousos, 1999; Hasko and Szabo, 1998), a mechanism by which catecholamines affect the class of immune response elicited in response to challenge.

In antigen-presenting cells, norepinephrine and epinephrine has been shown to inhibit the production of IL-12 in human whole blood cultures stimulated with lipopolysaccharide (LPS) *ex vivo* via stimulation of β-adrenoceptors on monocytes (Elenkov et al., 1996). IL-12 enhances IFN-γ and inhibits IL-4 synthesis by T-cells, which results in inhibition of Th1-cell development and promotion of Th2-cell differentiation (see figure 2-11). Inhibition of IL-12 production by catecholamines may thus represent one of the major mechanisms by which epinephrine and norepinephrine affect Th1/Th2 balance. Beside IL-12, catecholamines and β-adrenoceptor agonists inhibit the production of TNF-α by LPS-treated monocytes (Severn et al., 1992; van der Poll et al., 1994), microglial cells (Hetier et al., 1991), and astrocytes (Nakamura et al., 1998) and by human mast cells stimulated with IgE (Bissonnette and Befus, 1997). Mostly via this inhibition of TNF-α, also the production of IL-1 is suppressed by catecholamines (Koff et al., 1986; Van der Poll and Lowry, 1997). This effect is additionally potentiated by catecholamines enhancing the production of IL-10 (Elenkov et al., 1996;

Siegmund et al., 1998; van der Poll et al., 1996; Van der Poll and Lowry, 1997), a type 2 cytokine produced by antigen presenting cells. One other cytokine, of which the production is also up-regulated by catecholamines is IL-6 (Maimone et al., 1993; Norris and Benveniste, 1993). IL-6 exerts both pro-and anti-inflammatory effects, but possesses mostly Th2-type activities.

These actions of catecholamines are in close accordance with their effects on type 1 and type 2 cytokines. Since Th2 cells do not express β2-adrenoceptors (Sanders et al., 1997), catecholamines are most probable not able to affect the production of type 2 cytokines by Th2 cell directly. Thus, catecholamines mainly affect Th1 cells. For example, β2-adrenoceptor agonists have been shown to inhibit IFN-γ production by Th1 cells, but do not affect IL-4 production by Th2 cells (Borger et al., 1998; Sanders et al., 1997). Changes in cytokine patterns in turn affect the immunoglobulin-isotype produced by B-cells. In humans, IFN-γ producing Th1 cells induce B cells to produce IgG1, whereas IL-4 producing Th2 cells induce B cells to produce IgE and IgG4 (Fearon and Locksley, 1996).

Taken together, catecholamines on the one hand do not have a direct effect on the secretion of cytokines from Th2 cells hand, but facilitate the production of type 2 cytokines by antigen presenting cells. On the other hand, they inhibit the production of type 1 cytokines by both antigen presenting cells and Th1 cells. Thus, all effects of catecholamines on cytokine production of antigen presenting cells aim at attenuating cellular immunity and facilitating humoral immunity.

2.3.3.2.5 Effector functions

Lymphocyte proliferation:

T-cell proliferation induced by mitogens has repeatedly been shown to be inhibited by catecholamines or β-adrenoceptor agonists (Chambers et al., 1993; Hadden et al., 1970). Since $CD8^+$ T-cells have a higher number of β-adrenoceptors, the proliferative response of $CD8^+$ T-cells has been found to be inhibited to a greater extent than $CD4^+$ T-cells (Bartik et al., 1993). This anti-proliferative effect of catecholamines has been linked to an increase of cyclic adenosine monophosphate (cAMP) in lymphocytes (Carlson et al., 1989). Because elevation of cAMP in turn inhibits the secretion of the co-stimulatory cytokine IL-2 by T-cells (Bartik et al., 1993), inhibition of T-cell proliferation might at least in part be due to the inhibition of the production of IL-2 (Elenkov et al., 2000).

Cytotoxic T-lymphocyte (CTL) activity:

Only a few studies are available investigating the effects of catecholamines on CTL function. For example, Cook-Mills et al. found anti-MOPC-215 tumor cytotoxicity by mouse splenic CTLs to be suppressed by epinephrine, norepinephrine, and isoproterenol (Cook-

Mills et al., 1995). Dobbs et al. observed restraint stress to induce suppression of herpes simplex virus-specific CTL activation in mice, an effect which could be prevented by an peripherally acting β-adrenoceptor antagonist (Dobbs et al., 1993). Furthermore, *in vitro* development of memory CTL activity in mice was found to be significantly inhibited by increasing cAMP via a β-adrenoceptor agonist (Cook-Mills et al., 1995). Contrary, Hatfield et al. observed increased CTL-mediated cytotoxicity in mixed lymphocyte culture (BALB/c mice), but only, when catecholamines or β-adrenoceptor agonists were added at the initiation of a 5-day sensitization phase (Hatfield et al., 1986). These findings suggest that catecholamines on the one hand exert enhancing effects on the initiation of CTL responses, while they on the other hand inhibit effector cell function, i.e. CTL cytotoxicity and CTL memory development (Elenkov et al., 2000).

Antibody production / humoral immunity:

Regarding humoral immunity, norepinephrine is generally known to enhance B-cell antibody production via stimulation of β2-adrenoceptors. This effect is achieved by several mechanisms. First, catecholamines have been found to induce an increase in the frequency of B-cells differentiating into antibody-secreting cells (Sanders, 1998; Sanders, 2006). Seconds, agents that elevate cAMP up-regulate the expression of B7 molecules on B-cells, a molecule which determines the effectiveness of T-cell-B-cell interaction (Watts et al., 1993). Furthermore, for activation of conjugated B-cells, a critical threshold level of intracellular cAMP must be given (Pollok et al., 1991). Stimulation of β2-adrenoceptor during Th-2-B-cell interaction may help to augment cAMP levels above this critical threshold (Sanders, 1998). Third, catecholamines may influence B-cell functions by their effects on type1 and type 2 cytokine production (see section 2.3.3.2.4). In facilitating IL-4, IL-6, and IL-10 production (Coqueret et al., 1994) and inhibiting IFN-γ production (Coqueret et al., 1995), catecholamines selectively inhibit Th1 functions and cellular immunity and mediate a Th2 shift that potentiates humoral immunity.

2.3.3.2.6 Summary of catecholamine effects on immune functions

Taken together, catecholamines modulate lymphocyte trafficking, in that they mobilize NK-cells and granulocytes in the short-time, thereby reducing the risk for infections for example during acute stress (Benschop et al., 1996). Regarding cytokine production and release, catecholamines have been shown to cause a Th1/Th2 shift, resulting in potentiation of humoral immunity (Chrousos, 2000; Elenkov and Chrousos, 1999). These effects are facilitated by catecholamines inhibiting lymphocyte proliferation and CTL effector functions (Elenkov et al., 2000) on the one hand, and enhancing T-cell-B-cell interaction and B-cell antibody production on the other (Sanders, 1998).

2.3.3.3 Immune System – to – Brain Communication

In 1975, Besedovsky et al. reported that antigenic challenge to the immune system causes serum levels of glucocorticoids to increase to levels three times higher than normal (Besedovsky et al., 1975). Starting with this observation, evidence accumulated that the communication between nervous system and immune system is not unidirectional, but that the immune system itself sends signals to the nervous system that alter physiological and behavioral states. In the context of PNE and PNI, the activation of the HPA axis with subsequent secretion of glucocorticoids by cytokines is of special interest: These cytokine actions constitute a negative feedback loop ('cytokine-HPA axis feedback circuit'), in which an activated immune system is finally reset to a state of decreased or baseline activity. Therefore, instead of interfering with antigen presentation, clonal expansion of activated lymphocytes, or the initial production of cytokines, the rather late phenomenon of increased glucocorticoid levels are thought to prevent inappropriate, excessive expansion and activity of immune cells and overproduction of cytokines (Besedovsky and del Rey, 1996; Besedovsky and del Rey, 2000).[4]

2.3.4 Mechanisms and Modulation of Hormone-to-Immune System Signaling

In the previous sections, effects of stress as well as effects of single stress mediators on the immune system were described. As outlined, for glucocorticoids and catecholamines to modulate immune functions, the major requirement is the presence of receptors in and on immune cells and immune tissues, respectively. In the following sections, the molecular mechanisms of how hormone-receptor binding influences immune functions will be summarized (2.3.4.1: Mechanisms of hormone-to-immune system signaling). Subsequently, potential pathways of modulation of these mechanisms will be described (2.3.4.2: Modulation of hormone-to-immune system signaling).

2.3.4.1 Mechanisms of Hormone-to-Immune System Signaling

To facilitate insight into possible pathways by which glucocorticoids and catecholamines can influence immune cell functions, first the activation of the main effector cells of the innate immune response by LPS will be elaborated exemplarily in more detail.

[4] As this way of communication is not in the focus of the present thesis, the interested reader is referred to Besedovsky and del Rey (1996 and 2000) for further information, such as the different proposed pathways of cytokine-to-brain-signaling.

2.3.4.1.1 Monocyte activation and cytokine production

Basically, cells of innate immunity recognize pathogen-associated molecular patterns (PAMPs; Medzhitov and Janeway, 1997a; Medzhitov and Janeway, 1997b; Uthaisangsook et al., 2002), which are shared by large groups of pathogens. PAMPs are recognized by immune cells via a set of molecules as well as receptors referred to as pattern-recognition molecules (PRMs) and pattern-recognition receptors (PRRs; (Medzhitov and Janeway, 1997a). Binding of PRRs or PRMs to PAMPs is the first step in activating functions of innate immunity (see also section 2.3.1.2.2: recognition of pathogens: PAMPs and PRRs/PRMs). One example for a pathogen-associated molecular pattern is a component of the cell wall of gram-negative bacteria, namely lipopolysaccharide (LPS; Kitchens, 2000; Uthaisangsook et al., 2002). In more detail, LPS is recognized by the pattern-recognition receptors of the Toll receptor family. In humans, at least ten of these so-called Toll-like receptors (TLR) have been identified so far (Chuang and Ulevitch, 2001). For LPS recognition by monocytes and macrophages, specifically TLR-4 is thought to play an important role (Medzhitov et al., 1997). Before the discovery of TLR-4, LPS was only known to complex with soluble receptors (e.g., lipopolysaccharide binding protein (LBP) or the soluble form of the monocyte surface protein CD14 (sCD14)) and that this complex binds to the surface molecule CD14 of monocytes and macrophages. However, as CD14 does not possess a cytoplasmic domain, it could not be resolved how LPS could activate the subsequent cascade of effects. But TLRs – as type I trans-membrane proteins – consist not only of an extracellular leucine-rich repeat domain, but also do have a cytoplasmic domain. This cytoplasmic domain is homologous to the cytoplasmic domain of the human IL-1 receptor (IL-1R), hence it is referred to as the Toll/IL-1R homology (TIR) domain (Kopp and Medzhitov, 1999) and the intracellular cascade following activation of monocytes and macrophages by LPS is similar to the cascade activated by IL-1 (Muzio and Mantovani, 2000). This cascade is shown in figure 2-12.

In detail (see Caamano and Hunter, 2002), upon interaction with LPS bound to CD14, the intracellular domain of TLR-4 binds to the adaptor protein myeloid differentiation factor-88 (MyD88). The interaction of the TIR domain of TLR and the TIR domain of MyD88 recruits the IL-1R associated kinase (IRAK), which becomes phosphorylated and binds to TNF-receptor-associated-factor-6 (TRAF6). This binding activates the nuclear factor-kappaB (NF-κB)-inducing kinase (NIK), which next phosphorylates IκB kinase (IKK). IKK in turn phosphorylates and degrades inhibitory κB-α. Normally, the protein IκB-α inhibits activation of the transcription factor NF-κB by binding to NF-κB in the cytoplasm of cells. Upon dissociation of NF-κB from IκB, a nuclear localization signal (NLS) becomes unmasked and NF-κB translocates to the nucleus, where it binds to κB-responsive DNA elements. Binding of NF-κB to the κB-responsive DNA elements then induces the transcription of a large array of monocyte and macrophage products, such as TNF-α (McKay and Cidlowski, 1999), which

is usually the first cytokine to appear at an inflammatory site (Hesse et al., 1988; van Deventer et al., 1990).

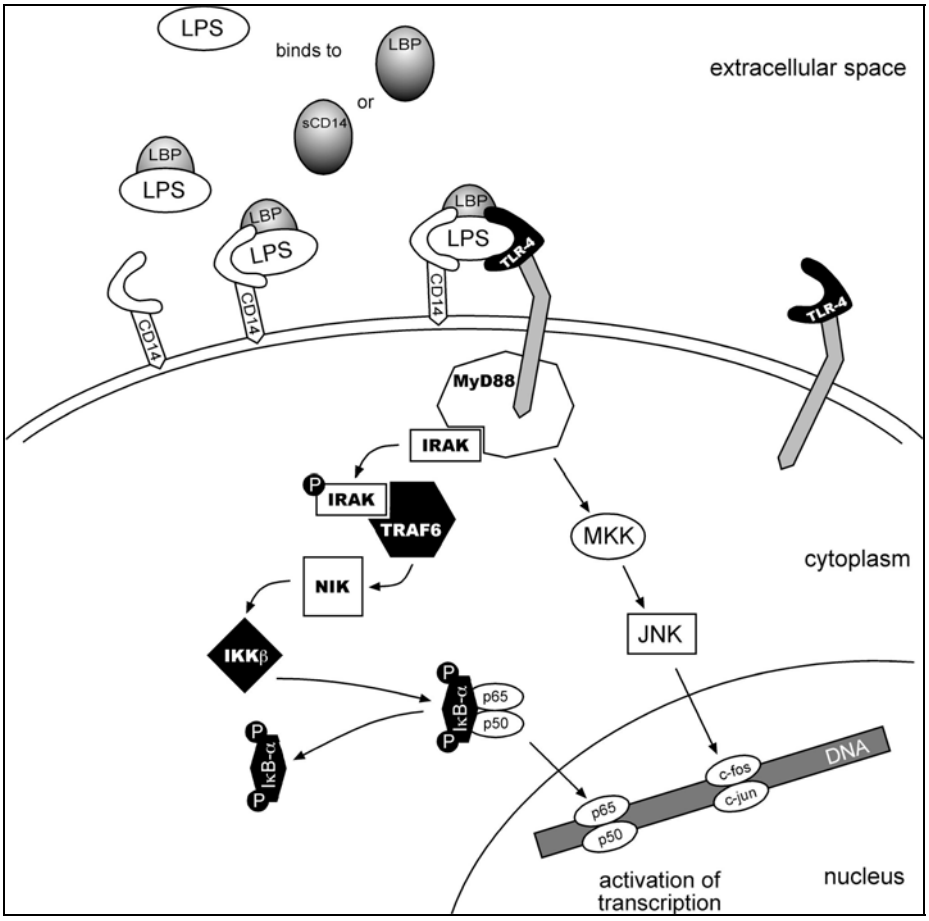

Fig. 2-12: Mechanism of monocyte activation by LPS (taken from Rohleder, 2003). NF-κB is depicted in the most frequently occurring heterodimeric complex between the p50 and p65 subunits.

However, NF-κB is found in virtually every cell of the immune system, hence a much greater number of genes transcriptionally regulated by NF-κB-DNA binding exist. Among these are growth factors (relevant cell types: monocytes, macrophages, myeloid cells), such as granulocyte/macrophage colony stimulating factor (GM-CSF), pro-inflammatory cytokines (B-cells, T-cells, monocytes, macrophages, etc.), especially IL-1, IL-2, IL-6, IL-8, and TNF-α, cell adhesion molecules (endothelial cells, mucosal cells, megakaryocytes), such as inter-cellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM), acute phase proteins (hepatocytes), for example serum amyloid A precursor protein and complement factors B and C4, but also transcriptional regulators including the protein IκB-α (for review see (McKay and Cidlowski, 1999). Such new synthesis of IkB-α causes retention of NF-κB in the cytoplasm and attenuation of NF-κB-mediated transcriptional activation (Baldwin, 1996), and provides a feedback mechanism for modulating the extent and duration of inflammatory responses by a cell.

In parallel to this pathway, binding of LPS complex to TLR and consecutive binding to MyD88 may also activate activator protein 1 (AP-1). The transcription factor AP-1 is a dimeric

complex that in mammalian cells most commonly comprises members of the Jun und Fos protein. Several cascades of AP-1 activation are known resulting in increased synthesis of the AP-1 components Jun and Fos and/or in phosphorylation of these components. Figure 2-12 depicts exemplarily the activation of the catalytic activities of the Jun N-terminal kinase (JNK) via the MAP kinase (MKK) cascade (Karin and Gallagher, 2005; Karin et al., 2001). Once activated, AP-1 has similar effects as NF-κB, with AP-1 regulating gene expression both independently of and synergistically with NF-κB (McKay and Cidlowski, 1999). Thus, AP-1 is a critical regulator of for example cell proliferation, cell survival, cell death, DNA repair and metabolism (Karin and Gallagher, 2005).

2.3.4.1.2 Glucocorticoid signal transduction

As shown in the previous chapters, glucocorticoids exert a wide range of effects on the immune system and these effects are mediated by glucocorticoid/type-II receptors (GR) and mineralocorticoid/type-I receptors (MR). Regarding immune cells and tissues, the former appears to be expressed predominantly (Armanini et al., 1985; Lowy, 1989; McEwen et al., 1997).

The main mechanism by which glucocorticoids exert their effects on immune functions is the inhibition of the NF-κB and AP-1 activation cascades described above (McKay and Cidlowski, 1999). For a more detailed insight in these mechanisms, the following three chapters first describe the activation of glucocorticoid receptors, followed by a summary of the so-called classic and alternative mechanism of glucocorticoid receptor action.

Glucocorticoid receptor activation

The glucocorticoid receptor is found in the cytoplasm as part of an assembly consisting of the receptor itself, two molecules of heat shock protein (HSP)90 and one molecule each of HSP70 and/or HSP56, as well as other, lesser know proteins, such as p23 (Pratt, 1993; Smith and Toft, 1993). Interestingly, in this complex of glucocorticoid receptor and HSP90, the receptor undergoes simultaneous conversion from a non-steroid binding but DNA binding state (transformed receptor) back to the steroid binding, non-DNA binding state (untransformed receptor; Scherrer et al., 1990). HSP90 in this regard seems to be required for strong ligand binding activity. But once hormone binding has occurred, it can be sustained in the absence of HSP90 (Bresnick et al., 1989).

As lipophilic substances, glucocorticoids (GC) are able to cross the cell membrane readily to interact with the intracellular glucocorticoid receptor (GR). Ligand binding induces conformational change in the GR molecule that has a number of functional consequences (Bamberger et al., 1996). First, the GR-GC complex dissociates from the HSP complex and is no longer able to re-associate with it (Hutchison et al., 1994). Furthermore, the partially

phosphorylated receptor protein becomes hyper-phosphorylated (Bodwell et al., 1993) and finally, now unmasked nuclear localization signals (NLS) within the ligand-binding domain of the receptor (Picard and Yamamoto, 1987) cause nuclear translocation of the GR-GC complex (Akner et al., 1994). Within the nucleus, the hormone-activated glucocorticoid receptor can act in two ways, referred to as classic and alternative mechanism of glucocorticoid receptor action.

Classic mechanism of glucocorticoid receptor action

The classic mechanism of GR action is characterized by GRs interacting with specific DNA sequences. A receptor homodimer binds to short DNA sequences termed glucocorticoid response elements (GREs) in the promoter region of glucocorticoid-responsive genes. When bound to the GRE, the GR homodimer interacts with components of the basic transcription machinery, either directly or indirectly via bridging factors. This interaction is sufficient to stabilize the pre-initiation complex on the promoter and thus to enhance transcription of GR-regulated genes by RNA polymerase II. In addition, binding of the GR homodimer to the GRE can induce a rearrangement of the chromatin structure in the respective promoter region, thus allowing other transcription factors to bind to the previously inaccessible DNA. Furthermore, in some promoters, binding of the activated GR to so-called negative glucocorticoid response elements (nGREs) causes inhibition rather than enhancement of transcription (Bamberger et al., 1996; McKay and Cidlowski, 1999).

Regarding the immune system, GRs are known to repress cytokine genes, for example IL-1, IL-2, IL-6, IL-8, and TNF-α genes, as well as cell adhesion molecule genes, such as ICAM-1 (reviewed in McKay and Cidlowski, 1999). Interestingly, the promoters of these (pro-inflammatory) genes do not contain nGREs nor do they have any other GR-binding site, yet they are repressed by glucocorticoids (Bamberger et al., 1996; Cato and Wade, 1996). But what they do carry are sites for NF-κB and AP-1. It has therefore been proposed that immune suppression is mediated by glucocorticoid-induced expression of the inhibitory protein IκB-α, and in 1995, Scheinman et al. as well as Auphan et al. showed that glucocorticoids in fact induce the transcription of the gene encoding IκB-α and effectively down-regulate NF-κB associated gene products (Auphan et al., 1995; Scheinman et al., 1995a). In this regard, an increase in protein synthesis leads to the rapid turnover of IκB-α protein associated with pre-existing NF-κB complexes. In the presence of an activator, such as LPS, newly released NF-κB re-associates with the glucocorticoid induced IκB-α, thus reducing the amount of NF-κB translocating to the nucleus. Additionally, newly synthesized IκB-α may enter the nucleus and inhibit NF-κB-DNA binding. Both pathways effectively down-regulate NF-kB associated gene products (Scheinman et al., 1995a).

Alternative mechanism of glucocorticoid receptor action

The alternative mechanism of glucocorticoid receptor action involves physical interaction of the GR with other transcription factors, such as AP-1 and NF-κB. Regarding NF-κB, comparing the genes regulated by GR with NF-κB-induced genes (see former sections), clearly reveals GR and NF-κB to be physiological antagonists. Accumulating evidence exists that the activated GR specifically interferes with the trans-activation potential of the NF-κB p65 subunit (De Bosscher et al., 1997; De Bosscher et al., 2000b; Ray and Prefontaine, 1994). The mammalian NF-κB/Rel family of proteins consists presently of five members, namely, Rel (c-Rel), p65 (Rel A), Rel B, p50 (NFKB1; precursor: p105), and p52 (NFKB2; precursor: p100). The members p50 and p52 lack transcriptional activation domains and their homodimers are thought to act as repressors. In contrast, p65, Rel B, and c-Rel carry transcriptional activation domains, and with the exception of Rel B, they are able to form homo- and heterodimers with the other members of the protein family. In general, the designation NF-κB refers to the most frequently occurring and ubiquitously expressed heterodimeric complex between the p50 and p65 subunits (Caamano and Hunter, 2002; Wulczyn et al., 1996). Another important physical interaction occurs between the GR and AP-1. GRs can interact with both Fos and Jun subunits of the AP-1 heterodimer, although it appears that Fos may be the preferential target for GRs. This interaction alters in turn the interaction of both transcription factors with DNA, resulting in reciprocal repression of AP-1 and GR transactivation functions (Hsu et al., 1993; Jonat et al., 1990; Kerppola et al., 1993; Konig et al., 1992; Schule et al., 1990; Yang-Yen et al., 1990). As NF-κB and AP-1 can synergize in the pro-inflammatory pathway, GR repression of AP-1 may be an additional and important indirect mechanism for suppressing NF-κB-mediated immune responses. Figure 2-13 summarizes the classic and alternative mechanism of glucocorticoid actions by means of IκB-α upregulation and negative GR-NF-κB interaction.

Beside the two outlined mechanisms, also labelled the IκB-α upregulatory model and the protein-protein interaction model, a third model of glucocorticoid-mediated repression of NF-κB- and AP-1-driven genes is discussed, namely the cofactor squelching or competition model (De Bosscher et al., 2000a; McKay and Cidlowski, 1999). Cofactors are essential coactivators of transcription and the competition model proposes that transcription factors have to compete for limiting amounts of such essential coactivators. At present, specifically the cofactor CREB-binding protein (CBP)/p300 is being examined as a potential candidate, but so far the findings are contradictory and some laboratories report data supporting (Lee et al., 1998; Sheppard et al., 1998), others data countering the competition model (De Bosscher et al., 2000b; Pfitzner et al., 1998).

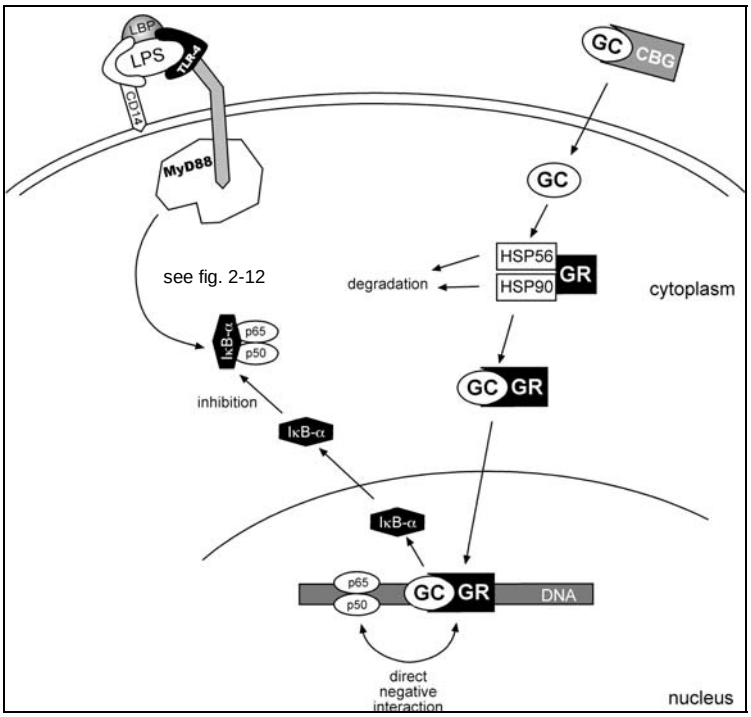

Fig. 2-13: Mechanisms of glucocorticoid inhibition of monocyte activation via IκB-α upregulation and negative GR-NF-κB interaction (modified from Rohleder, 2003).

2.3.4.1.3 Catecholamine signal transduction

Norepinephrine and epinephrine transduce their biological information through stimulation of adrenoceptors. Adrenoceptors directly activate G-proteins, which consist of three distinct classes of subunits, α, β, and γ, with the α-subunit conferring the effector specificity. The different α-subunit genes have been divided into four major subfamilies, G_s, G_i, G_q, and G_{12}. Different types of adrenoceptors thereby couple to different G-proteins: β-adrenoceptors couple to G_s protein, α1-adrenoceptor to G_i proteins and α1-adrenoceptors to G_q protein. Coupling of β-adrenoceptors to G_s proteins activates adenylate cyclase (AC), which in turn increases intracellular cAMP. Contrary, coupling of α1-adrenoceptors to G_i proteins inhibits AC and thus subsequently the formation of cAMP from ATP. The α1-adrenoceptor coupling to G_q proteins activates another intracellular effector, namely phospholipase C (PLC), which increases inositol triphosphate (IP_3) and diacylglycerol (DAG). Once these second messengers are generated, cAMP activates protein kinase A (PKA), DAG activates protein kinase C (PKC), and IP_3 mobilizes Ca^{2+} from intracellular stores. The latter is further linked to the Ca^{2+}/calmodulin (Ca^{2+}/CaM) pathway, which – like PKA – subsequently transfers signals to the nucleus (Elenkov et al., 2000).

As outlined above (see section 2.3.3.2.4), for example activation of β2-adrenoceptors on immune cells has been shown to be associated with increased levels of cAMP (Fedyk et al., 1996) as well as the inhibition of TNF-α and IL-12 (Elenkov et al., 1996; Severn et al., 1992; van der Poll et al., 1994) and an increase of IL-10 production (Elenkov et al., 1996; Siegmund et al., 1998; van der Poll et al., 1996; Van der Poll and Lowry, 1997).

In parallel to glucocorticoids, several lines of evidence exist of catecholamine signal transduction pathways interfering with activity of transcription factors, such as NF-κB and AP-1. Thereby, Haraguchi et al. suggested that all three pathways, i.e. the cAMP/PKA, the PKC, and the Ca^{2+}/CaM pathway, modulate these transcription factors by regulating their phosphorylation status (Haraguchi et al., 1995). Regarding NF-κB, elevated levels of cAMP are generally known to inhibit its activation, for example by cAMP inhibiting the binding of NF-κB to the NF-κB-DNA site (Chen and Rothenberg, 1994; Neumann et al., 1995; Tsuruta et al., 1995). Furthermore, the cAMP/PKA pathway induces impaired nuclear translocation and DNA binding of p65, probably due to a retarded degradation of IκB-α (Neumann et al., 1995; Paliogianni et al., 1993). Alternatively, this pathway may also inhibit NF-κB transcription by phosphorylating the transcription factor CREB, which then competes with p65 for limited amounts of CBP (Parry and Mackman, 1997). Contrary, NF-κB activity can also be stimulated via the Ca^{2+}/CaM pathway enhancing inactivation of the inhibitory protein IκB-ϙ (Frantz et al., 1994). It is also conceivable that CREB competes with AP-1 for binding at the AP-1 site, thus displacing it (Elenkov et al., 2000). This proposed mechanism is supported by findings of cAMP to also inhibit the binding of NF-AT (nuclear factor of activated T-cell), a nuclear factor that contains AP-1 protein (Paliogianni and Boumpas, 1996).

2.3.4.1.4 Summary of mechanisms of hormone-to-immune system signaling

The former sections clearly showed that glucocorticoids and catecholamines modulate the immune system at the transcriptional level. Furthermore, both stress mediators exert at least some of their immune effects via interference with other transcription factors, such as NF-κB and AP-1. In this regard, especially the inhibition of NF-κB activity by glucocorticoids is instrumental for glucocorticoid receptor anti-inflammatory mechanisms, while the inhibition of Th1 cytokines by glucocorticoids is essentially explained by the action of AP-1 (Refojo et al., 2001). However, the data presented in the last result section (4.4) of this thesis will be on the effects of endocrine stress responses – and specifically of missing cortisol stress responses in patients with Addison's disease – on NF-κB activity.

2.3.4.2 Modulation of Hormone-to-Immune System Signaling

Given the complexity of glucocorticoid and catecholamine signal transduction pathways, it is conceivable to expect various mechanisms and modulators to interfere with these pathways at different levels and thus to change the transcriptional output. Among these are extracellular and intracellular hormone availability, receptor expression level, hormone binding affinity, and repression by other transcription factors (Bamberger et al., 1996). These and other determinants of hormone sensitivity will be summarized in the

following sections exemplarily by means of factors influencing glucocorticoid sensitivity. The overview will follow the glucocorticoid-induced cascade of events described above in section 2.3.4.1.2 leading to modulation of transcription.

2.3.4.2.1 Extracellular hormone availability

Glucocorticoid concentrations vary not only due to stress, also other factors influence glucocorticoid availability. For example, circadian variation in glucocorticoids has been shown to be sufficient to cause a corresponding cyclic variation in lymphocyte function, i.e. release of IFN-γ and proliferative response to challenge, in humans (Hiemke et al., 1995; Petrovsky and Harrison, 1995; Petrovsky et al., 1994), a finding supported by the observation of diurnal changes in circulating T-cell subsets being comparable to those induced by prednisolone (Fukuda et al., 1994). Furthermore, in blood cortisol is largely bound to corticosteroid-binding globulin (CBG, synonym: transcortin). Another 15-20% is bound less tightly to albumin, leaving only about 5% of circulating cortisol as unbound (Pearson-Murphy, 2000). These 5% of unbound or free cortisol are thought of as the biologically active fraction, based on the concept known as the “free hormone hypothesis” (Mendel, 1992). Free cortisol levels may thus vary depending on factors influencing hepatic CBG production, such as oestrogen. In this regard, CBG levels have been shown to be increased for example in women under oral contraceptive medication (Crook, 1997; Wiegratz et al., 1995). Contrary, short-term treatment with IL-6 was shown to induce rapid decreases of CBG levels (Tsigos et al., 1998), which would translate into increased availability of glucocorticoids during infection and thereby a higher efficacy of immune suppression.

2.3.4.2.2 Intracellular hormone availability

Once glucocorticoids have entered a target cell, two enzymes inside this target cell catalyze the inter-conversion of active glucocorticoids (cortisol/corticosterone) to inactive forms (cortisone, 11-dehydrocorticosterone) and vice versa, interrupting GR-GC binding and thus determining glucocorticoid access to the glucocorticoid receptor. These enzymes are 11 beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and 11β-HSD2, with 11β-HSD1 producing the active and 11β-HSD2 producing the inactive form (“cortisol-cortisone shuttle”; Rook, 1999). Among other tissues, 11β-HSD2 is found in the kidney, where it converts cortisol into inactive cortisone to stop the mineralocorticoid receptor from binding cortisol (Walker, 1994). As a result, mineralocorticoid functions are almost exclusively mediated by aldosterone, although this hormone is present at much lower concentrations. 11β-HSD1, converting cortisone back to cortisol, is present in essentially all tissues with the liver being probably the major site of this conversion (Stewart and Whorwood, 1994). Interestingly, exposure of cells expressing both enzymes to IL-1β or TNF-α was shown to cause a marked

increase in expression of 11β-HSD1 (Escher et al., 1997). This finding points to a mechanism by which cytokines counterbalance their own pro-inflammatory effect by determining the anti-inflammatory action of glucocorticoids.

Interestingly, Balzi et al. (Balzi et al., 1994) recently identified a yeast transporter protein (PDR5P; see also LEM1, STS1, YDR1P) that was shown to actively and specifically export glucocorticoids from the cell (Kralli et al., 1995). If a mammalian glucocorticoid-transporter homolog exists, such a protein would surely represent another important regulator of intracellular hormone availability.

2.3.4.2.3 Glucocorticoid receptor expression level

Another factor modulating the effect of a given concentration of glucocorticoids is the number of glucocorticoid receptors available (Vanderbilt et al., 1987). This expression of GR varies between different tissues, with the thymus probably expressing one of the highest numbers of receptors per cell (Miller et al., 1990). If this variation in GR numbers also do have regulatory consequences, is not known yet. So far the most potent regulator of GR expression appears to be glucocorticoids themselves (Burnstein et al., 1991; Silva et al., 1994). With glucocorticoids down-regulating GR expression – for example by reducing its half-life (McIntyre and Samuels, 1985) –, a short-loop feedback mechanism is constituted that may protect tissues from excessive glucocorticoid levels (Bamberger et al., 1996).

Furthermore, a large body of evidence exists of cytokines influencing GR expression. With the exception of one study finding TNF-α to decrease GR number (Franchimont et al., 1999), cytokines, i.e. pro-inflammatory and type1 cytokines, are generally found to up-regulate transcription of the glucocorticoid receptor.[5] This findings are contradicted by studies differentiating between the two isoforms of glucocorticoid receptors. The human glucocorticoid receptor contains a total of 10 exons. By alternative splicing of exon 9, two GR isoforms are produced (Encio and Detera-Wadleigh, 1991), the fully functional GRα isoform and the GRβ isoform without hormone-binding activity (Hollenberg et al., 1985). These two forms are able to form heterodimers, but only GRα homodimers are functional. Thus, GRβ is thought to act as an endogenous inhibitor of glucocorticoid action (Bamberger et al., 1995). In parallel to these findings, treatment of human peripheral blood mononuclear cells (PBMCs) with IL-2 and IL-4 was shown to increase the expression of GRβ by more than 100% (Leung et al., 1997), while TNF-α and IL-1β causes a relative over-expression of GRβ by increasing GRα by 150% and GRβ by 350% (Webster et al., 2001). While the former data

[5] Pariante et al. (1999): IL-1α; Costas et al. (1996): TNF-α; Verheggen et al. (1996): IL-1β, TNF-α, LPS; Rakasz et al. (1993): IL-1β, IL-6, TNF-α; Masera et al. (2000): IL-6; Sartori et al. (1998): IL-2; Salkowski and Vogel (1992b): LPS; Salkowski and Vogel (1992a): IFN-γ; Franchimont et al. (1999): IL-10, TNF-α.

point to cytokines increasing GR expression, the latter studies let assume that they predominantly increase the negative isoform GRβ and thus down-regulate glucocorticoid sensitivity of target cells. Cytokines may thus counter-regulate actions of glucocorticoids themselves on GR.

2.3.4.2.4 Hormone binding affinity

Irrespective of the number of GR proteins, glucocorticoid-binding affinity of the GR is another factor potentially modulating signal transduction. For example, all point mutations within the coding-region for the ligand-binding domain have been found to be associated with decreased ligand-binding affinity (Ashraf and Thompson, 1993; Hurley et al., 1991). Additionally, factors influencing the assembly of the GR-HSP complex, which serves to keep the GR in ligand-friendly high affinity conformation (Pratt, 1993), negatively affected the binding affinity of GR. Such factors are ATP/energy depletion (Hu et al., 1994; Orti et al., 1992), decreased expression of HSP90 (Picard et al., 1990), and mutations of HSP90 (Cadepond et al., 1994; Nathan and Lindquist, 1995). Contrary, Huizenga et al. reported of GR polymorphisms, detected in approximately 6% of 216 volunteers, to be associated with an increased glucocorticoid sensitivity (Huizenga et al., 1998) and Ning and Sanchez observed stabilized GR-HSP complexes and thus increased hormone-binding affinity in response to the immunosuppressive drug FK506, which is also a ligand for HSP56 (Ning and Sanchez, 1995).

Rather complex are the effects of cytokines on hormone binding affinity. While for example each of the cytokines IL-1β, IL-6, and TNF-α were reported to increase binding affinities (Falus et al., 1995; Masera et al., 2000; Rakasz et al., 1993), using various combinations of these three cytokines were reported to decrease hormone binding affinity (Rakasz et al., 1993; Verheggen et al., 1996). As *in vivo* additionally sequences of events (e.g. in inflammation: TNF-α > IL-1 > IL-6; (Chrousos, 1995) have to be considered, the picture becomes even more complex and no general consequences regarding the effects of cytokines on glucocorticoid-binding affinity of GR can be drawn.

2.3.4.2.5 Glucocorticoid receptor phosphorylation and nuclear translocation

Before nuclear translocation of the GR-GC complex takes place, the GR becomes hyper-phosphorylated. To date it is not clear whether phosphorylation of GR influences its activity. Selective mutations of single, multiple, or all phosphorylation site of the mouse (Mason and Housley, 1993) as well as the human (Almlof et al., 1995) GR only modestly reduced the transcriptional activity of the receptor. Furthermore, when diverse phosphatases, phosphatase inhibitors, and kinases were analyzed with regard to their effect on GR function,

no consistent pattern of enhancement or inhibition could be established (Kuiper and Brinkmann, 1994; Orti et al., 1992).

In unmasking a nuclear localization signal (NLS), glucocorticoids may accelerate the nuclear translocation of the GR-GC complex (Akner et al., 1994). Furthermore, both cyclosporin A (Renoir et al., 1995), a binding agent of the immunosuppressive mediator immunophilin, and the HSP56-binding drug FK506 (Hutchison et al., 1993; Ning and Sanchez, 1993) were shown to enhance glucocorticoid-mediated transcription at low hormone concentrations. Contrary, treatment of L929 mouse fibroblasts with IL-1α resulted in decreased GR translocation to the nucleus (Pariante et al., 1999).

2.3.4.2.6 DNA-GRE binding

Once the GR-GC complex translocated to the nucleus, it will bind to DNA and specific GREs or nGREs. Thereby, again various factors may negatively or positively regulate this binding and hence attenuate or augment transcription. For example, several proteins, such as a promoter present in rat liver cells termed ATP-stimulated GR translocation promoter (ASTP; Okamoto et al., 1993), increase GR binding to DNA, but so far none of these proteins has been analyzed in terms of their expression pattern and functional relevance *in vivo* (Bamberger et al., 1996). Interestingly, activation of the PKA pathway (see section 2.3.4.1.3: catecholamine signal transduction) has been reported to augment transcription initiated by glucocorticoids (Espinas et al., 1995; Rangarajan et al., 1992). Findings of PKA activators not being correlated with changes in GR phosphorylation suggest the effect of PKA being mediated by phosphorylation of factors interacting with the GR (Moyer et al., 1993).

Regarding immune related parameters, Pariante et al. not only found IL-1α to decrease GR translocation to the nucleus, but consequently also to decrease GR-mediated gene transcription (Pariante et al., 1999). Contrary but also in L929 cells, Costas et al. showed TNF-α to increase transcriptional activity (Costas et al., 1996). This latter finding may reflect again a mechanism by which cytokines potentiate the counter-regulation of inflammatory processes by glucocorticoids.

2.3.4.2.7 Interaction with other nuclear factors

It was already outlined above that activated GR interferes with the transcription factor NF-κB (De Bosscher et al., 1997; De Bosscher et al., 2000b; Ray and Prefontaine, 1994). But NF-κB may repress the DNA-binding activity of the GR to the same extent. Again, it is the p65 subunit, which is involved in this functional antagonism (Caldenhoven et al., 1995; McKay and Cidlowski, 1998; Ray and Prefontaine, 1994; Scheinman et al., 1995a). As a second mechanism of GR repression by NF-κB, NF-κB has been shown to increase the

expression of the receptor isoform GRβ, thus increasing the ratio of GRβ:GRα, impairing GR transactivation, and eventually causing glucocorticoid resistance (Webster et al., 2001).

This mutual antagonism of NF-κB and the GR as well as the reciprocal repression of AP-1 and GR transactivation functions (see section 2.3.4.1.2) imply that during inflammation the glucocorticoid sensitivity of the respective tissue should decrease. This conclusion is substantiated by findings in steroid-resistant asthmatics of reduced GR-DNA binding due to abnormal AP-1-GR interactions (Adcock et al., 1995a; Adcock et al., 1995b).

2.3.4.2.8 Summary of modulation of hormone-to-immune system signaling

The previous sections clearly showed that glucocorticoid sensitivity of target tissue can be modulated at nearly every level of the glucocorticoid signal transduction pathway. Regarding immune related parameters, contradicting results hamper a general conclusion. Some evidence points to cytokines counter-regulating their own action by increasing glucocorticoid sensitivity (via increasing 11β-HSD1 expression, GRβ transcription, glucocorticoid binding affinity, and GR-mediated gene transcription). Contrary, other findings suggest that cytokines counter-regulate glucocorticoid effects, as combinations of the cytokines IL-1β, IL-6, and TNF-α decreased binding affinity and IL-1α was associated with attenuated GR translocation and GR-mediated gene transcription. This is supported by findings of both NF-κB and AP-1 to also repress GR-mediated gene transcription. However, practically none of the outlined data come from *in vivo* studies. Therefore it is impossible to predict the overall result of different combinations of moderators on glucocorticoid signaling, even less so over the course of e.g. an inflammatory or stress response.

3 PROBLEM FORMULATIONS

Especially the last section revealed a general problem in psychoneuroimmunology research: Much data comes from approaches scarcely to be linked to the situation in human whole organism. But especially if the interest is of clinical nature, it is important to be able to decide on the role of endocrine stress responses for health and disease.

As outlined in section 2.1, an endocrines stress response is basically thought of as being primarily protective (Benschop et al., 1996; Besedovsky and del Rey, 1996). Section 2.3 gave further evidence to this positive role in regard to the immune system. Consequently, usually only dysfunctions of stress systems have been associated with negative or pathophysiological consequences (Chrousos, 1998a).

But based on which data are such conclusions drawn? In human research, mostly just associations can be revealed between, for example, endocrine dysfunctions and distinctive features of the immune system. To decide on direction and above all relevance of such associations, results of – in the broadest sense – fundamental research have to be consulted. This implies effects of stress mediators being investigated in animals or *in vitro*. Next, a logical set of criteria has to be applied to analyze the role of glucocorticoids and catecholamines in the stress response. Sapolsky, Romero, and Munck (Sapolsky et al., 2000) for example applied the following three criteria:

1 The criterion of time course
(When does an effect occur?)

2 The criteria of hormone subtraction and replacement
(What happens if a glucocorticoid and/or catecholamine stress response is eliminated? Does administering of stress or/and basal levels of the respective hormone restore the stress response?)

3 The criterion of homeostasis
(Which action, i.e., permissive, suppressive, stimulating, or preparative action, makes more physiological sense in terms of restoring homeostasis?)

However, logical reasoning does not overcome the general problem of the arguable explanatory power of many of the underlying data regarding the human whole body situation. Surely the question, to what extent data from animal research can be assigned to humans, is a very common one, but it is nevertheless a legitimate one. Further, *in vitro* approaches may reveal important basic mechanisms and help to interpret *in vivo* findings. But to what extent can they also predict and explain events in this highly complex situation? These open issues notwithstanding, the criteria of hormone subtraction and replacement are unquestionably essential for gaining insights into how endocrine stress responses affect the immune system.

3.1 ADDISON'S DISEASE: A METHOD

The most evident answer for these problems is to investigate the criteria of hormone subtraction and replacement in humans. In this regard, several approaches are conceivable and most of them are widely utilized. In parallel to animal and *in vitro* studies, also human subjects may be treated with hormone antagonists and agonist. An example for a glucocorticoid antagonist is mifepristone (RU486), which blocks the glucocorticoid receptor. The synthetic glucocorticoid dexamethasone in turn is a glucocorticoid agonist, which consequently also inhibits glucocorticoid secretion by negative feedback mechanism. To both RU486 and dexamethasone several objections may be raised. For example, RU486 does not specifically block the glucocorticoid receptor as it also act as an antiprogestin and thus blocks the progesterone receptor (Mahajan and London, 1997). Furthermore, though it does not evoke a glucocorticoid-like effect, because the RU486-GR complex fails to interact properly with the basal transcription machinery (Bamberger and Chrousos, 1995; Chrousos et al., 1988; Mao et al., 1992), in some cases the RU486-bound glucocorticoid receptor still mediates transrepression of AP-1 and NF-κB (Heck et al., 1994; Liu et al., 1995; Scheinman et al., 1995b), whereas it has antagonistic effects in others (Caldenhoven et al., 1995; Jonat et al., 1990). Dexamethasone on the other hand shows a higher binding affinity to the glucocorticoid receptor, does not bind to CBG and is thus retained in high amounts, and blocks HPA activity predominantly on the pituitary level (De Kloet et al., 1998). But the most prominent objection concerning antagonists and agonists in general is the fact that this kind of treatment mostly is limited in duration of time. Hence this approach does not allow for considering allostatic processes elicited by longer lasting stress system dysfunctions and especially not for considering the extend to which these allostatic processes are capable of counter-balancing dysfunctions. Consequently, it is questionable whether such short time treatments may allow predictions regarding the clinical relevance of glucocorticoids and catecholamines for normal functioning of the immune system. An alternative approach should thus account for long-term effects of stress system dysfunctions. In animal research, adrenalectomy is a common method in this regard. A similar albeit therapeutically approach can be found in patients with adrenocortical adenoma, carcinoma, or dysplasia. Bi-lateral adrenalectomy in turn will result in primary adrenal insufficiency or Addison's disease. As outlined in section 2.2, patients with Addison's disease – independent of etiology – do not produce any cortisol due to destroyed (missing) adrenals and therefore have to be treated with glucocorticoid replacement (Arlt and Allolio, 2003; Betterle et al., 2002; Oelkers, 1996; Ten et al., 2001). The therapy thereby only provides basal cortisol supply, while it does not provide for additional doses during stress. This represents an unique physiological situation satisfying all prerequisites for an approach investigating the role of stress and stress mediators in human health and disease.

3.2 PROBLEM FORMULATIONS

The present thesis was guided by the question of the clinical relevance of regular stress responses or more precisely by the question: Is a regular endocrine stress response mandatory for physical health or does the organism, at least to a certain extent, compensate for it? As outlined above, investigation of patients with Addison's disease can be seen as the most promising approach in this regard. But despite this patient population being obviously quite interesting for PNI research, no stress-related endocrine and immune data are available so far. Therefore, the aim of the present work had to be to first define the general conditions of the 'method' Addison's disease's. This concerns the hypothesized missing cortisol stress response and even more essentially, the basal cortisol levels theoretically restored by glucocorticoid replacement therapy.

3.2.1 Study 1: Basal Endocrine State

Establishing basal cortisol levels is all the more important as the adequacy of replacement therapy to mimic diurnal cortisol rhythm was already repeatedly questioned. In most cases, patients with Addison's disease are reported to be over-treated (Lovas and Husebye, 2003; Lovas et al., 2002; Peacey et al., 1997). This is not only of special interest from a patients' point of view as over-treatment has been associated with long-term adverse consequences, such as decreases in bone mineral density (Zelissen et al., 1994). Deviations in circadian free cortisol rhythm has also been connected repeatedly to various clinical and psychiatric diseases (Giubilei et al., 2001; Pruessner et al., 2003). Over-treatment will further most probably affect the sensitivity of target tissue to glucocorticoid signals, which is important to consider when interpreting stress effects on immune parameter. Unfortunately, so far in Addison's disease the determination of the appropriate dose is mainly based on clinical judgement and no objective assessment has proven to be reliable for monitoring replacement quality. For this reason, the first study of the present thesis was undertaken not only to determine the adequacy of replacement therapy, but at the same time to test the usability and reliability of salivary free cortisol measures in patients with Addison's disease.

3.2.2 Study 2: Stress Effects in Addison's Disease

3.2.2.1 Psychosocial Stress: Endocrine and Cardiovascular Response

The second study first of all aimed at establishing endocrine and cardiovascular stress responses in Addison's disease, primarily the missing cortisol response. But missing cortisol stress responses are not the sole distinctive feature to be expected. Endogenous and locally acting cortisol is critical in the last step of the catecholamine biosynthesis (Bornstein

et al., 1995), consequently in Addison's disease very low epinephrine levels have been observed (Bornstein et al., 1995). These findings suggest that beside a missing cortisol stress response, patients with Addison's disease may also not show stress responses in epinephrine. This leaves only norepinephrine to regularly respond to stress with an increased release. As both catecholamines and glucocorticoids are known to be important activators and mediators, respectively, of the cardiovascular stress response (Galosy et al., 1981; Sambhi et al., 1965; Sapolsky et al., 2000), investigation of heart rate and blood pressure changes during stress in Addison's disease may provide first evidences of the compensatory capacity of the human organism.

However, physiological changes in response to hormone subtraction just indicate the *necessity* of the respective hormone for these effects. Only subsequent hormone replacement will also reveal the *sufficiency* of the hormone for these effects. Therefore, a pilot study in healthy subjects was conducted to determine the dose of hydrocortisone *iv* best mimicking normal stress-induced increases in free cortisol levels (=0.03mg/kg). In the course of study 2, this dose was also tested in patients with Addison's disease regarding its adequacy to restore stress levels of free cortisol and their potentially modulatory effects on cardiovascular parameters.

3.2.2.2 Psychosocial Stress: Immunological Consequences

The second aim of study two was to investigate the immunological consequences of missing endocrine stress responses in Addison's disease. As glucocorticoids show pronounced anti-inflammatory actions, missing cortisol stress responses should result in facilitated pro-inflammatory processes in patients with Addison's disease. This hypothesis is supported by observations of patients being prone to diseases like bronchial asthma (Green and Lim, 1971) and various allergies (Carryer et al., 1960). To gain insight into the immunological consequences of endocrine dysfunctions, stress effects on three common immune parameters were investigated: Percentages of cell subtypes, which provide general information about immune system activation (Dhabhar, 2000), as well as LPS-stimulated IL-6 production and PHA-stimulated cell proliferation, which provide information about the responsiveness of cells of innate immunity and adaptive immunity, respectively (Vedhara et al., 1999).

It may further be hypothesized that glucocorticoid replacement therapy influences the sensitivity of target cells to glucocorticoid signaling, even more so if patients with Addison's disease are actually over-treated. Therefore, additionally the sensitivity of LPS-stimulated IL-6 production and PHA-stimulated cell proliferation to the inhibitory signal of the synthetic glucocorticoid dexamethasone was measured. Furthermore, hydrocortisone injections were utilized to decide on glucocorticoids being necessary or actually sufficient.

3.2.2.3 Psychoneuroimmunology: Mediative Role of NF-κB

Beside operationalizing glucocorticoid sensitivity by adding dexamethasone *ex vivo* to stimulated whole blood or peripheral blood mononuclear cells, also measuring NF-κB activity in patients with Addison's disease may provide insights into long-term adaptive processes regarding glucocorticoid signaling. As outlined above, the transcription factor NF-κB is found in virtually every cell of the immune system regulating a great number of immune related genes. Transcriptional activity of NF-κB and glucocorticoid receptors usually leads to the production of immune mediators with contrary actions. Furthermore, as promoters of most pro-inflammatory genes do not contain negative glucocorticoid response elements (Bamberger et al., 1996; Cato and Wade, 1996), it is thought that glucocorticoids exert their anti-inflammatory action by activated glucocorticoid receptors interfering with NF-κB activity (De Bosscher et al., 1997; De Bosscher et al., 2000b; Ray and Prefontaine, 1994). Hence, NF-κB can be regarded as important mediator of immune-suppressive glucocorticoid actions. The fourth aim of the present work was to investigate this mediative role of NF-κB by measuring NF-κB-DNA binding activity before and repeatedly after stress in patients with Addison's disease.

In the following chapter (chapter 4), the results of the accomplished studies will be presented. Aligned formally to a manuscript, for each of the four problem formulation first a short introduction will be given summarizing the respective theoretical background. This is followed by information on subjects, study design, and methods. Adjacent to the presentation of results, findings will be discussed in the context of the respective problem formulation. Subsequent to the result chapter, data will be pulled together and discussed in a greater context (chapter 5: Summary, general discussion, and outlook).

4 RESULTS

4.1 ADDISON'S DISEASE: BASAL ENDOCRINE STATE

Salivary Free Cortisol Measurement in Addison's Disease – A Reliable Method for Glucocorticoid Replacement Therapy Surveillance

4.1.1 Abstract

Background: Addison's disease is caused by bilateral destruction of the adrenal cortex. The resulting failure to produce steroid hormones requires life-long replacement therapy with mineralocorticoids and glucocorticoids. In both over and under-treated patients substantial morbidities develop. While obesity, osteoporosis, and impaired glucose tolerance are found in patients over-treated with glucocorticoids, incipient crisis and impairment of wellbeing is observed in patients with insufficient steroid replacement regimens. Despite the significant iatrogenic consequences, the choice of the appropriate replacement dose is mainly based on clinical judgment rather than careful monitoring of steroid levels in the patient. The present study therefore investigated whether repeated measurements of free cortisol levels in saliva provide a tool for objectively assessing the adequacy of replacement therapy in patients with Addison's disease.

Methods: In 27 patients with Addison's disease, salivary cortisol was measured repeatedly over two days at time-points related to individual medication intake.

Results: All patients show markedly elevated free cortisol levels compared to reference values. Salivary free cortisol levels in patients taking cortisone showed less pronounced fluctuating courses compared to the hydrocortisone group. In the latter, a non-linear relationship between dose and salivary cortisol levels was found. Salivary cortisol levels rose with increasing replacement doses up to 20mg hydrocortisone, while lower cortisol levels were found with hydrocortisone doses of 25mg.

Conclusions: Since salivary free cortisol levels are a valid reflection of free cortisol levels in blood, monitoring of salivary cortisol levels provides an easy approach to objectively assess the adequacy of replacement therapy in patients with Addison's disease. The present data provide firm evidence that in current replacement regimens, most patients are over-treated. In order to avoid treatment-induced morbidity, cortisol levels should be monitored more closely with non-invasive sampling strategies.

4.1.2 Introduction

Primary adrenal insufficiency, also labeled Addison's disease, is caused by bilateral destruction or impaired function of the adrenal cortex, whereupon adrenal insufficiency manifests itself as clinical disease if the functional adrenal mass falls below 10% (Ten et al., 2001). This reduction of adrenocortical cell mass is responsible for the characteristic deficiency of glucocorticoids and mineralocorticoids, more specifically cortisol and aldosterone, respectively (Marzotti and Falorni, 2004).

Addison's disease is a relatively rare disease with prevalence rates ranging from 10 to 140 per million population, varying between countries (Marzotti and Falorni, 2004; Oelkers, 1996; Ten et al., 2001). The incidence is 4.7-6.2 per million in white populations (Arlt and Allolio, 2003). On average, the age at diagnosis peaks in the fourth decade of life, with women more frequently affected than men (Arlt and Allolio, 2003).

Addison's disease can have various etiologies. Betterle et al. (Betterle et al., 2002) offer a classification, in which they distinguish between autoimmune adrenalitis, infectious adrenalitis, Addison's disease due to neoplastic diseases, adrenal hemorrhage, or adrenal thrombosis, drug-induced adrenal insufficiency, neonatal causes, and genetic causes of Addison's disease. Many of the listed causes are very rare. In non-developed countries, infectious diseases (e.g., tuberculosis) are the major cause of Addison's disease. In developed countries, autoimmune adrenal destruction is the major cause, affecting approximately 90% of all patients with primary adrenal insufficiency (Winqvist et al., 1996).

The treatment of patients with Addison's disease accounts for the missing glucocorticoids and mineralocorticoids. Mineralocorticoid replacement consists of oral administration of fludrocortisone in a single daily dose of 50-200μg. The dose is guided by measurements of blood pressure, serum potassium, and plasma renin activity (Betterle et al., 2002). Additional markers can be serum sodium concentrations and appearance of peripheral oedema (Arlt and Allolio, 2003). Optional is the replacement of dehydroepiandrosterone (DHEA) to increase well-being and mood, taken daily as one dose of 25-50mg DHEA in the morning (Arlt and Allolio, 2003). Glucocorticoid replacement is usually given in two or three daily doses, with a half to two-thirds of the daily dose administered in the morning to mimic the circadian secretion pattern of cortisol (Arlt and Allolio, 2003). The dosage is based on the normal daily cortisol production rate of 5.7±0.3mg/m^2·day (Kerrigan et al., 1993), which translates to about 10-20mg/m^2·day of oral hydrocortisone (Ten et al., 2001), accounting for step-down losses from absorption, hepatic processing, and metabolic bioavailability. Initially, the treatment starts with doses of 25mg hydrocortisone (15 + 10mg) or 37.5mg cortisone acetate (25 + 12.5mg; Oelkers, 1996). The daily dose may be decreased to 20 or 15mg of hydrocortisone to prevent over-replacement, which was repeatedly shown to be associated to weight gain, osteoporosis and impaired

glucose tolerance (al-Shoumer et al., 1995; Florkowski et al., 1994; Zelissen et al., 1994). On the other hand, under-replacement bears the risk of incipient crisis and impairment of wellbeing. The goal should be to use the smallest dose that relieves the patient's symptoms (Oelkers, 1996).

In Addison's disease, the determination of the appropriate dose is mainly based on clinical judgment, taking into account signs and symptoms potentially suggestive of glucocorticoid over-replacement or under-replacement. So far, no objective assessment has proven to be reliable for monitoring replacement quality. Cortisol levels can be measured in plasma. But since cortisol concentrations vary throughout the day, the diagnostic usefulness of random plasma samples is limited. Measuring cortisol levels repeatedly over the day on the other hand is unpleasant for patients as it is associated with repeated venipunctures or an in-dwelling catheter. Additionally, some endogenous and synthetical corticosteroids cross-react in the cortisol assay and should therefore be avoided within 24 hours of testing (Grinspoon and Biller, 1994). Taken together, this hampers a close surveillance of replacement therapy by plasma samples. Urinary 24 hrs free cortisol excretion has been advocated for monitoring replacement. However, since after HC administration cortisol-binding globulins are rapidly saturated, renal cortisol excretion is transiently but clearly increased (Monson, 1997). Urinary cortisol should therefore only be used as a marker for HC over-treatment (Peacey et al., 1997). Adrenocorticotropic hormone (ACTH) cannot be used as a criterion for dose adjustment, since in primary adrenal insufficiency it is invariably high before the morning dose and rapidly declines with increasing cortisol concentrations after glucocorticoid ingestion (Feek et al., 1981; Scott et al., 1978).

Furthermore, in blood cortisol is largely bound to corticosteroid-binding globulin (CBG, synonym: transcortin). Another 15-20% is bound less tightly to albumin, leaving only about 5% of circulating cortisol as unbound (Pearson-Murphy, 2000). These 5% of unbound or free cortisol are thought of as the biologically active fraction, based on the concept known as the "free hormone hypothesis" (Mendel, 1992). Since only the free cortisol fraction acts upon target tissues, leading to the known broad spectrum of physiological effects of cortisol, measures of total cortisol levels in plasma have to be interpreted with caution. Not only can total cortisol levels be increased as a result of hepatic cortisol-binding globulin production due to e.g. oestrogen, but, regarding cortisol effects on target tissues, it is rather debatable whether measures of total cortisol levels are conclusive at all. Therefore, in clinical and especially endocrinological research the measure of free cortisol is usually preferred. Moreover, since almost twenty years, free cortisol can be measured in saliva. The main advantage of saliva samples is that they offer a non-invasive method to measure free cortisol levels. Thus free cortisol levels can be investigated repeatedly and in short time intervals without inconvenience for patients, and even at patients' homes. Additionally, free cortisol

levels are found to be unaffected by conditions which alter the amount of CBG and/or albumin (e.g., oestrogens) and hence influence the measurement of total cortisol (Gozansky et al., 2005; Kirschbaum and Hellhammer, 1989). Due to the wide-spread usage of free cortisol measures in saliva in research, normal values for free cortisol levels over the day exists (Westermann et al., 2004; Wust et al., 2000) and different types of assays with low cross-reactivity to most endogenous and synthetic corticosteroids are commercially available.

Specifically in patients with Addison's Disease, the measurement of free cortisol levels in saliva would represent a valuable alternative approach in that it allows for closely controlling the adequacy of glucocorticoid replacement. This adequacy was repeatedly questioned. For example, Peacey et al. found by measuring cortisol in serum and urine, 75% of patients on conventional glucocorticoid therapy to be over-treated (Peacey et al., 1997). Additionally, Lovas et al. reported of persistent complaints of fatigue, weariness, and reduced stress tolerance in many patients with Addison's disease on standard replacement therapy (Lovas et al., 2002). Therefore, Lovas and Husebye concluded that none of the glucocorticoid replacement regimes satisfactorily mimic the normal diurnal rhythm of cortisol (Lovas and Husebye, 2003). This is of special interest, since not only over-treatment in patients with Addison's Disease has been associated with long-term adverse consequences like decreases in bone mineral density (Zelissen et al., 1994). Deviations in circadian free cortisol rhythm – far less pronounced than to be expected in patients with Addison's disease due to replacement therapy – has also been connected repeatedly to various clinical and psychiatric diseases, like Alzheimer's disease (Giubilei et al., 2001) or depression (Pruessner et al., 2003).

However, it has been shown that various factors, e.g. gender or health status, influence the circadian rhythm of free cortisol in non-Addisonians (Kudielka and Kirschbaum, 2003; Wust et al., 2000). Beside the dose of glucocorticoid replacement, it is conceivably to hypothesize that some of these factors may also influence salivary cortisol levels in patients with Addison's disease. For example, gender may influence the course of cortisol levels as a result of hepatic cortisol-binding globulin production due to oestrogen (Grinspoon and Biller, 1994). Contrary, as outlined above, free cortisol levels are found to be unaffected by conditions which alter the amount of CBG and/or albumin (Gozansky et al., 2005; Kirschbaum and Hellhammer, 1989). Also the type of medication may influence cortisol levels. While hydrocortison is chemically identical to endogenous cortisol, cortisone compounds first have to be activated to form cortisol by the hepatic enzyme 11 beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1). Therefore, the absorption curve of cortisone is blunted and delayed compared to that of hydrocortisone (Feek et al., 1981), which also may result in different salivary cortisol courses.

So far, salivary cortisol measurement has already been proven to be a useful method in diagnosing Cushing's syndrome (Trilck et al., 2005). The aim of the present study was to apply this method also to patients with Addison's disease and further, to investigate the impact of factors like gender, type and dosage of replacement therapy, which are known to influence total cortisol levels in plasma, on free cortisol levels in saliva.

4.1.3 Methods

4.1.3.1 Subjects

A total of thirty-seven patients with Addison's Disease were recruited at the university hospital at Duesseldorf and via advertisement in the journal "Glandula". Complete endocrine data were available for twenty-seven patients. The sample consisted of 17 women and 10 men between 19 and 63 years of age (mean: 45.11, SD: 10.6). Male and female patients did not differ significantly in age (t_{25}=-0.74, p=.46). Disease duration varied between 0.5 and 41.0 years (mean: 11.05, SD: 10.9) and differed significantly between male (mean: 16.60, SD: 13.1) and female (mean: 7.78, SD: 8.0) patients (t_{25}=-2.18, p=.039), consistently with reported differences in gender distribution and mean age at diagnosis depending on the cause of Addison's disease (Betterle et al., 2002). Of all patients, 18 patients (66,6%) were diagnosed with autoimmune Addison's disease due to the presence of autoantibodies (i.e., adrenocortical autoantibodies (ACAs), steroid cell antibodies (StCA); see (Peterson et al., 2000) and/or according to the criteria for diagnosing APS-1 or APS-2 (Neufeld et al., 1981), one patient was diagnosed with infectious Addison's disease due to tuberculosis, and two with adrenal insufficiency due to Cushing's disease. Six patients (22.2%) could not further specify their diagnosis of primary adrenal insufficiency and the co-morbidities did not fulfil criteria for classification to autoimmune Addison's disease. Most patients are prescribed hydrocortisone (HC; n=21), six patients took cortisone acetate (cortisone). Glucocorticoid replacement was given in two doses in 15 patients and in three doses in 12 patients. The pattern of glucocorticoid replacement varied widely between the patients. In 27 patients, 15 different patterns were found (see table 4-1). Controlling for gender, both weight and body mass index (BMI) did not correlate with first dose of hydrocortisone and total daily dose of hydrocortisone (dose1 HC: r_{18}=-.06, p=.81; r_{18}=-.03, daily dose of HC: p=.91; r_{18}=-.11, p=.66; r_{18}=-.03, p=.92; respectively).

In further analyses, only the first two doses, independently of the time of day of the second dose were included. The mean time interval between dose1 and dose2 was 6h58min (standard deviation (SD): 1h56min, range: 4hrs to 11.5hrs).

Tab. 4-1: Patterns of glucocorticoid replacement (HC: hydrocortisone, C: cortisone acetate).

dose 1 (mg)	dose 2 (mg)	dose 3 (mg)	n
10	5	-	2 HC
		5	1 HC
15	5	-	1 HC
		5	4 HC
	10	-	2 HC
		5	3 HC
20	5	-	1 HC
		5	1 HC
	10	-	3 HC
		10	1 HC
25	5	5	1 HC
	10	5	1 HC
18,75	12,5	-	1 C
25		-	4 C
37,5	25	-	1 C

4.1.3.2 Experimental Protocol

Patients were asked to collect six saliva samples at two consecutive days at home using the Salivette device (Sarstedt, Nümbrecht, Germany). Because of the widely varying patterns of glucocorticoid replacement, time-points at which patients collected saliva samples were related to the time-points of medication intake: The first sample (sample 1) had to be taken one hour after taking the first substitution dose (dose1), sample 2 between sample 1 and 3, sample 3 immediately before taking dose2, and samples 4, 5, and 6 at one, two, and four hours after taking dose2, respectively. At day 2, patients were asked to skip the second dose but collect saliva samples at the same time-points as at day 1. The time interval of one hour after medication intake was chosen to avoid contamination of saliva samples with residues of the orally taken medication. The patients were further asked not to eat, drink (except water) or brush their teeth during a phase of 30 minutes prior to each sampling to avoid false high free cortisol concentrations.

4.1.3.3 Biochemical Analysis

Free cortisol levels in saliva were measured using a commercially available chemiluminescence assay (IBL, Hamburg, Germany). Samples were measured in duplicates and averaged for subsequent statistical analyses. Samples with >100nmol/l free cortisol were measured again diluted 1:10 in assay buffer and included only if two more measures in duplicates yielded identical results.

4.1.3.4 Statistical Analysis

Data were analyzed using the Statistical Package for the Social Science Version 11.0.2 (SPSS Institute, Chicago, IL). Time effects and group differences in free cortisol levels

throughout the day(s) were calculated by repeated measures analysis of variance with within-group levels representing the time-points of saliva sampling. All data were tested for normality prior to statistical analysis using the Kolmogorov-Smirnov test and Greenhouse-Geisser corrections for repeated measures were calculated where appropriate. Furthermore, partial correlations controlling for type of medication and Pearson's correlations were computed. Values of $p<0.05$ were considered significant.

4.1.4 Results

4.1.4.1 Courses of Salivary Cortisol Levels

At day1, salivary cortisol levels changed significantly over time showing decreases from time-point one to three and from four to six, and increases from time-point three to four. The overall time effect including all six time-points was significant with $F_{5,130}=34.89$; $p<.001$. Though at day2 the overall time effect also was significant ($F_{5,130}=30,59$; $p<.001$), salivary cortisol levels did not show significant changes from sample 3 on (time effect calculated for sample 3 to sample 6: $F_{3,78}=1.03$; $p=.34$). Thereby, day1 and day2 differed significantly ($F_{1,130}=6.09$, $p=.02$), but no time-by-day effect was found ($F_{5,130}=2.51$, $p=.11$). Figure 4-1 depicts the course of salivary cortisol levels at day1 and day2 over the six time-points with reference to replacement dose1 and dose2 in comparison to normal values.

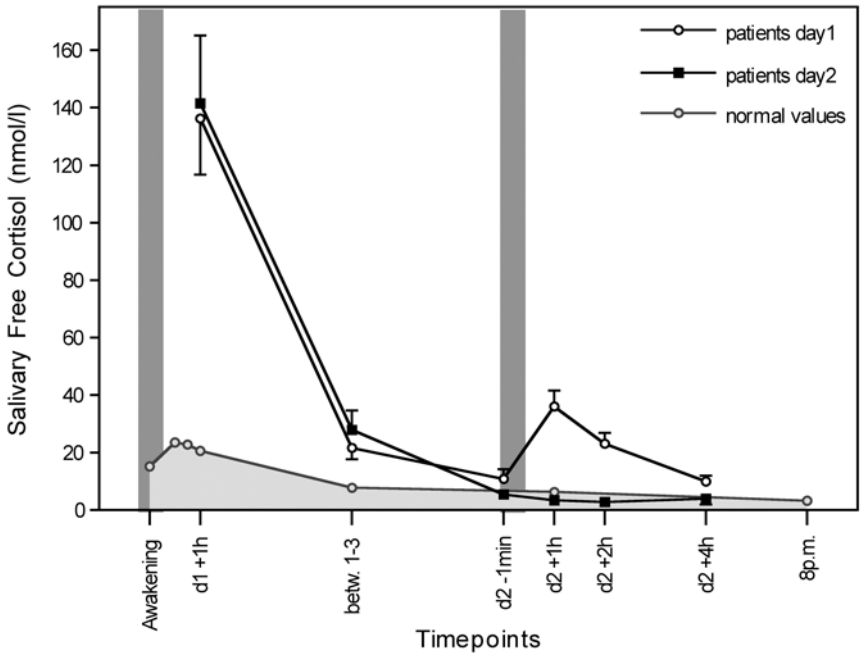

Fig. 4-1: Means and standard errors (SE) of salivary cortisol levels at day1 and day2 (day2: patients were asked to skip dose2 (d2); dose1 and dose2 indicated by grey bars; d1 +1h: one hour after dose1, appr. 8a.m.; betw. 1+3: between sample 1 and sample 3, appr. 11a.m.; d2 +1min: immediately before dose2, appr. 2p.m.; d2 +1h, d2 +2h, and d2 +4h: one, two, and four hour after dose2, resp., appr. 3p.m., 4p.m. 6p.m.) compared to normal values and standard errors (grey line with shaded area under the curve; timepoints: at awakening, awakening +30min, awakening +45min, awakening +60min, 11a.m., 3p.m., and 8p.m.; Wust et al., 2000; Westermann et al., 2004).

4.1.4.2 Salivary Cortisol Levels: Etiology, Gender, and Time Interval

With six time-points at two days as within-subjects factors, no significant effects were found with between-subjects factors etiology or gender, except significant time effects (etiology: group: $F_{3,23}$=.45, p=.72; day: $F_{1,115}$=1.13, p=.30; time: $F_{5,115}$=8.40, p=.005; group-by-time: $F_{15,115}$=.77, p=.54; group-by-day: $F_{3,115}$= 0.22, p=.88; time-by-day: $F_{5,115}$=.36, p=.61; group-by-time-by-day: $F_{15,115}$=.18, p=.95 – gender: group: $F_{1,25}$=.68, p=.42; day: $F_{1,125}$=4.02, p=.056; time: $F_{5,125}$=37.46, p<.001; group-by-time: $F_{5,125}$=.98, p=.34; group-by-day: $F_{1,125}$= 3.73, p=.065; time-by-day: $F_{5,125}$=2.82, p=.09; group-by-time-by-day: $F_{5,125}$=1.06, p=.33).

With increases in time intervals between sample 1 and 2 (time1>2) or between sample 1 and 3 (time1>3), salivary cortisol levels decreased (time1>2 x sample 2: r=-.46, p=.015; time1>3 x sample 3: r=-.41, p=.033).

4.1.4.3 Salivary Cortisol Levels: Glucocorticoid Replacement

Mean salivary cortisol levels did not differ significantly between the hydrocortisone and the cortisone group, but showed a trend to attenuated levels in the cortisone group and a trend to an interaction between type of medication and time (group: $F_{1,25}$=3.31, p=.08; medication-by-time: $F_{5,125}$=3.82, p=.05). Mean cortisol levels at day1 and day2 differed significantly (day: $F_{1,125}$=7.69, p=.01), as well as salivary cortisol levels over time (time: $F_{5,125}$=22.14, p<.001). The interactions between day and time as well as day and time and type of medication also did not reach statistical significance (day-by-time: $F_{5,125}$=.87, p=.39; day-by-time-by-group: $F_{5,125}$=1.51, p=.23). Figure 4-2 depicts the course of salivary cortisol levels at day1 for the groups hydrocortisone (n=21) and cortisone (n=6).

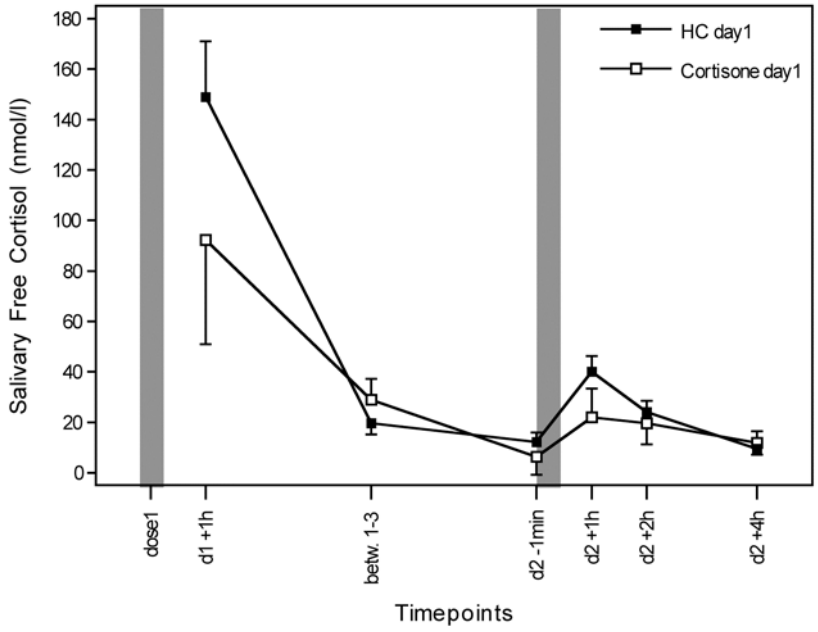

Fig. 4-2: Salivary cortisol levels at day1 for groups hydrocortisone and cortisone (means and SE).

Next, partial correlations between doses and the appropriate indices of the course of salivary cortisol levels were computed controlling for type of medication. Generally, the first replacement dose did not correlate with salivary cortisol levels at time-points 1, 2, or 3, nor with decreases from time-point 1 to 2 (decr1>2) or decr1>3 (averaged for day1 and day2; r=.28, p=.17; r=.07, p=.72; r=-.11, p=.59; r=.28, p=.17; r=.30, p=.14; resp.). Dose2 at day1 did not correlate with salivary cortisol levels at time-points 4, 5, or 6, nor with decr4>5 or decr4>6 (r=.20, p=.32; r=.32, p=.11; r=.08, p=.71; r=-.03, p=.90; r=.16, p=.44; resp.), but did correlate positively with increases in salivary cortisol levels from sample 3 to 4 (r=.42, p=.035), indicating more pronounced increases with higher replacement doses.

To further test for effects of replacement doses, two separate repeated measures ANOVAs were computed for dose1 and dose2. Regarding dose1, salivary cortisol levels of samples 1 to 3 were again averaged for day1 and day2. Furthermore, due to the group and group-by-time trend in type of medication, four hydrocortisone replacement groups (10mg, 15mg, 20mg, and 25mg) as well as one cortisone replacement group (mean: 26.04mg, SD: 6,2) were included as between-subjects factors. Cortisone dosages were not further divided into sub-groups due to the low number of cases. Regarding dose2, salivary cortisol levels of samples 4 to 6 as within-subjects factors and hydrocortisone replacement doses 5mg and 10mg and one cortisone group (mean: 14.58mg, SD: 5.1) as between-subjects factors were included. Following the first replacement dose, the five groups differed significantly overall and in their course of salivary cortisol levels (group: $F_{4,22}$=2.90, p=.045; time: $F_{2,44}$=39.54, p<.001; group-by-time: $F_{8,44}$=3.21, p=.028). In salivary cortisol levels following dose2, no significant group effects of replacement doses were found (group: $F_{2,24}$=2.25, p=.13; time: $F_{2,48}$=10.04, p=.001; group-by-time: $F_{4,48}$=.90, p=.44). Figure 4-3 depicts the course of salivary cortisol levels depending on replacement doses.

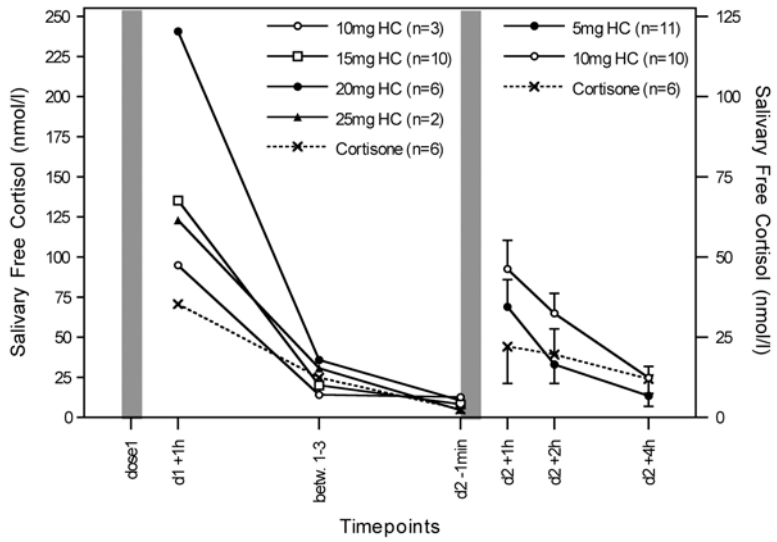

Fig. 4-3: Salivary cortisol levels broken down by replacement doses (left side: mean cortisol levels and SE averaged for day1 and day2; please note that for the sake of clarity, no SE are shown; right side: mean cortisol levels and SE of day1).

4.1.5 Discussion

The present study shows salivary cortisol levels in patients with Addison's disease to be notably elevated compared to standard values. Furthermore, on average seven hours after the first replacement dose, the effect of the replacement medication on salivary cortisol levels vanished and cortisol levels did not vary anymore thereafter. Generally, gender and etiology did not influence the course of salivary cortisol levels, whereas cortisone acetate resulted in less pronounced and less varying salivary cortisol levels compared to hydrocortisone. Comparing the dosages of glucocorticoid replacement, increasing morning hydrocortisone doses resulted in higher cortisol levels up to 20mg, while two patients taking 25mg hydrocortisone showed lower levels comparable to 15mg hydrocortisone. Again, cortisone produced the lowest levels one hour after medication intake. No effects of the second doses on subsequent salivary cortisol levels were found.

The present findings of salivary cortisol levels clearly point to an over-replacement of patients with Addison's disease. This is in line with prior findings and strengthens the skepticisms expressed repeatedly regarding the adequacy of replacement regimes (Howlett, 1997; Lovas and Husebye, 2003; Peacey et al., 1997), especially because chronic over-replacement is associated with substantial morbidity, including impaired glucose tolerance, obesity, and osteoporosis (al-Shoumer et al., 1995; Florkowski et al., 1994; Zelissen et al., 1994).

The results of the present study regarding the effects of type of medication and dosage on salivary cortisol levels are in line with previous findings in total plasma cortisol and free urinary cortisol. As in plasma cortisol, the salivary cortisol curve of cortisone is blunted and delayed compared to that of hydrocortisone (Feek et al., 1981). The missing linear association between hydrocortisone doses and resulting salivary cortisol levels supports findings showing no beneficial effects of hydrocortisone doses greater than 20mg a day. For example, Wichers et al. did not find decreases in well-being in patients taking 15 or 20mg hydrocortisone per day, but long-term negative effects of higher replacement doses on bone metabolism (Wichers et al., 1999).

Furthermore, higher hydrocortisone doses just resulted in steeper decreases of salivary cortisol levels, while already three hours after medication intake no substantial differences in salivary cortisol levels between the various doses can be observed. Additionally, comparison of salivary cortisol levels at the second day of investigation with normal values shows that on average 7 hours after the first replacement dose, levels of patients with Addison's disease fall below levels of healthy subjects. This is in line with data on pharmacokinetics and pharmacodynamics of cortisol: Since plasma half-life of cortisol is less than 2 hours, even peak total cortisol levels of 1000nmol/l will result in non-detectable levels after approximately eight hours (Howlett, 1997). These results support a change in

replacement regimes to smaller doses taken more frequently, which is a conclusion also drawn from several other studies. For example, both Howlett and Peacey et al. suggested from 24-hour urinary free cortisol and serum cortisol measures that optimal replacement is achieved with thrice daily regimes with 10mg/5mg/5mg (rising/lunch/evening; Howlett, 1997; Peacey et al., 1997).

Comparison of salivary cortisol courses between hydrocortisone and cortisone suggests that the latter might be preferable due to the less pronounced fluctuations in salivary cortisol. However, only six patients took cortisone, therefore these results have to be interpreted with caution. Since cortisone has to be activated to form cortisol by 11β-HSD1 and thus variable action of this enzyme could result in variable und unpredictable cortisol effects, some authors oppose the use of cortisone. On the other hand, no evidence exists that this is a clinical problem, other than in rare cases of 11β-HSD1 deficiency (Lovas and Husebye, 2003).

Taken together, the present results clearly circumstantiate the findings by others of patients with Addison's disease to be over-treated. They endorse recommendations of replacement regimens which allow for lower but more frequent doses (Howlett, 1997; Peacey et al., 1997) or alternatively, a combination of one higher dose of glucocorticoid replacement in the morning combined with sustained-release tablets (Lovas and Husebye, 2003). In each case, resulting cortisol levels should be under close surveillance to avoid over- and under-treatment, both being associated with serious health risks. In this regard, the measurement of salivary cortisol has been proven to be a useful method to assess the adequacy of glucocorticoid replacement therapy. The method is reliable, non-invasive, convenient for the patients, inexpensive, without limitations to the number of samples to be collected, and (with the exception of prednisolone) cross-reactivities in commercially available assays are under 2%. It is thus most suitable for a tight treatment surveillance and accomplishes the prerequisites for individually adjusted glucocorticoid therapy.

From a methodological point of view, the present results regarding the over-treatment of patients clearly question the comparability of the basal hormonal situation in patients with Addison's disease and healthy persons. As basal glucocorticoid levels exert permissive functions in stress responses (Sapolsky et al., 2000), distinctive features in patients with Addison's disease may not be ascribable solely to the missing cortisol stress response but rather to glucocorticoid (over-)treatment, which results in non-physiological high 'basal' glucocorticoid levels and evokes compensatory mechanisms in other bodily systems.

The straightest implication of the present results for further investigations concerns the study design. For studying stress effects in patients with Addison's disease in comparison to healthy controls, patients should avoid ingestion of glucocorticoid replacement several hours prior to study. Otherwise patients will show already at baseline cortisol levels

far higher than those healthy subjects will show in response to a laboratory stressor. Again, this will hamper a comparison of patients and healthy subjects as well as the interpretation of results. Futhermore, variations in free cortisol levels between patients also render it impossible to utilize a defined dose of hydrocortisone for mimicking cortisol stress responses of healthy subjects.

4.2 ADDISON'S DISEASE: ENDOCRINOLOGICAL RESPONSE TO PSYCHOSOCIAL STRESS

Endocrine and Cardiovascular Stress Responses in Addison's Disease

4.2.1 Abstract

Background: Many findings in psychoneuroendocrinology (PNE) and psychoneuroimmunology (PNI) raise the question, which consequences for health are to be expected if endocrine stress responses in humans are altered permanently and independently of context. We see investigation of patients with Addison's disease as a valuable approach in this regard. Since so far no experimental data on endocrine and cardiovascular stress response patterns in these patients exist, the present study aims at establishing these patterns and additionally, at testing an *i.v.* injection of 0.03mg/kg hydrocortisone (HC) in patients for its appropriateness at mimicking normal free cortisol stress responses.

Methods: 31 patients and 18 healthy subjects were exposed to the "Trier Social Stress Test" (TSST). Saliva and blood samples were collected before and repeatedly afterwards for determination of salivary cortisol, plasma epinephrine, and plasma norepinephrine levels. At the same time-points, blood pressure and heart rate were measured. 16 patients were treated with HC after TSST.

Results: Patients with Addison's disease did not show stress responses in cortisol and epinephrine, while norepinephrine levels did increase in response to TSST. HC injection in patients resulted in salivary free cortisol levels comparable to those of healthy subjects. No differences between patients and healthy subjects as well as no HC effects were found in blood pressure and heart rate stress responses.

Conclusions: The present data for the first time show experimentally the expected dysregulated endocrine stress response patterns in patients with Addison's disease. These findings provide a basis for investigating the effects of permanent and context independent alterations of stress response patterns on other bodily systems, such as the immune system. This knowledge may further provide valuable insights to what extend an organisms is able to compensate dysregulations in these systems.

4.2.2 Introduction

4.2.2.1 Stress Response in Healthy Subjects

Numerous studies have shown that psychosocial stress is associated with an increased activity of the hypothalamus-pituitary-adrenal (HPA) axis as well as the

sympathetic-adrenal-medullary (SAM) axis (for review see Biondi and Picardi, 1999; Mason, 1968). Basically, changes in hormone secretory patterns in response to a stressor can be divided into two consecutive but distinct responses, the so called first wave and the second wave. In the first wave, occurring within seconds after the onset of a stressor, among others an enhanced secretion of catecholamines (epinephrine and norepinephrine) from the sympathetic nervous system, the hypothalamic release of corticotropin-releasing hormone (CRH) and (approximately 10 seconds later) enhanced secretion of pituitary adrenocorticotropic hormone (ACTH) can be observed. Minutes later, the second wave involves the stimulation of glucocorticoid release (Sapolsky et al., 2000).

Stress-induced increases in the secretion of glucocorticoids and catecholamines have been shown to be associated with a broad range of effects on various bodily systems, including metabolic, cardiovascular, immunological, and cognitive effects (for review see (Sapolsky et al., 2000). For catecholamines, Cannon coined the term “fight-or-flight response” to describe the tasks performed by catecholamines secreted in response to a challenge (Cannon, 1935). This term summarizes the major effects of catecholamines, namely the rapid activation of the cardiovascular system and the rapid mobilization of glucose. Both effects are important in terms of preparing and enabling the organism to fight or to flight. Activation of the cardiovascular system involves elevated arterial pressure, heart rate, and cardiac output, diversion of blood to muscle via constriction of mesenteric and renal vessels and dilation of vessels supplying skeletal muscle (Galosy et al., 1981). Blood glucose levels are elevated rapidly by mobilization from existing stores and by inhibition of further storage through a rapid insulin resistance. This makes sure that energy is diverted from storage sites to the exercising muscle. Additionally, catecholamines have been found to markedly enhance memory for emotionally laden details (Cahill et al., 1994), an effect again making sense biologically and evolutionarily. Regarding the immune system, catecholamines have a wide array of effects. For example, they increase the number of circulating natural killer (NK)-cells and granulocytes, i.e., immune cells of innate immunity, and thus help reducing the risk for infections in cases of tissue damage (Benschop et al., 1996).

These stress-induced rapid events are followed by increases in glucocorticoids. Consequently, important effects of stress-induced increased glucocorticoid levels include the prolongation of catecholamine effects. For example, since glucocorticoids are known to also increase blood pressure and cardiac output (Sambhi et al., 1965), they prolong the cardiovascular effects prior initiated by catecholamines. This is also the case in energy metabolism, where glucocorticoids synergize with catecholamines and glucagon to stimulate lipolysis and to elevate circulating glucose concentrations by stimulating glycogenolysis and gluconeogenesis. Furthermore, stress-induced glucocortiocoids inhibit peripheral glucose utilization and stimulate liver glycogen deposition. Since this restoring of glycogen levels

takes hours, glucocorticoids also help preparing the organism for subsequent stressors (Sapolsky et al., 2000). The mostly suppressive effects of glucocorticoids on the immune system are thought of as restraining defense reactions to stress, which would themselves become damaging if left uncontrolled (Besedovsky and del Rey, 1996). In other words, glucocorticoids “sculpt” the immune response by inhibiting superfluous or autoimmune-prone components (Besedovsky et al., 1991).

In summary, all of the described actions of stress mediators suggest that an endocrine stress response is basically protective, adaptive, and improving chance of the individual for survival. On the other hand, given such a complex interplay, it is also conceivable to anticipate serious health consequences if stress systems show malfunctions. In fact, HPA axis dysfunctions have been associated repeatedly with various pathophysiologic states, including psychiatric, endocrine, and inflammatory disorders and/or susceptibility to such disorders (for review see Chrousos, 1998a).

However, one question remains to be answered: What happens in this complex network of interactions if parts of the endocrine stress response are missing permanently and independently of context? Does the organism compensate for the missing response or is a regular endocrine stress response mandatory for health?

4.2.2.2 Stress Response in Patients with Addison’s Disease

To answer such questions, mostly adrenalectomized animals were investigated (e.g., Jacobson and Sapolsky, 1993; Keller et al., 1983; Rocha, 1985) or receptor blockers were administered (e.g., Hermann et al., 1995; Sheridan et al., 1998) in the past. However, both approaches hold their specific problems. Approaches like adrenalectomy are only realizable in animals, hence transfer of results to humans is limited. On the other hand, in pharmacological approaches the treatment is mostly temporary, limited to one hormone, and often also affects basal hormone concentrations. Thus, again only limited conclusions concerning the effects of a permanently missing endocrine stress response can be drawn.

We see investigation of patients with Addison’s disease as a valuable alternative approach in this regard. To the best of our knowledge no data are available to date which describe the endocrine stress responses in these patients. Therefore, the present work aims at investigating the endocrine stress responses in these patients, laying the groundwork for further studies.

Generally, Addison’s disease is a primary adrenal insufficiency caused by destroyed adrenal cortical cells (for review see Arlt and Allolio, 2003; Betterle et al., 2002; Oelkers, 1996; Ten et al., 2001). Due to these destroyed steroid producing cells, patients with Addison’s disease are no longer able to produce glucocorticoids and mineralocorticoids. Consequently, the treatment of these patients accounts for the two mainly missing hormones cortisol and aldosterone. However, glucocorticoid replacement therapy only aims at restoring

basal cortisol levels and mimicking its circadian rhythm. But theoretically, patients with Addison's disease also can not show a regular cortisol stress response. This is in parallel with findings in animal studies, where after adrenalectomy (ADX) whether stress nor ACTH induce any changes in corticosterone levels (Desser-Wiest, 1976). Nevertheless, to date it is unclear if patients with Addison's disease show the same response pattern found in ADX animals.

Furthermore, endogenous and locally acting cortisol is critical in the last step of the catecholamine biosynthesis, since it has been shown to induce the enzyme phenylethanolamine-N-methyl-transferase (PNMT), which converts norepinephrine to epinephrine (Bornstein et al., 1995). Without cortisol, norepinephrine will not be converted to epinephrine and in contrast to norepinephrine, the adrenal medulla is the sole source of circulating epinephrine in all mammalian species, including humans. However, norepinephrine released from the adrenal medulla usually makes up to only 35% of the levels measured in the bloodstream. The remaining 65% are a small proportion of norepinephrine released locally from sympathetic nerve terminals and reaching the bloodstream (Goldstein, 2000; Kvetnansky and McCarty, 2000; Pollard, 2000). Hence, in Addison's disease low epinephrine levels accompanied by elevated norepinephrine levels have been reported (Bornstein et al., 1995). Therefore, also a low to non-existing epinephrine stress response has to be expected in these patients accompanied by – albeit generally elevated norepinephrine levels – a regular norepinephrine stress response compared to healthy controls.

Based on the findings outlined above of stress-induced cortisol and catecholamines affecting cardiovascular parameters, we were further interested in the effect of the suspected altered endocrine stress pattern in patients with Addison's disease on stress responses in blood pressure and heart rate. Postural hypotension is a typically clinical sign in developing Addison's disease (Ten et al., 2001) and of mineralocorticoid under-treatment, since mineralocorticoid deficiency leads to dehydration and hypovolaemia, resulting in low blood pressure, postural hypotension, and sometimes even in prerenal failure (Arlt and Allolio, 2003). On the other hand, hypertension, bradycardia, and suppressed renin levels are clinical signs of over-treatment with mineralocorticoids (Ten et al., 2001). Nevertheless, combined mineralocortioid and glucocorticoid replacement in Addison's disease has been shown to reconstitute the diurnal rhythm of blood pressure (Fallo et al., 1994) and to reverse cardiac dysfunction (Fallo et al., 1999). Glucocorticoids contribute to this improvement not only by mineralocorticoid receptor binding, but also by permissive effects on catecholamine action (Allolio et al., 1994). Therefore, given an adequate glucocorticoid and mineralocorticoid replacement therapy, at least no baseline differences before stress in patients with Addison's disease are to be expected.

Beside Addison's disease being a potentially valuable methodological approach, endocrine and cardiovascular stress responses in Addison's disease also are interesting from a patients' point-of-view. Many patients with Addison's disease on standard replacement therapy have persistent complaints of fatigue, wariness, and reduced stress tolerance, which in many cases reduce their ability to maintain work (Lovas et al., 2002). We were therefore also interested in confirming these findings in regard to fatigue. Primarily, these findings may concern mostly the adequacy of replacement therapy, which itself was repeatedly questioned (Howlett, 1997; Lovas and Husebye, 2003; Peacey et al., 1997). Nevertheless, it is conceivable to hypothesize these complains being at least partly caused by dysfunctional endocrine stress responses.

4.2.2.3 Aim of the Study

The aim of the present study was to establish endocrine and cardiovascular stress response patterns in patients with Addison's disease. Furthermore, we were interested whether an *i.v.* 0.03mg/kg hydrocortisone in patients with Addison's disease adequately mimics a salivary cortisol stress response usually seen in healthy subjects. This dose was chosen based on a pilot study in healthy subjects with various doses of hydrocortisone injected in the late afternoon, a time of day characterized by low salivary free cortisol levels. Additionally, cardiovascular variables were tested for treatment effects. For catecholamines, no effect of treatment was expected, since for conversion of norepinephrine to epinephrine cortisol is needed in high concentrations locally in the adrenal medulla (chromaffin cells, Bornstein et al., 1995).

4.2.3 Methods

4.2.3.1 Subjects

A total of 36 patients with Addison's disease (AD) and 21 healthy subjects (HS) were investigated. Patients were further subdivided into two age- and gender-matched groups. Due to missing endocrine data, the final sample consisted of 31 patients and 18 healthy subjects. Of the two patient groups, one group was treated with 0.03mg/kg hydrocortisone (HC) *i.v.* (Sigma, Berlin) after a psychosocial stress test (AD-HC: n=16), while the second group as well as all healthy subjects received an injection of 4ml saline (NaCl; AD-NaCl: n=15; HS-NaCl: n=18). The AD-HC group consisted of 11 females and five males, the AD-NaCl group of 12 females and three males, and the HS-NaCl group of 11 females and seven males (χ^2=1.38, p=.50). Mean age of all subjects was 44.59 years (standard deviation (SD): 8.6). The three groups did not differ in age ($F_{2,48}$=0.10, p=.91; AD-HC: mean=43.81, SD=8.5; AD-NaCl: mean=44.87, SD=7.9; HS-NaCl: mean=45.06, SD=9.8) or body mass index (BMI; $F_{2,48}$=0.77, p=.47; AD-HC: mean=23.89, SD=4.3; AD-NaCl: mean=23.98, SD=2.6; HS-NaCl:

mean=25.40, SD=4.6). Based on the existence of autoantibodies (i.e., adrenocortical autoantibodies (ACAs), steroid cell antibodies (StCA); see Peterson et al., 2000) or co-morbidities fulfilling criteria for classification to Autoimmune Polyglandular Syndrom (APS) type 1 or type 2 (Neufeld et al., 1981), 21 patients were diagnosed with autoimmune Addison's disease (67.7%). In four patients the cause for Addison's disease was former Cushing's disease (12.9%) and six patients could not provide sufficiently detailed information for differential diagnosis (19.4%).

4.2.3.2 Experimental Protocol

Subjects reported to the lab at 1p.m. and were examined for past or current health problems by a physician. Patients were asked to postpone their second glucocorticoid replacement dose usually taken around 2p.m. (mean=13:49h, SD=1h57min) to avoid unpredictable and un-physiologically high salivary free cortisol levels (see section 4.1), hampering the comparability of patients and healthy subjects. After catheter insertion, attaching the ambulatory blood pressure monitoring system ('Quiet Track', Welch Allyn/Tycos Instruments, Arden, NC) and a resting period of 45 minutes, a first blood (9ml ethylenediamine tetraacetic acid (EDTA)-tubes, Sarstedt, Nümbrecht, Germany) and saliva (Salivette, Sarstedt, Nümbrecht, Germany) sample was collected and blood pressure and heart rate were measured. Subsequently, subjects were exposed to the psychosocial stress test "Trier Social Stress Test" (TSST), which consists of a three minute preparation period, a five minute free speech and a five minute mental arithmetic task in front of an audience (Kirschbaum et al., 1993). Additional blood and saliva samples were collected as well as blood pressure and heart rate were obtained 1, 10, 20, 30, 45, 60, 90, and 120 minutes after stress exposure. After time-point +1, HC or saline was injected. This time-point was chosen since the stress test has a duration of 13 minutes and the maximum cortisol stress response is to be expected 15-20 minutes after stress onset. The study protocol was approved by the ethics committee of the University of Düsseldorf, and written informed consent was obtained from all subjects.

4.2.3.3 Fatigue Assessment

During the resting period, all subjects were ask to self-report their fatigue measured by the Multidimensional Fatigue Inventory (MFI; Smets et al., 1995). Beside general fatigue, the MFI covers the dimensions physical fatigue, mental fatigue, reduced activity, and reduced motivation.

4.2.3.4 Biochemical Analyses

Saliva samples: Free cortisol levels in saliva were measured using a commercially available chemiluminescence assay (IBL, Hamburg, Germany). Samples were measured in duplicates and averaged for subsequent statistical analyses.

Blood samples: Plasma was separated at 4°C and stored at -80°C. Plasma concentrations of norepinephrine and epinephrine were determined by high-performance liquid chromatography with electrochemical detection (Smedes et al., 1982). Epinephrine levels under detection limit were labeled 5pg/ml representing half of the lowest standard.

4.2.3.5 Statistical Analysis

Data were analyzed using the Statistical Package for the Social Science Version 11.0.2 (SPSS Institute, Chicago, IL). Student's *t* tests were applied for comparing the scores of the five MFI-scales and baseline (-1min) endocrine and cardiovascular measures between patients and healthy subjects (AD vs. HS). For endocrine and cardiovascular parameters, time (i.e. TSST) effects and group differences were calculated by repeated measures analysis of variance (ANOVA) with within-group levels representing the nine time-points of blood/saliva sampling or blood pressure and heart rate measures. For group or group-by-time effects, two between-group levels for catecholamines (AD vs. HS) and three between-group levels for cortisol levels and cardiovascular measures (AD-HC, AD-NaCl, and HS-NaCl) were included. All data were tested for normality prior to statistical analysis using the Kolmogorov-Smirnov test and Greenhouse-Geisser corrections for repeated measures were calculated where appropriate. To test for effects of treatment, univariate ANOVAs were calculated with salivary free cortisol levels at time-point +10 (cort+10) as well as with increases in salivary free cortisol levels from time-point -1 to +10 (incr_cort1>3; calculated by subtracting salivary free cortisol levels at time-point -1 from levels at +10) as dependent and the three groups as independent variables. In cases of significant group effects, additionally Scheffé post-hoc tests were computed to specify treatment effects. Values of $p<0.05$ were considered significant.

4.2.4 Results

4.2.4.1 Fatigue Assessment

Student's *t* tests revealed significant group differences for the two MFI scales general fatigue and physical fatigue (general fatigue: $t_{45}=3.23$, $p=.002$; physical fatigue: $t_{45}=2.56$, $p=.014$, mental fatigue: $t_{45}=0.65$, $p=.52$; reduced activity: $t_{45}=.43$, $p=.67$, reduced motivation: $t_{45}=-0.38$, $p=.70$). In both cases, patients showed higher fatigue scores than healthy subjects (see figure 4-4).

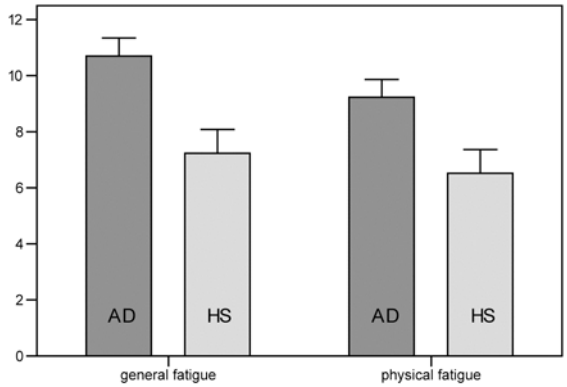

Fig. 4-4: General and physical fatigue (means and standard errors (SE)) in patients with Addison's disease (AD, n=30) and healthy subjects (HS, n=17), measured by the Multidimensional Fatigue Inventory.

4.2.4.2 Baseline Differences

At baseline, only salivary free cortisol levels and epinephrine levels differed significantly between patients and healthy subjects (cortisol: t_{47}=-3.67, p=.001; norepinephrine: t_{47}=1.42, p=.16; epinephrine: t_{47}=-4.81, p<.001; systolic blood pressure: t_{44}=0.39, p=.70; diastolic blood pressure: t_{44}=0.07, p=.95, heart rate: t_{44}=0.92, p=.36).

4.2.4.3 Salivary Free Cortisol Stress Response

Repeated measures ANOVA with salivary free cortisol levels as within-group levels revealed significant group, time, and group-by-time effects (group: $F_{2,46}$=22.65, p<.001; time: $F_{8,368}$=48.06, p<.001; group-by-time: $F_{16,368}$=13.98, p<.001). Subsequent Scheffé post-hoc tests showed all three groups to differ significantly from each other (HC-AD vs. NaCl-AD: $mean_{diff}$=4.81, SD=1.6, p=.014, HC-AD vs. HS-NaCl: $mean_{diff}$=-5.47, SD=1.5, p=.003, AD-NaCl vs. HS-NaCl: $mean_{diff}$=-10.28, SD=1.5, p<.001).

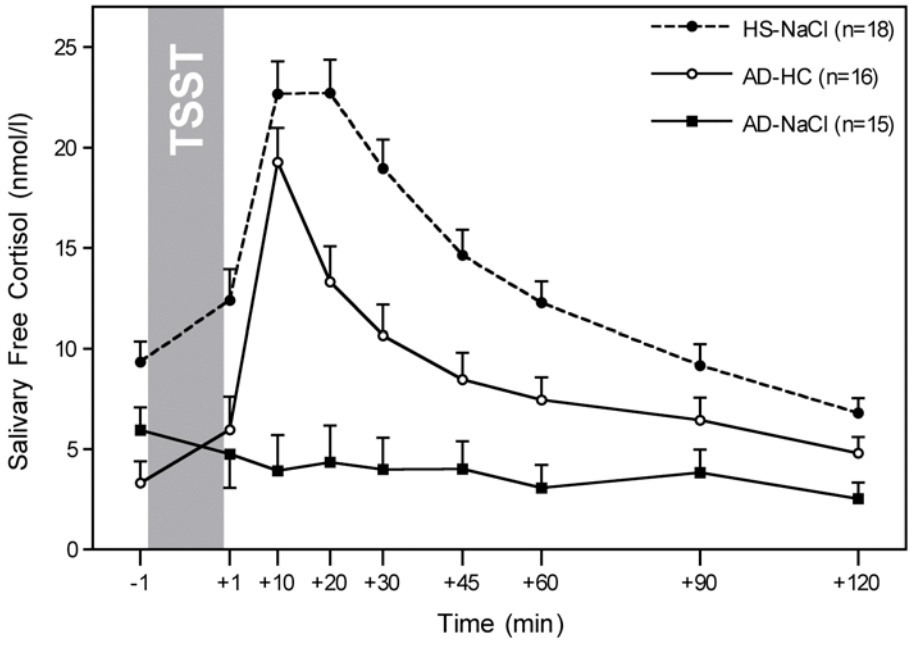

Fig. 4-5: Effects of TSST, group (AD vs. HS) and treatment (HC vs. NaCl) on salivary free cortisol levels (mean, SE).

4.2.4.4 Effects of Treatment on Salivary Free Cortisol Levels

In both, salivary free cortisol levels at time-point +10 as well as increases in salivary free cortisol levels from time-point -1 to +10, the AD-NaCl group did differ significantly from the two other groups. Hydrocortisone injection resulted in non-significant group differences between healthy subjects and patients (*cort+10:* $F_{2,46}$=33.50, p<.001 – Scheffé: AD-HC vs. AD-NaCl: $mean_{diff}$=15.34, SD=2.5, p<.001; AD-HC vs. HS-NaCl: $mean_{diff}$=-3.40, SD=2.4, p=.36; AD-NaCl vs. HS-NaCl: $mean_{diff}$=-18.74, SD=2.4, p<.001; *incr_cort1>3:* $F_{2,46}$=30.90, p<.001 – Scheffé: AD-HC vs. AD-NaCl: $mean_{diff}$=17.98, SD=2.5, p<.001; AD-HC vs. HS-NaCl: $mean_{diff}$=2.63, SD=2.4, p=.54; AD-NaCl vs. HS-NaCl: $mean_{diff}$=-17.35, SD=2.4, p<.001).

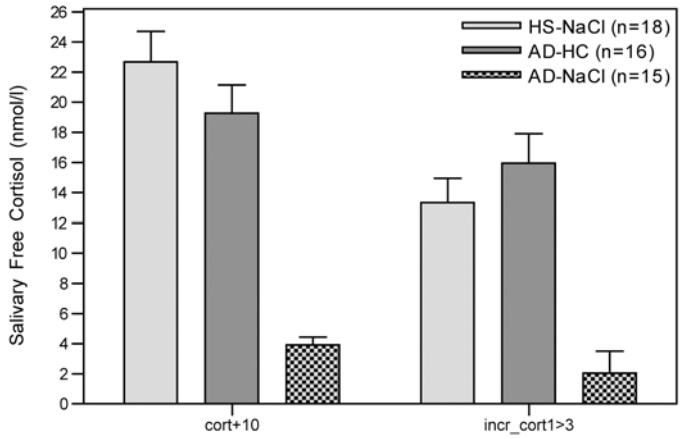

Fig. 4-6: Group differences in salivary free cortisol levels at time-point +10 (cort+10; left side; mean, SE) and in increases from time-point -1 to +10 (incr_cort1>3; right side; mean, SE).

4.2.4.5 Catecholamine Stress Response

As outlined above, no treatment effects in catecholamine stress responses in patients are to be expected for physiological reasons (Bornstein et al., 1995). Regarding catecholamines, repeated measures ANOVAs were therefore computed with two between-group levels representing patients with Addison's disease (n=31) and healthy subjects (n=18).

Despite higher norepinephrine levels in patients throughout the investigation, no significant group or group-by-time effects were found (group: $F_{1,47}$=2.43, p=.13; group-by-time: $F_{8,376}$=1.00, p=.42). Nevertheless, TSST caused increases in norepinephrine levels indicated by a significant time effect (time: $F_{8,376}$=12.36, p<.001).

For epinephrine, significant group, TSST, and group-by-time effects were found (group: $F_{1,47}$=33.59, p<.001; time: $F_{8,376}$=10.74, p<.001; group-by-time: $F_{8,376}$=6.87, p=.002). Figure 4-7 depicts norepinephrine and epinephrine levels before and after TSST.

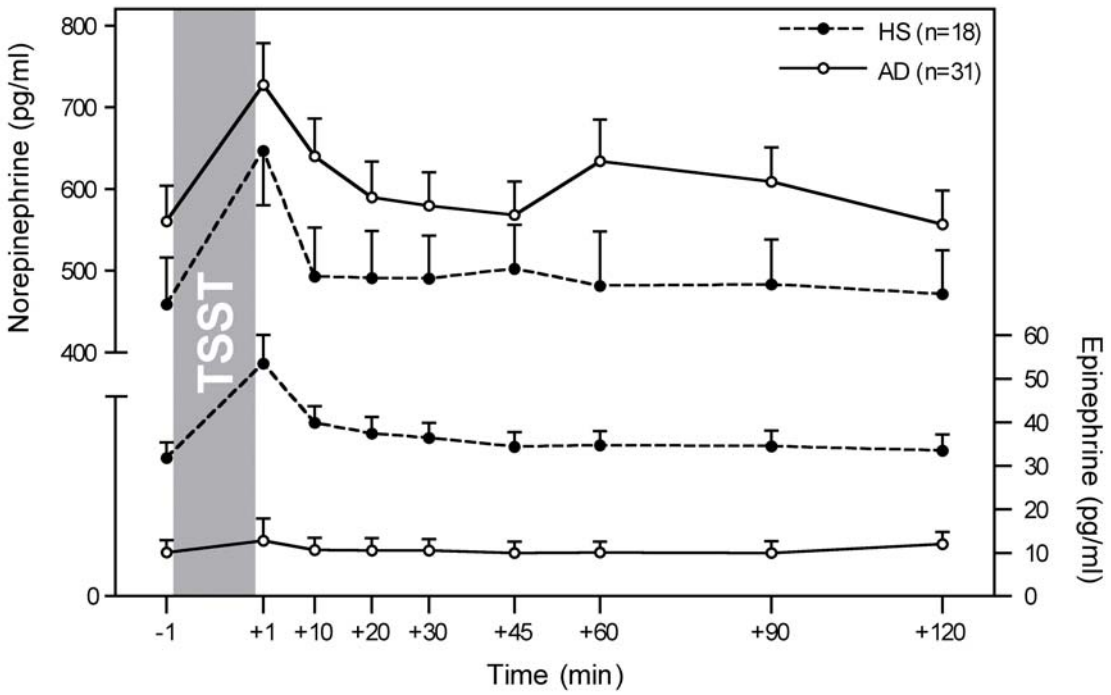

Fig. 4-7: Effects of TSST and group (AD vs. HS) on plasma norepinephrine (top) and epinephrine (bottom) levels (mean, SE).

4.2.4.6 Cardiovascular Stress Response

For blood pressure and heart rate, only significant time effects but no group or group-by-time effect were found (systolic blood pressure – group: $F_{2,36}$=0.82, p=.45; time: $F_{8,288}$=5.93, p<.001; group-by-time: $F_{16,288}$=0.98, p=.47; diasystolic blood pressure – group: $F_{2,36}$=0.14, p=.87; time: $F_{8,288}$=2.35, p=.036; group-by-time: $F_{16,288}$=0.62, p=.81; heart rate – group: $F_{2,36}$=1.13, p=.34; time: $F_{8,288}$=4.97, p<.001; group-by-time: $F_{16,288}$=0.76, p=.67). Figure 4-8 depicts the course of systolic and diastolic blood pressure and figure 4-9 the course of heart rate over the period of investigation.

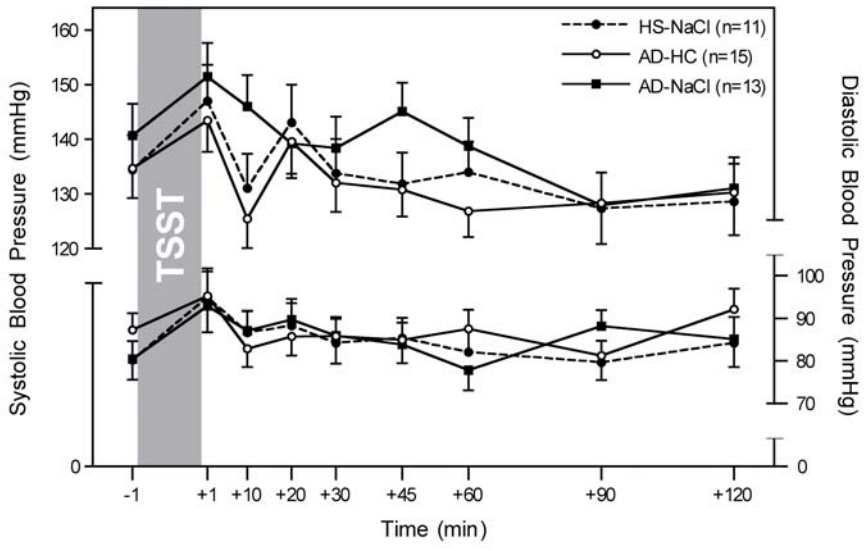

Fig. 4-8: Effects of TSST, group (AD vs. HS) and treatment (HC vs. NaCl) on systolic (top) and diastolic (bottom) blood pressure (mean, SE).

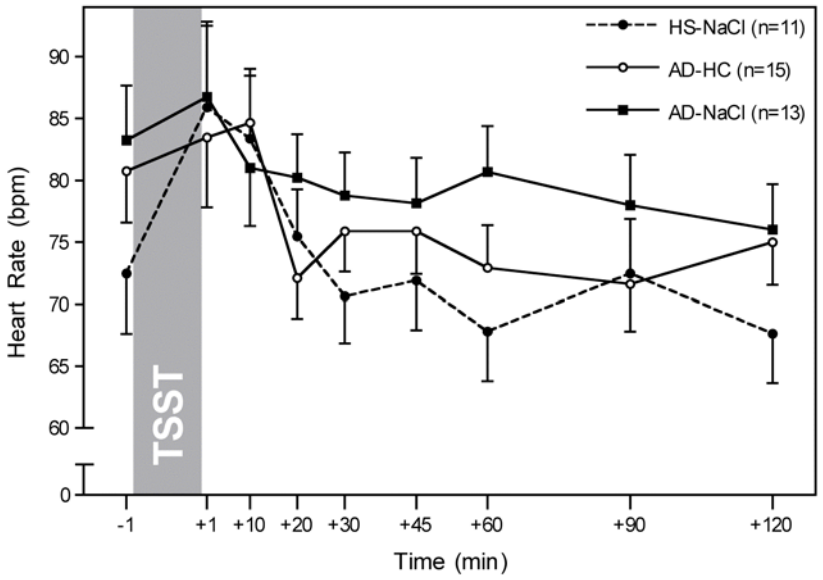

Fig. 4-9: Effects of TSST, group (AD vs. HS) and treatment (HC vs. NaCl) on heart rate (mean, SE).

4.2.5 Discussion

In summary, patients with Addison's disease showed significantly more general and physical fatigue compared to healthy subjects. Regarding endocrine and cardiovascular parameters, no baseline differences were found in norepinephrine, blood pressure or heart rate, but significantly lower salivary free cortisol and epinephrine levels in patients with Addison's disease. The TSST induced significant increases in salivary free cortisol levels in healthy subjects, while patients did not show any response. The injection of 0.03mg/kg hydrocortisone in patients with Addison's disease resulted in salivary cortisol increases and maximum levels comparable to those found in healthy subjects. No significant differences were found in norepinephrine stress responses, despite generally higher norepinephrine levels in patients. In epinephrine, healthy subjects showed a typical stress response pattern, while patients showed very low levels throughout the investigation period and no stress response. Furthermore, no distinctive features were found in blood pressure and heart rate responses to the TSST.

The present findings of elevated general and physical fatigue scores in patients with Addison's disease are in line with former findings (Lovas et al., 2002) and with subjective self-reports of patients. Interestingly, contrary to subjective self-reports no differences in mental fatigue, activity and motivation were found in patients compared to healthy subjects. This may be explained in part by patients' efforts to master their life despite their disease and by a tendency to rather admit physical or unspecific problems than mental or intentional problems.

Regarding salivary free cortisol levels, patients with Addison's disease showed the expected missing stress response. These findings confirm data from studies investigating the effects of adrenalectomy on corticosteroid stress response (Desser-Wiest, 1976). Treatment

of patients with 0.03mg/kg hydrocortisone resulted in stress-induced cortisol increases and maximum salivary cortisol levels mimicking those of healthy subjects. However, patients clearly showed a more pronounced decrease in salivary cortisol levels thereafter. Contrary to one-time hydrocortisone treatment in patients with Addison's disease, in healthy subjects, HPA-reactivity is adjusted to stress duration and may show several episodes of secretion. This demonstrates that treatment of patients with hydrocortisone at one time-point is only to a certain extent able to mimic context-dependent HPA-reactivity controlled by negative feedback circuit.

The findings of very low epinephrine levels accompanied by a trend to elevated norepinephrine levels are also in line with prior findings and emphasize the role of glucocorticoids in normal functioning of chromaffin cells and their capacity at the transcriptional level to express PNMT, the enzyme that converts norepinephrine to epinephrine (Wong et al., 1995). Our data regarding basal catecholamine levels confirm other findings in patients with Addison's disease (Bornstein et al., 1995), in experimental animals with reduced glucocorticoid synthesis (Wurtman, 1966), and after adrenalectomy (Eisenhofer et al., 1995; Merke et al., 2000). One study additionally included stress stimulation in patients with isolated glucocorticoid deficiency due to unresponsiveness to adrenocortiocotropin hormone (ACTH; Zuckerman-Levin et al., 2001). The authors found a minimal stress response in epinephrine and slightly increased norepinephrine responses compared to healthy subjects. Again, this is in line with the present findings. Plasma norepinephrine derives to a high proportion from nerve terminals of the sympathetic nervous system and to a lower extend from the adrenal medulla (Goldstein, 2000; Kvetnansky and McCarty, 2000; Pollard, 2000). This raises the question where the slightly increased norepinephrine levels come from. Based on the data outlined above and on findings of decreased norepinephrine in 21-hydroxylase deficient mice (Bornstein et al., 1999), it was repeatedly suggested to be more likely an intra-adrenal effect. However, to which extent elevated norepinephrine levels may compensate almost missing epinephrine levels is unknown.

As expected, the present study further found no significant differences in resting blood pressure or heart rate in patients with Addison's disease compared to healthy subjects. This suggests glucocorticoid and mineralocorticoid replacement therapy to restore cardiac functions as demonstrated by Fallo et al. (Fallo et al., 1999; Fallo et al., 1994). Furthermore, no differences in stress responses in these parameters as well as no effects of hydrocortisone treatment were found. Therefore, it may be speculated that increased norepinephrine levels are at least partly able to compensate for missing epinephrine and cortisol responses in patients with Addison's disease. Also permissive actions of cortisol (i.e., basal cortisol levels induced by replacement therapy) on catecholamine stress effects may

explain these findings to a certain extend (Allolio et al., 1994). The missing effects of hydrocortisone treatment in patients may be attributable to dosage and/or frequency being too low to exert effects or by effects appearing later than two hours after treatment. Studies investigating the role of cortisol in hypertension usually treat subjects with higher doses for longer periods of time (e.g., 6-8mg/day intravenously or 50mg every 6h orally over a 5 day administration period; (Whitworth et al., 1995) and observe increases in systolic pressure of the order of 15mmHg (Kelly et al., 1998).

Taken together, the present data in patients with Addison's disease show for the first time the dysregulated endocrine stress response pattern expected from literature. No effects of missing cortisol and epinephrine stress responses on blood pressure and heart rate were found, hinting to mechanisms able to compensate dysregulated endocrine stress responses.

Treatment of patients with 0.03mg/kg hydrocortisone i.v. proved to adequately mimic acute free cortisol stress responses found in healthy subjects. In further investigations, such treatment may prove helpful at attributing observed effects to a specific stress mediator. It cannot be concluded from these data that patients should be treated with additional doses of hydrocortisone during stress. First of all, hydrocortisone was injected in this study and the respective dose of orally taken hydrocortisone is unknown and untested. Furthermore, there is increasing evidence that patients tend to be over-treated (Howlett, 1997; Lovas and Husebye, 2003; Peacey et al., 1997). Chronic over-treatment in turn was repeatedly associated with increased risks of impaired glucose tolerance, obesity, and osteoporosis (al-Shoumer et al., 1995; Florkowski et al., 1994; Zelissen et al., 1994). Increasing the daily dose of glucocorticoid replacement might further heighten the risk for such morbidities.

However, these findings provide a basis for investigating the effects of permanently and context independently altered stress response patterns on other bodily systems, such as the immune system, in future studies. Studying such effects in patients with Addison's disease may provide valuable insights in the interplay of endocrine stress systems in human whole organism and the extend to which an organisms is able to compensate dysregulations in these systems. This knowledge may help to further evaluate findings in psychoneuroendocrinology and psychoneuroimmunology of altered stress response patterns being associated with immunological diseases or psychiatric disorders.

4.3 ADDISON'S DISEASE: PSYCHOSOCIAL STRESS AND IMMUNOLOGICAL CONSEQUENCES

Stress and Addison's Disease:
Implications of Dysregulated Stress Systems for Health and Disease in Humans

4.3.1 Abstract

Background: Dysfunctions in endocrine stress responses were repeatedly implicated with immunological diseases. Studies investigating such interactions between stress and immunological diseases usually only reveal associations. To decide on direction and possible mechanisms, findings from approaches like adrenalectomy, receptor-(ant)agonists treatment, or *in vitro* studies have to be consulted. Such approaches hold their own restrictions regarding the transfer of results to situations in the human whole organism. As an alternative approach, we investigated patients with Addison's disease to gain new insights in the effects of permanently and context independently missing cortisol and epinephrine stress responses.

Methods: 31 patients with Addison's disease and 16 healthy subjects were investigated. All subjects were exposed to the psychosocial stress paradigm "Trier Social Stress Test" (TSST). Percentages of leukocyte subtypes in peripheral blood, LPS-stimulated IL-6 production, PHA-stimulated cell proliferation, as well as the respective glucocorticoid sensitivities were measured before and repeatedly after stress (10, 60, and 120 minutes after TSST). 16 age- and gender-matched patients were treated with an injection of 0.03mg/kg hydrocortisone *i.v.* after stress, while all other subjects received placebo injection (saline).

Results: In healthy subjects, stress resulted in significant changes in blood cell counts and LPS-stimulated IL-6 production. No baseline differences in immune parameters were found between patients with Addison's disease and healthy subjects. Stress in patients with Addison's disease resulted in courses of blood cell counts and LPS-stimulated IL-6 productions significantly different from healthy subjects. Treatment with hydrocortisone only influenced the course of LPS-stimulated IL-6 production. No influence of disease or hydrocortisone treatment were found in cell proliferation parameters.

Conclusions: Investigation of patients with Addison's disease proved to be a valuable alternative approach for PNI research. The present findings confirm glucocorticoids to play an important role in leukocyte migration and stimulated cytokine production, while T-cell proliferation appeared to be glucocorticoid-independent. The distinctive features in blood cell counts and LPS-stimulated IL-6 production point to patients with Addison's disease being at an increased risk for infectious as well as autoimmune diseases.

4.3.2 Introduction

The field of psychoneuroimmunology (PNI) addresses the way in which nervous system and immune system interact with each other, and how these interactions influence the state of health of an individual. In their editorial to volume I of the "Neuroimmune Biology Book Series", Berczi and Szentivanyi pointed out that there is even much more to this interaction than 'bi-directional'. It is as a "truly multi-directional, all-inclusive systemic regulatory network formed by the nervous-, endocrine- and immune systems, which controls all bodily functions of higher animals and man" (Berczi and Szentivanyi, 2001). Within the field of PNI, one area has attracted considerable attention: The effects of stress on the immune system and hence on health and disease. To date, it is well-known that psychosocial stress is associated with an increased activity of the hypothalamus-pituitary-adrenal (HPA) axis as well as the sympathetic-adrenal-medullary (SAM) axis (for review see Biondi and Picardi, 1999; Mason, 1968). The major end products of these two axes are the hormones cortisol (HPA axis) and epinephrine and norepinephrine (SAM axis). Stress-induced increased release of these mediators was in turn repeatedly demonstrated to have a wide range of effects on the immune system. In cases of acute stress, these effects are thought of as primarily protective. For example, the fast release of catecholamines (epinephrine and norepinephrine), occurring within seconds after onset of a stressor, increases the number of circulating natural killer (NK)-cells and granulocytes, i.e., cells of innate immunity, and thus helps reducing the risk for infections in cases of tissue damage (Benschop et al., 1996). These stress-induced rapid events are followed by increases in glucocorticoids. The mostly suppressive effects of glucocorticoids on the immune system are thought of as restraining defense reactions to stress, which would themselves become damaging if left uncontrolled (Besedovsky and del Rey, 1996). On the other hand, given the complex interplay of hormones and immune system, it is also conceivable to anticipate serious health consequences if stress systems show malfunctions. In fact, HPA axis dysfunctions have been associated repeatedly with various pathophysiologic states, including psychiatric, endocrine, and inflammatory disorders and/or susceptibility to such disorders (for review see (Chrousos, 1998a). Thus, contrary to an endocrinological response to acute stress, chronic stress may result in HPA and SAM axes dysfunctions (e.g., missing cortisol stress responses), which in turn are associated with negative health outcomes.

Such conclusion are usually drawn by combining research results originating from different backgrounds. For example, Sapolsky, Romero, and Munck (Sapolsky et al., 2000) applied three criteria to analyze the role of glucocorticoids and catecholamines in stress response: the criterion of time course (When does an effect occur?), the criteria of hormone subtraction and replacement (What happens if a glucocorticoid and/or catecholamine stress response is eliminated? Does administration of stress or/and basal levels of the respective

hormone restore the stress response?), and the criterion of homeostasis (Which action, i.e., permissive, suppressive, stimulating, or preparative action, makes more physiological sense in terms of restoring homeostasis?).

However, independent of applied criteria, combining research results from different backgrounds raises three intertwined questions: (1)To what extend can results from animal research be combined with results from human research? (2) To what extend can effects of hormones *per se* be combined with effects of hormones secreted due to stress? (3) To what extend can results from *in vitro* approaches be combined with results from studies in whole organisms?

These questions are especially prominent in human research. For example, if interests are of clinical nature, patients with immunological diseases may be investigated and their endocrine and immunological stress responses compared with responses of healthy subjects. Without a longitudinal design, no causal associations can be revealed in such cases: Stress may exacerbate already existing immune dysfunctions or immune dysfunctions may influence endocrine stress responses. To hypothesize on direction, underlying mechanisms, and relevance of association for health and disease, findings from approaches like adrenalectomy, usage of receptor-(ant)agonists, or *in vitro* studies have to be consulted (see figure 4-10). By doing that, conclusions are drawn based on findings all limited in terms of transferability to the human whole organism situation.

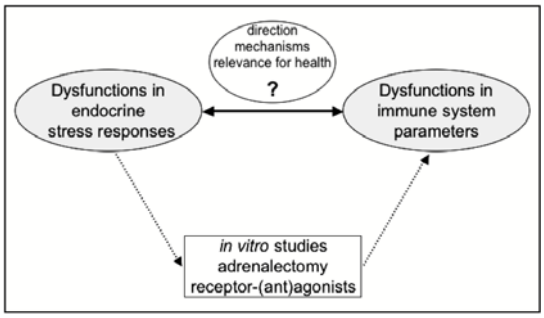

Fig. 4-10: Decisions on direction, underlying mechanisms and relevance of associations between stress response and health in humans can be reached based on results originating from other approaches like adrenalectomy, use of receptor-antagonists or -agonists, and *in vitro* studies.

4.3.2.1 Aim of the Study

Nevertheless, to define the role or stress hormones for normal functioning of the immune system, two important criteria are hormone subtraction and hormone replacement (Sapolsky et al., 2000). Therefore and since our focus is on human research, we aimed at finding a model for studying these criteria in human whole organism, thereby overcoming the shortcomings of alternative approaches like adrenalectomy, use of receptor-(ant)agonists, and *in vitro* studies. Such an approach would then allow for answering important questions in PNI research: What happens in the human whole organism, if parts of the endocrine stress response are missing permanently and independently of context? Are regular endocrine stress responses in fact mandatory for health? Or does the organism compensate for these

missing parts? And if compensatory mechanisms exists, to what extend are they able to compensate for dysregulations in endocrine stress responses? Answering such questions is also of great clinical interest, since it may help to decide on the clinical relevance of distinctive features in endocrine stress responses found in patients with immunological diseases.

We see investigation of patients suffering from Addison's disease as such an alternative approach providing valuable new evidences for human PNI research. Patients with Addison's disease do not produce any cortisol due to destroyed adrenals and therefore receive glucocorticoid replacement (for review see Arlt and Allolio, 2003; Betterle et al., 2002; Oelkers, 1996; Ten et al., 2001). This therapy only provides basal cortisol supply, while no provisions are made for additional doses during stress. Despite the apparent advantages compared with the above mentioned approaches, to the best of our knowledge no data on endocrine stress responses in these patients were available so far. We therefore first investigated such responses in patients with Addison's disease, thereby laying the groundwork necessary for answering the questions outlined above (see section 4.2). Our studies revealed missing cortisol and epinephrine stress responses and normal to slightly elevated norepinephrine levels in these patients. Furthermore, we showed an *i.v.* injection of 0.03mg/kg hydrocortisone after a psychosocial stress test of 13 minutes duration to accurately mimic the acute stress response in salivary free cortisol levels seen in healthy subjects.

Based on these endocrinological findings, we investigated in the present study the effects of missing cortisol and epinephrine stress responses on the immune system in patients with Addison's disease. In this regard we repeatedly measured percentages of immune cell subsets, lipopolysaccharide (LPS)-stimulated IL-6 production and phytohemagglutinin (PHA)-stimulated cell proliferation, as well as the respective glucocorticoid sensitivity in response to a psychosocial stress test in patients with Addison's disease and healthy subjects. Proportion of immune cells in circulation were chosen as an estimation of immune system activation, as decreased numbers of cells in peripheral blood can point to immune activation due to trafficking of these cells to inflammatory sites (Dhabhar, 2000). LPS-stimulated IL-6 production in whole blood (De Groote et al., 1992) and PHA-stimulated cell proliferation in peripheral blood mononuclear cells (PBMCs) provide information about reactivity of innate immunity and cellular immunity, respectively (Vedhara et al., 1999). Since sensitivity to the effects of glucocorticoids differ between individuals and even between different tissues of individuals (DeRijk and Sternberg, 1997), both parameters were additionally tested for their glucocorticoid sensitivity. To further distinguish between effects of missing cortisol from effects of missing epinephrine, the effect of treatment with 0.03mg/kg hydrocortisone i.v. on these immune measures were tested in patients.

4.3.3 Methods

4.3.3.1 Subjects

A total of 34 patients with Addison's disease (AD) and 20 healthy subjects (HS) were investigated. Due to missing data, the final sample consisted of 31 patients with Addison's disease and 16 healthy subjects. 16 age- and gender-matched patients were treated with an injection of 0.03mg/kg hydrocortisone *i.v.* (HC; Sigma, Berlin, Germany) after a psychosocial stress test, while 15 patients and 16 healthy subjects received a placebo injection (saline, NaCl; Sigma, Berlin, Germany). Mean age of subjects was 44.74 years (standard deviation (SD)=8.6) and mean body mass index (BMI) 24.11kg/m^2 (SD=3.6). Distribution of age and BMI did not differ between the three groups (age: $F_{2,46}$=0.96, p=.39; AD-HC: mean=43.75, SD=8.4; AD-NaCl: mean=47.27, SD=8.4; HS-NaCl: mean=43.38, SD=9.0. BMI: $F_{2,46}$=0.60, p=.55; AD-HC: mean=23.54, SD=3.8; AD-NaCl: mean=23.90, SD=2.2; HS-NaCl: mean=24.88, SD=4.4). The group AD-HC consisted of 12 females and 4 males, the group AD-NaCl of 10 females and 5 males, and the group HS-NaCl of 11 females and 5 males (χ^2=0.28, p=.87).

Of all patients, 22 (71%) were diagnosed with autoimmune Addison's disease, based on the existence of autoantibodies (i.e., adrenocortical autoantibodies (ACAs), steroid cell antibodies (StCA); see Peterson et al., 2000) or co-morbidities fulfilling criteria for classification to Autoimmune Polyglandular Syndrom (APS) type 1 or type 2 (Neufeld et al., 1981). One patient suffered from Addison's disease due to former infection (infectious AD; 3.2%) and three due to former Cushing's disease (9,7%). Five patients could not provide sufficiently detailed information for differential diagnosis (primary adrenal insufficiency (PAI); 16.1%). Glucocorticoid replacement was hydrocortisone in 24 patients (mean=26.77mg/day, SE=1.2) and cortisone acetate in seven patients (mean=39.58mg/day, SE=5.0).

4.3.3.2 Experimental Protocol

Subjects reported to the laboratory at 1p.m. and were examined for past or current health problems by a physician. Patients were asked to postpone their second glucocorticoid replacement dose usually taken around 2a.m. (mean=13:52h, SD=1h54min) to avoid unpredictable and un-physiologically high salivary free cortisol levels (see section 4.1), hampering the comparability of patients an healthy subjects. After catheter insertion and a resting period of 45 minutes, a first blood sample was collected (5ml heparinized syringe, 2,7ml ethylenediamine tetraacetic acid (EDTA) Monovette, 9ml citrate Monovette; Sarstedt, Nümbrecht, Germany). Subsequently, subjects were expose to the psychosocial stress test "Trier Social Stress Test" (TSST), which consists of a three minute preparation period, a five minute free speech and a five minute mental arithmetic task in front of an audience

(Kirschbaum et al., 1993). Additional blood samples were collected 10, 60, and 120 minutes after stress exposure. Immediately after TSST, HC or saline was injected. This time-point was chosen since the stress test has a duration of 13 minutes and the maximum cortisol stress response is to be expected 15-20 minutes after stress onset. The study protocol was approved by the ethics committee of the University of Düsseldorf, and written informed consent was obtained from all subjects.

4.3.3.3 Biochemical Analyses

4.3.3.3.1 Blood cell counting

Four blood samples were collected for determination of leukocyte numbers in EDTA-tubes. A differential blood count was performed measuring absolute numbers of leukocytes, as well as number and percentage of monocytes, lymphocytes, and granulocytes. Cell counting was performed on a Coulter AcTdiff cell counter (Beckman-Coulter, Krefeld, Germany). Each sample was measured five times and results were averaged.

4.3.3.3.2 LPS-stimulated IL-6 production and glucocorticoid sensitivity

IL-6 production: Venous blood was collected at four time-points in heparinized sterile tubes and diluted 10:1 with saline. Diluted whole blood was then co-incubated with lipopolysaccharide (LPS; E. Coli, Difco, Augsburg, Germany) and six different concentrations of dexamethasone (DEX; Sigma, Berlin, Germany) on a 24-well plate (Greiner, Nürtingen, Germany). 400µl diluted whole blood was added to 50µl of LPS and 50µl of DEX. Final concentrations were 30ng/ml LPS and 0, 10^{-9}, 10^{-8}, $5x10^{-8}$, 10^{-7}, and 10^{-6} M DEX, respectively. After 18 hours of incubation at 37°C and 5% CO_2, plates were centrifuged for 10 minutes at 2000g at 4°C. Plasma supernatant was collected and stored at -80°C until assayed.

Cytokine assay: IL-6 in plasma supernatant was measured using ELISA employing the multiple antibody sandwich principle (BD Pharmingen, San Diego, CA, USA). The detection limit of the IL-6 ELISA was 4.7pg/ml. Plates were read by microplate reader (Anthos HTII, Anthos Labtec, Salzburg, Austria), and absorbance was transformed to cytokine concentration (pg/ml) using a standard curve computed by Anthos Winread 2.3 software (Anthos Labtec, Salzburg, Austria).

4.3.3.3.3 PHA-stimulated cell proliferation and glucocorticoid sensitivity

Cell proliferation: Venous blood was collected at four time-points in sterile citrate tubes. Cells were isolated immediately after blood draw by density gradient centrifugation (10ml Ficoll, Biochrom, Berlin, Germany). Cells were then transferred to a new 50ml tube (Greiner, Nürtingen, Germany), washed three time with PBS (PAA, Coelbe, Germany), and

counted on a coulter AcTdiff cell counter (Beckman-Coulter, Krefeld, Germany). Cell numbers were adjusted to 1 million cells diluted in 1ml culture medium (RPMI 1640 with 10% FCS and 1% Penicillin/Streptomycin; Biochrom, Berlin, Germany). For each subject, the four cell suspensions were then co-incubated in triplets with phytohemagglutinin (PHA, Sigma, Berlin, Germany) and six different concentrations of dexamethasone (DEX; Sigma, Berlin, Germany) on one 96-well plate (Greiner, Nürtingen, Germany). Cell suspension (45μl) was added to 45μl of PHA and 10μl of DEX. Final concentrations were 7.5μg/ml PHA and 0, 10^{-9}, 10^{-8}, $5x10^{-8}$, 10^{-7}, and 10^{-6} M DEX, respectively. Plates were incubated at 37°C and 5% CO_2.

Proliferation assay: After 48 hours of incubation, 10μl of BrdU labeling reagent (Roche Diagnostics, Penzberg, Germany) was added and plates were incubated another 12 hours at 37°C and 5% CO_2. Plates were then centrifuged at room temperature for 10 minutes at 300g. Supernatant was discarded and cells were dried. Proliferation was measured by a colorimetric immunoassay for quantification of cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis (cell proliferation ELISA; Roche Diagnostics, Penzberg, Germany). Plates were read by a microplate reader giving ODs (optical density) as output. Results of each triplet were averaged and blanks (cell suspension without PHA and DEX) were subtracted.

4.3.3.4 Statistical Analysis

As an index for glucocorticoid sensitivity, inhibitory concentrations 50% (IC_{50}) were calculated of each individual dose-response-curve for DEX inhibition of LPS-induced cytokine production and DEX inhibition of PHA-induced cell proliferation. The IC_{50} reflects the specific dexamethasone concentration required for 50% inhibition of maximum cytokine production observed after LPS-stimulation without DEX and of maximum cell proliferation observed after PHA-stimulation without DEX. IC_{50}-values were calculated for each individual dose-response curve (i.e., for all four time-points and both variables (LPS-stimulated IL-6 production and PHA-stimulated cell proliferation) for every individual subject) using GraphPad Prism version 4.00c for Macintosh (GraphPad Software, San Diego, USA).

Data were analyzed using the Statistical Package for the Social Science Version 11.0.2 (SPSS Institute, Chicago, IL). Student's *t* tests were applied for comparing baseline immune measures between patients and healthy subjects (AD vs. HS). Immune parameters tested are: numbers of white blood cells (WBCs); lymphocytes, granulocytes, and monocytes given in percentage of WBCs (LY%, GR%, and MO%, respectively); LPS-stimulated IL-6 production (IL-6-LPS), glucocorticoid sensitivity of LPS-stimulated IL-6 production (IL-6-IC_{50}), PHA-stimulated cell proliferation (CP-PHA), and glucocorticoid sensitivity of PHA-stimulated cell proliferation (CP-IC_{50}).

To test for time (i.e., TSST) effects in healthy subjects, repeated measures analysis of variance (ANOVA) were calculated with within-group levels representing the four time-points of blood sampling. To test for effects of Addison's disease on immune parameters, repeated measures ANOVAs were calculated with two between-group levels representing the groups AD-NaCl and HS-NaCl ('disease'). Next, a second set of repeated measures ANOVAs were calculated to test for effects of HC-treatment in patients with Addison's disease with AD-HC and AD-NaCl representing the two between-group levels ('treatment'). Third, repeated measures ANOVAs were calculated including all three groups (AD-HC, AD-NaCl, and HS-NaCl).

For dose-response curves of LPS-stimulated IL-6 production and PHA-stimulated cell proliferation, repeated measures ANOVAs were computed with the two within-group factors time (four levels: TSST -1, +10, +60, and +120 minutes) and dexamethasone (six levels: 0, 10^{-9}, 10^{-8}, $5x10^{-8}$, 10^{-7}, and 10^{-6} M DEX) and the three groups as between-group factor.

For repeated measures ANOVAs, numbers of white blood cells and ODs of cell proliferation were transferred to percent change to baseline. All data were tested for normality prior to statistical analysis using the Kolmogorov-Smirnov test and Greenhouse-Geisser corrections for repeated measures were calculated where appropriate. Values of $p<0.05$ were considered significant.

4.3.4 Results

4.3.4.1 Stress Effects in Healthy Subjects

In a first set of analyses, effects of stress on immune parameter in healthy subjects were tested. In numbers of white blood cells, most pronounced changes (i.e., increases) were found 2 hours after TSST (see fig. 4-11). The percentage of lymphocytes (LY%, % of white blood cells) increased immediately after TSST (+1 min), but decreased below baseline during the subsequent two hours. The opposite picture was found for granulocytes (see fig. 4-11). The percentage of granulocytes (GR%) decreased after TSST, but increased over baseline levels during the following 2 hours. No changes could be observed in the percentage of monocytes (MO%).

Regarding LPS-stimulated IL-6 levels, healthy subjects showed decreases after TSST (+1 min) and increases over baseline levels thereafter, while the glucocorticoid sensitivity of LPS-stimulated IL-6 production did not change over time (see fig. 4-14 and 4-15). Since glucocorticoid sensitivity of LPS-stimulated IL-6 production was shown to be dependent on gender (Rohleder et al., 2001), the course of IC_{50} was tested for differences between male (n=5) and female (n=11) healthy subjects. No statistically significant effects of gender were found (group: $F_{1,14}=0.02$, p=.90; time: $F_{3,42}=2.32$, p=.09; group-by-time: $F_{3,42}=1.16$, p=.31).

Furthermore, no effects of TSST were found in PHA-stimulated cell proliferation or glucocorticoid sensitivity of PHA-stimulated cell proliferation (see fig. 4-16). Table 4-2 summarizes the statistics of all results regarding stress effects in healthy subjects.

Tab. 4-2: Results of repeated measures ANOVAs regarding effects of TSST on immune parameters in healthy subjects. Significant results (p<.05) are highlighted in gray.

Immune parameter	Abbreviation	ANOVA	
white blood cells (x10^3)	WBC	$F_{3,45}$=11.59,	p<.001
lymphocytes (% of white blood cells)	LY%	$F_{3,45}$=7.54,	p=.005
granulocytes (% of white blood cells)	GR%	$F_{3,45}$=6.99,	p=.007
monocytes (% of white blood cells)	MO%	$F_{3,45}$=0.20,	p=.87
LPS-stimulated IL-6 levels	IL-6-LPS	$F_{3,45}$=5.69,	p=.003
glucocorticoid sensitivity of LPS-stimulated IL-6 production	IL-6-IC_{50}	$F_{3,45}$=1.56,	p=.23
PHA-stimulated cell proliferation	CP-PHA	$F_{3,39}$=0.82,	p=.43
glucocorticoid sensitivity of PHA-stimulated cell proliferation	CP-IC_{50}	$F_{3,39}$=0.83,	p=.43

4.3.4.2 Baseline Differences (AD vs. HS)

All immune parameter were tested for baseline differences between healthy subjects and patients with Addison's disease. In none of these parameters, patients with Addison's disease differed significantly from healthy subjects (see table 4-3).

Tab. 4-3: Student's *t* tests for baseline group differences between patients with Addison's disease and healthy subjects in immune parameters.

Immune parameter	Abbreviation	Student's *t* tests	
white blood cells (x10^3)	WBC	t_{43}=1.10,	p=.28
lymphocytes (% of white blood cells)	LY%	t_{43}=-0.48,	p=.63
granulocytes (% of white blood cells)	GR%	t_{43}=0.60,	p=.55
monocytes (% of white blood cells)	MO%	t_{43}=-0.75,	p=.46
LPS-stimulated IL-6 levels	IL-6-LPS	t_{45}=0.83,	p=.41
glucocorticoid sensitivity of LPS-stimulated IL-6 production	IL-6-IC_{50}	t_{45}=-0.34,	p=.74
PHA-stimulated cell proliferation	CP-PHA	t_{41}=0.71,	p=.48
glucocorticoid sensitivity of PHA-stimulated cell proliferation	CP-IC_{50}	t_{41}=-0.72,	p=.47

4.3.4.3 Stress-Induced Changes in Blood Cell Counts

For blood cell counts, three different sets of repeated measures ANOVAs were computed. First, stress effects on immune blood cell counts were compared between patients and healthy subjects. Significant changes in white blood cells over time were found. Additionally, significant group-by-time effects indicated differences in the trajectories of white blood cells (see. fig. 4-11), percentage of lymphocytes and percentage of granulocytes. Thereby, patients with Addison's disease did not show the sharp increases in white blood cells from time-point +60 min to +120 min as healthy subjects. And contrary to healthy subjects, patients with Addison's disease showed increases in lymphocytes and decreases in granulocytes over time (see fig. 4-12). No significant group differences in percentages of monocytes were found (see column A in table 4-4).

Next, effects of treatment in patients with Addison's disease were investigated. While patients with Addison's disease showed significant changes over time in all four blood cell counts, hydrocortisone injection (AD-HC) did not result in different time courses compared to placebo (AD-NaCl; see column B in table 4-4). Repeated measures ANOVAs with three between-group levels reflected these results (see column C in table 4-4).

Tab. 4-4: Results of repeated measures ANOVAs for blood cell counts (WBC%: white blood cells in % change from baseline; LY%, GR%, and MO%: lymphocytes, granulocytes and monocytes in % of white blood cells, respectively) with four within-group levels representing the four time-points and two between-group levels testing for group (column **A**; AD-NaCl vs. HS-NaCl) and treatment effects (column **B**; AD-HC vs. AD-NaCl), as well as three between-group levels comparing changes over time in blood cell counts between all three groups (column **C**; AD-HC vs. AD-NaCl vs. HS-NaCl). Significant results ($p<.05$) are highlighted in gray, trends ($p<.10$) are indicated by a superscript plus sign ($^{+}$).

		A: DISEASE AD-NaCl (n=14) / HS-NaCl (n=16)	**B:** TREATMENT AD-HC (n=15) / AD-NaCl (n=14)	**C:** DISEASE x TREAT. AD-HC / AD-NaCl / HS-NaCl
WBC%	group:	$F_{1,28}=0.11$, $p=.75$	$F_{1,27}=0.14$, $p=.71$	$F_{2,42}=0.08$, $p=.92$
	time:	$F_{3,84}=11.74$, $p<.001$	$F_{3,81}=5.63$, $p=.008$	$F_{3,126}=13.54$, $p<.001$
	group x time:	$F_{3,84}=4.07$, $p=.026$	$F_{3,81}=0.47$, $p=.61$	$F_{6,126}=3.57$, $p=.012$
LY%	group:	$F_{1,28}=1.51$, $p=.23$	$F_{1,27}=2.49$, $p=.13$	$F_{2,42}=1.26$, $p=.30$
	time:	$F_{3,84}=1.72$, $p=.19$	$F_{3,81}=11.09$, $p<.001$	$F_{3,126}=4.92$, $p=.013$
	group x time:	$F_{3,84}=10.74$, $p<.001$	$F_{3,81}=1.03$, $p=.35$	$F_{6,126}=7.49$, $p<.001$
GR%	group:	$F_{1,28}=1.90$, $p=.18$	$F_{1,27}=2.74$, $p=.11$	$F_{2,42}=1.44$, $p=.25$
	time:	$F_{3,84}=2.14$, $p=.14$	$F_{3,81}=17.04$, $p<.001$	$F_{3,126}=7.01$, $p=.003$
	group x time:	$F_{3,84}=11.87$, $p<.001$	$F_{3,81}=1.19$, $p=.31$	$F_{6,126}=8.75$, $p<.001$
MO%	group:	$F_{1,28}=0.34$, $p=.57$	$F_{1,27}=0.44$, $p=.51$	$F_{2,42}=0.26$, $p=.77$
	time:	$F_{3,84}=1.60$, $p=.21$	$F_{3,81}=4.83$, $p=.008$	$F_{3,126}=3.37$, $p=.028$
	group x time:	$F_{3,84}=2.52$, $p=.08^{+}$	$F_{3,81}=0.19$, $p=.86$	$F_{6,126}=1.35$, $p=.25$

Figure 4-11 depicts the courses of white blood cells (in % change to baseline) for the three groups AD-HC, AD-NaCl, and HS-NaCl. Figure 4-12 shows the courses of percentages

of lymphocytes and granulocytes for placebo-treated patients with Addison's disease and healthy subjects.

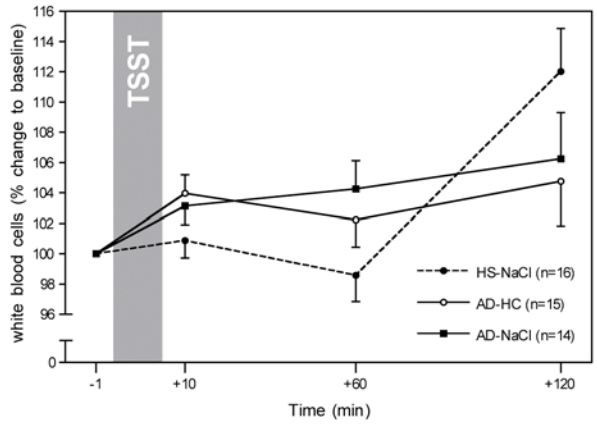

Fig.4-11: Effects of TSST, disease, and treatment on white blood cells counts (mean and standard errors (SE), given in % change to baseline).

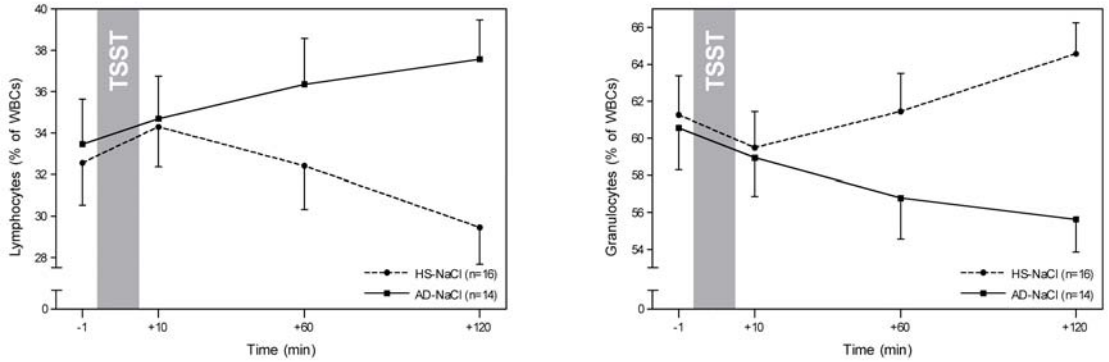

Fig. 4-12: Lymphocytes and granulocytes (in % of white blood cells; mean and SE) for placebo treated healthy subjects and patients with Addison's disease.

4.3.4.4 Stress-Induced Changes in LPS-Stimulated IL-6 Production and Glucocorticoid Sensitivity of LPS-Stimulated IL-6 Production

Dose-response-curves for LPS-stimulated IL-6 production were tested with repeated measures ANOVA and the three groups (AD-HC: n=16, AD-NaCl: n=15, and HS-NaCl: n=16) as between-group levels. Two within-group factors were included (time-points: TSST -1, +10, +60, and +60 minutes; dexamethasone: 0, 10^{-9}, 10^{-8}, $5x10^{-8}$, 10^{-7}, 10^{-6} M DEX) resulting in 24 within-group levels. All effects were highly significant (see table 4-5).

Tab. 4-5: LPS-stimulated IL-6 production – repeated measures ANOVA including groups AD-HC, AD-NaCl, and HS-NaCl as between-group levels and four time-points and six concentrations of dexamethasone as within-group levels. Significant results (p<.05) are highlighted in gray.

effect:	F-values	p-values	effect:	F-values	p-values
group	$F_{2,44}$=5.95	p=.005	group-by-time	$F_{6,132}$=6.85	p<.001
time	$F_{3,132}$=9.03	p<.001	group-by-DEX	$F_{10,220}$=4.25	p=.012
DEX	$F_{5,220}$=493.66	p<.001	time-by-DEX	$F_{15,660}$=12.69	p<.001

Figure 4-13 depicts the dose-response curves of each group broken down by four time-points. LPS-stimulated IL-6 levels without dexamethasone as well as glucocorticoid sensitivity can be seen to be influenced by stress, disease and hydrocortisone treatment.

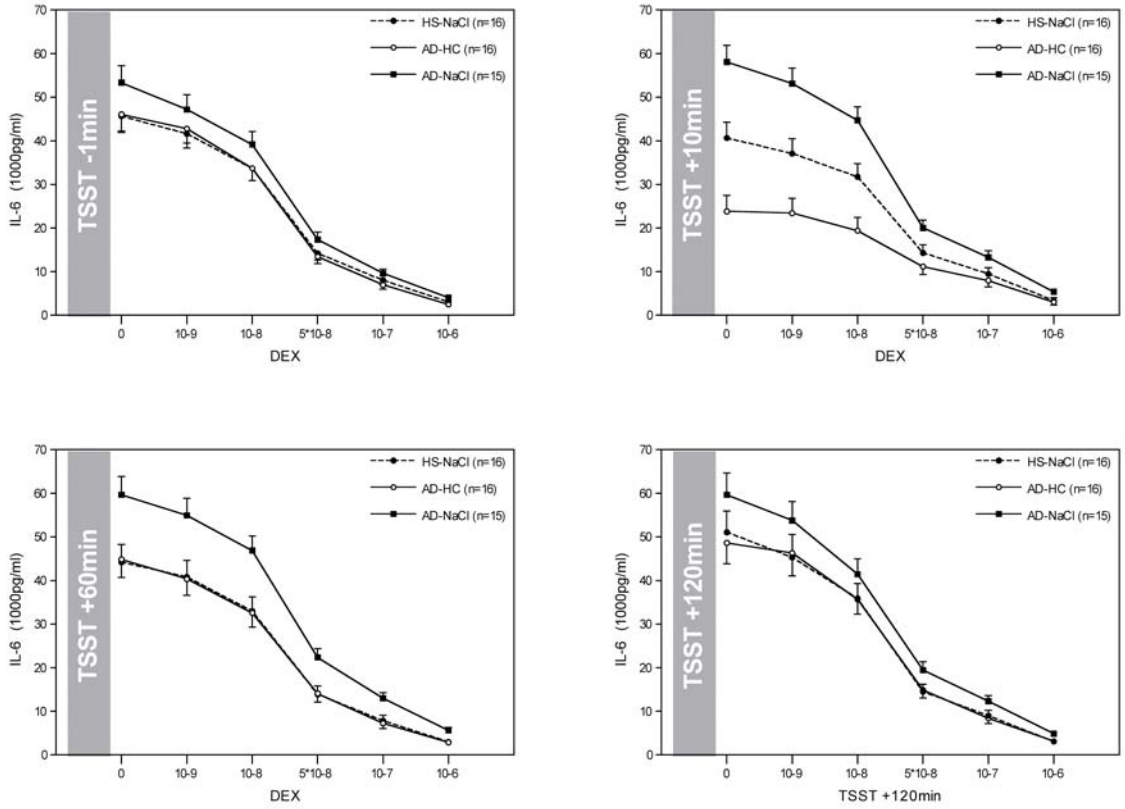

Fig. 4-13: Inhibition of LPS-stimulated IL-6 production (mean and SE) by increasing doses of dexamethasone for groups AD-HC, AD-NaCl, and HS-NaCl broken down by four time-points (TSST -1, +10, +60, and +120 minutes).

Because of the complexity of information contained in dose-response curves, they will be described in the following by two indices: LPS-stimulated IL-6 production without inhibition by dexamethasone (IL-6-LPS; indicated in figure 4-13 as 'DEX 0') as well as glucocorticoid sensitivity (IL-6-IC_{50}), representing the specific dexamethasone concentration required for 50% inhibition of LPS-stimulated IL-6 production without DEX.

As in blood cell counts, a three step approach was chosen to test the variables IL-6-LPS and IL-6-IC_{50} for effects of disease and hydrocortisone treatment. Patients with Addison's disease differed significantly in LPS-stimulated IL-6 levels showing higher levels than healthy subjects. No effects of disease on IC_{50} levels were found (see column A in table 4-6). Treatment with hydrocortisone in patients with Addison's disease resulted in significant group and group-by-time effects with pronounced decreases in LPS-stimulated IL-6 production and in a trend to group-by-time interaction with steep increases in IC_{50} levels 10 minutes after injection compared to placebo treated patients with Addison's disease (see column B in table 4-6). At time-points +60 and +120min, hydrocortisone treated patients showed LPS-stimulated IL-6 levels and IC_{50} levels comparable to healthy subjects (see figure

4-14 and 4-15). Repeated measures ANOVAs with three between-group levels reflected these results (see column C in table 4-6).

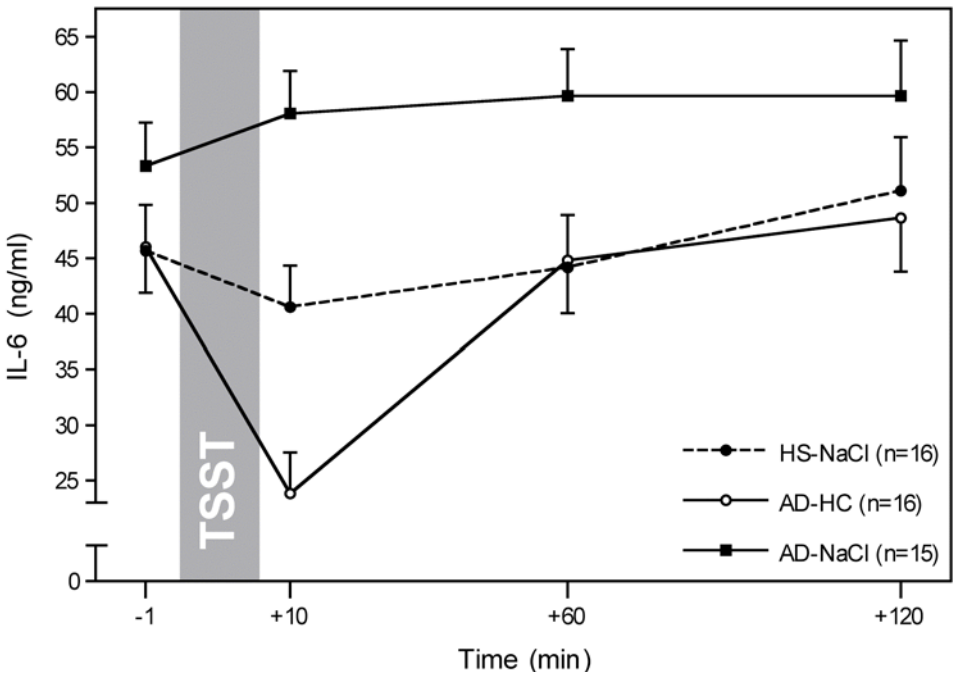

Fig. 4-14: Stress, group, and treatment effects on LPS-stimulated IL-6 production (mean and SE).

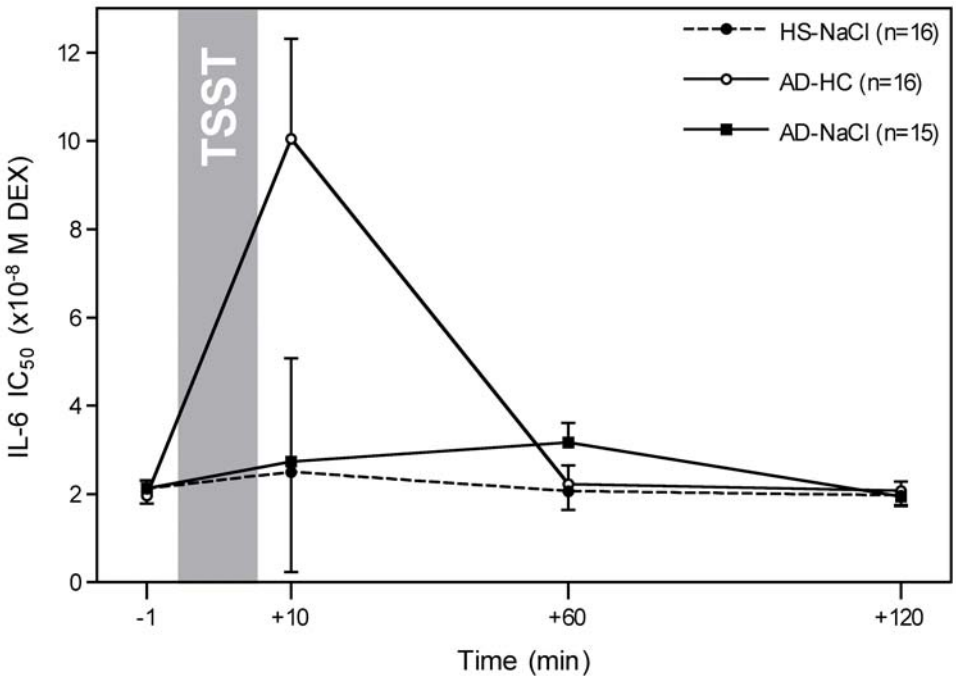

Fig. 4-15: Stress, group, and treatment effects on glucocorticoid sensitivity of LPS-stimulated IL-6 production (IC_{50}, in 10^{-8} M DEX; mean and SE). Glucocorticoid sensitivity is inversely related to IC_{50} levels.

Since LPS-stimulated IL-6 levels were pronouncedly decreased in hydrocortisone-treated patients at time-point +10 minutes, thereby affecting IC_{50} calculation, the set of repeated measures ANOVAs for IL-6-IC_{50} were computed again with IL-6-LPS levels at +10 minutes as covariate. Only significant time effects remained and formerly significant group-by-time interaction vanished (see table 4-6, third row).

Tab. 4-6: Results of repeated measures ANOVAs for LPS-stimulated IL-6 production and glucocorticoid sensitivity of LPS-stimulated IL-6 production (IL-6-IC_{50}) with four within-group levels representing the four time-points and two between-group levels testing for group (column **A**; AD-NaCl vs. HS-NaCl) and treatment effects (column **B**;AD-HC vs. AD-NaCl), as well as three between-group levels comparing changes over time between all three groups (column **C**; AD-HC vs. AD-NaCl vs. HS-NaCl). The last row (IL-6_c-IC_{50}) shows the respective results of an ANCOVA with LPS-stimulated IL-6 production at time-point +10 minutes as covariate. Significant results (p<.05) are highlighted in gray, trends (p<.10) are indicated by a superscript plus sign ($^+$).

		A: DISEASE AD-NaCl (n=15) / HS-NaCl (n=16)	**B:** TREATMENT AD-HC (n=16) / AD-NaCl (n=15)	**C:** DISEASE x TREAT. AD-HC / AD-NaCl / HS-NaCl
IL-6-LPS	group:	$F_{1,29}$=5.80, p=.023	$F_{1,29}$=10.19, p=.003	$F_{2,44}$=5.77, p=.006
	time:	$F_{3,87}$=3.23, p=.030	$F_{3,87}$=9.22, p<.001	$F_{3,132}$=13.42, p<.001
	group x time:	$F_{3,87}$=2.45, p=.074$^+$	$F_{3,87}$=9.76, p<.001	$F_{6,132}$=6.59, p<.001
IL-6-IC_{50}	group:	$F_{1,29}$=0.95, p=.34	$F_{1,29}$=2.31, p=.14	$F_{2,44}$=2.93, p=.064$^+$
	time:	$F_{3,87}$=2.42, p=.095$^+$	$F_{3,87}$=4.25, p=.046	$F_{3,132}$=4.79, p=.032
	group x time:	$F_{3,87}$=1.47, p=.24	$F_{3,87}$=3.59, p=.066$^+$	$F_{6,132}$=3.62, p=.032
IL-6_c-IC_{50}	group:	$F_{1,28}$=0.00, p=.96	$F_{1,28}$=0.00, p=.98	$F_{2,43}$=1.37, p=.27
	cov:	$F_{1,28}$=3.27, p=.081$^+$	$F_{1,28}$=1.61, p=.22	$F_{2,43}$=1.23, p=.27
	time:	$F_{3,84}$=0.21, p=.83	$F_{3,84}$=5.83, p=.021	$F_{3,129}$=6.19, p=.015
	group x time:	$F_{3,84}$=0.44, p=.66	$F_{3,84}$=0.02, p=.91	$F_{6,129}$=1.04, p=.36
	cov x time:	$F_{3,84}$=0.54, p=.60	$F_{3,84}$=3.30, p=.078$^+$	$F_{3,129}$=3.47, p=.067$^+$

4.3.4.5 Stress-Induced Changes in PHA-Stimulated Cell Proliferation and Glucocorticoid Sensitivity of PHA-Stimulated Cell Proliferation

In parallel to LPS-stimulated IL-6 production, dose-response-curves for PHA-stimulated cell proliferation were tested with repeated measures ANOVA. No significant effects of group and no significant interactions were found. Only stress (i.e., TSST -1, +10, +60, and +120 minutes) and treatment of cells *in vitro* with increasing doses of dexamethasone (0, 10^{-9}, 10^{-8}, $5x10^{-8}$, 10^{-7}, 10^{-6} M DEX) resulted in significant changes in PHA-stimulated cell proliferation (see table 4-7).

Tab. 4-7: PHA-stimulated cell proliferation – repeated measures ANOVA including groups AD-HC (n=15), AD-NaCl (n=14), and HS-NaCl (n=14) as between-group levels and four time-points and six concentrations of dexamethasone as within-group levels. Significant results (p<.05) are highlighted in gray.

effect:	F-values	p-values
group	$F_{2,40}$=0.35	p=.71
time	$F_{3,120}$=12.18	p<.001
DEX	$F_{5,200}$=325.91	p<.001
group-by-time	$F_{6,120}$=0.52	p=.74
group-by-DEX	$F_{10,200}$=0.54	p=.70
time-by-DEX	$F_{15,600}$=1.63	p=.20
group-by-time-by-DEX	$F_{30,600}$=1.43	p=.23

While TSST resulted in significant increases over time, no effects of disease or hydrocortisone treatment were found in PHA-stimulated cell proliferation and glucocorticoid sensitivity of PHA-stimulated cell proliferation (see table 4-8).

Tab. 4-8: Results of repeated measures ANOVAs for PHA-stimulated cell proliferation (in % change of baseline; CP-PHA) and glucocorticoid sensitivity of PHA-stimulated cell proliferation (CP-IC_{50}) with four within-group levels representing the four time-points and two between-group levels testing for group (column **A**; AD-NaCl vs. HS-NaCl) and treatment effects (column **B**;AD-HC vs. AD-NaCl), as well as three between-group levels comparing changes over time between all three groups (column **C**; AD-HC vs. AD-NaCl vs. HS-NaCl). Significant results (p<.05) are highlighted in gray.

		A: DISEASE AD-NaCl (n=14) / HS-NaCl (n=14)	**B:** TREATMENT AD-HC (n=15) / AD-NaCl (n=14)	**C:** DISEASE x TREAT. AD-HC / AD-NaCl / HS-NaCl
CP-PHA	group:	$F_{1,26}$=0.27, p=.61	$F_{1,27}$=0.00, p=.98	$F_{2,40}$=0.18, p=.84
	time:	$F_{3,78}$=3.28, p=.041	$F_{3,81}$=4.34, p=.009	$F_{3,120}$=4.76, p=.005
	group x time:	$F_{3,78}$=0.23, p=.82	$F_{3,81}$=0.17, p=.90	$F_{6,120}$=0.21, p=.96
CP-IC_{50}	group:	$F_{1,26}$=0.37, p=.55	$F_{1,27}$=0.32, p=.57	$F_{2,40}$=0.25, p=.78
	time:	$F_{3,78}$=3.23, p=.047	$F_{3,81}$=5.58, p=.011	$F_{3,120}$=5.81, p=.004
	group x time:	$F_{3,78}$=0.44, p=.65	$F_{3,81}$=0.48, p=.58	$F_{6,120}$=0.55, p=.71

Figure 4-16 depicts the courses of PHA-stimulated cell proliferation and glucocorticoid sensitivity of PHA-stimulated cell proliferation broken down by group and treatment (AD-HC vs. AD-NaCl vs. HS-NaCl).

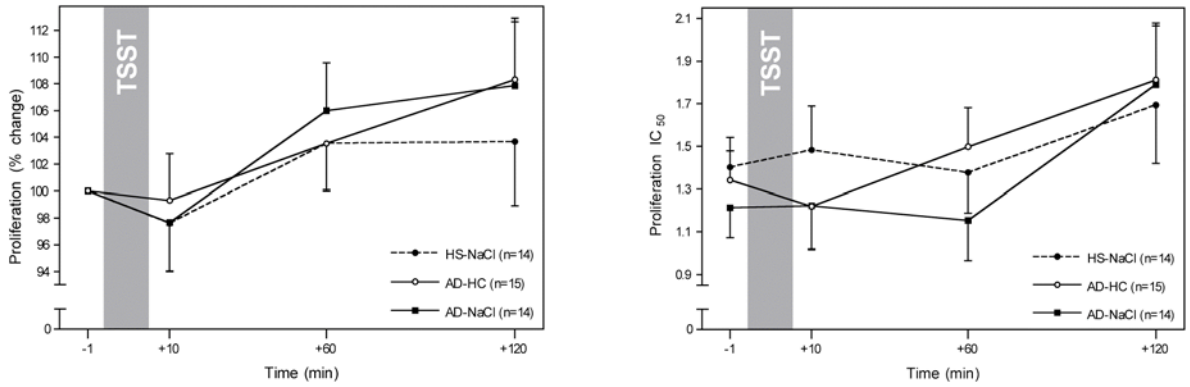

Fig. 4-16: Stress, group, and treatment effects on PHA-stimulated proliferation and IC_{50} levels (*10^{-8}M DEX).

4.3.4.6 Summary of Results

In healthy subjects, stress resulted in significant changes in blood cell counts and LPS-stimulated IL-6 production. No baseline differences in immune parameters were found between patients with Addison's disease and healthy subjects. Stress in patients with Addison's disease resulted in courses of blood cell counts and LPS-stimulated IL-6 production significantly different from healthy subjects. Treatment with hydrocortisone only influenced the course of LPS-stimulated IL-6 production. No influences of disease or hydrocortisone treatment were found in cell proliferation parameters.

4.3.5 Discussion

In the present study, stress responses in three different immune parameters were tested and compared between patients with Addison's disease and healthy subjects. Furthermore, treatment of patients with hydrocortisone i.v. were tested for its ability to restore

possibly changed immune stress responses. In the following, results will be first discussed separately for each of the three immune parameters.

4.3.5.1 Blood Cell Counts

Stress resulted in significant changes in numbers of white blood cells over time. Patients with Addison's disease showed slow increases in numbers of white blood cells, while healthy subjects showed most pronounced increases between one and two hours after stress. Although numbers or percentages of specific subsets of peripheral blood mononuclear cells not necessarily correlate with function of the respective cell (Anesi et al., 1994), blood cell counts provide information about immune system activation, as decreased numbers of cells in peripheral blood point to immune activation due to trafficking of these cells to inflammatory sites (Dhabhar, 2000). To determine which subsets of cells may have caused observed changes in white blood cells, percentages of lymphocytes, granulocytes, and monocytes were measured. While healthy subjects showed an increase in percentage of lymphocytes in response to stress, percentage of lymphocytes decreased below baseline in the following two hours. The opposite trajectory was found in granulocytes. Here, percentages decreased in an acute response to stress to subsequently increase over baseline levels thereafter. No changes in percentage of monocytes were found. These findings reflect patterns found by others, indicating catecholamines being the primary mediator of acute stress-induced changes in numbers of cell subtypes, while glucocorticoids seem to play a role at later time-points. After catecholamine administration, typically two phases are recognized (for review see Benschop et al., 1996): A quick (<30 minutes) mobilization of lymphocytes, followed by an increase in granulocyte numbers with decreasing lymphocyte numbers. Thereby, changes in lymphocytes seem to be mainly mediated via activation of β_2-adrenoceptors, whereas granulocytes increases involve α-adrenoceptor stimulation. Regarding later time-points, studies by Dhabhar et al. showed glucocorticoids to play a role in distribution of white blood cells (Dhabhar et al., 1996). The authors observed significantly increased lymphocyte numbers in adrenalectomized rats two hours after being stressed by a vehicle injection, while intact animals showed significant decreases. Injection of corticosterone in adrenalectomized rats resulted in significantly decreased lymphocyte numbers two hours later. This redistribution of cells is probably due largely to alterations in cell adhesion molecules (Sapolsky et al., 2000), since glucocorticoids can regulate the expression of cell adhesion molecules in lymphocytes, thereby altering cell trafficking and hence cell infiltration at sites of inflammation (Cronstein et al., 1992; for review see Pitzalis et al., 2002).

As may be expected from these findings, patients with Addison's disease showed early stress responses in lymphocytes and granulocytes comparable to healthy controls,

indicating norepinephrine being able to compensate missing epinephrine responses. But at one and two hours after stress, differences in trajectories were found between patients and healthy subjects, indicating glucocorticoids to play a role at these later time-points. These later trajectories seem to be a prolongation or missing termination of earlier events, in that lymphocytes continued increasing and granulocytes continued decreasing. Nevertheless, hydrocortisone treatment of patients did not restore these patterns, as would have been suggested. This may at least in part be due to the dose of 0.03mg/kg being injected as a bolus. This treatment resulted in salivary free cortisol levels being comparable between patients and healthy controls immediately after stress, but patients showed faster decreases in cortisol levels thereafter. Thus, cortisol effects may have been terminated earlier in patients than in healthy subjects. Furthermore, patients are treated with glucocorticoid replacement two to three times a day with mean doses of 26.77mg hydrocortisone and 39.58mg cortisone acetate per day. It may therefore be hypothesized that this treatment alters the sensitivity of lymphocytes to glucocorticoid regulation of cell adhesion molecule expression. Thus, as in untreated patients, lymphocytes are recruited from the marginal pool and the spleen by catecholamines (Benschop et al., 1996), but do not exit peripheral blood due to missing glucocorticoid induction of cell adhesion molecules.

In healthy subjects, changes in blood leukocyte distribution are interpreted as migration of specific leukocytes to the “battle stations” and “communication stations” (Dhabhar et al., 1996), i.e., immune compartments like lymph nodes, lung, skin, and mucosa, where they are important for the ability of the immune system to perform its surveillance and effector functions. In turn, missing lymphocyte redistribution to other immune compartments, as found in patients with Addison’s disease, may attenuate the ability of the immune system to respond to infections and thus represent an enhanced health risk.

4.3.5.2 LPS-Stimulated IL-6 Production

Cells of innate immunity recognize pathogen-associated molecular patterns (PAMPs; Medzhitov and Janeway, 1997a; Medzhitov and Janeway, 1997b; Uthaisangsook et al., 2002), which are shared by large groups of pathogens, as for example lipopolysaccharide (LPS), a component of the bacterial cell wall. PAMPs in turn are recognized by a set of molecules as well as receptors referred to as pattern-recognition molecules (PRMs) and pattern-recognition receptors (PRRs), which are germline encoded, i.e., they arise over evolutionary time due to selection by pathogens at the populational levels (Medzhitov and Janeway, 1997a). Binding of PAMPs to PRRs or PRMs is the first step in activating functions of innate immunity. Based on these mechanisms, stimulation of immune cells *in vitro* by LPS measures their ability to produce cytokines, if challenged by an infection, and thereby informs about “what could be”. In this regard, LPS stimulation of whole blood appears to reproduce the natural environment better than stimulation of PBMCs (De Groote et al., 1992).

The cytokine IL-6 is one of the major pro-inflammatory cytokines and has a wide range of important functions in the immune system. It is produced and released by a variety of cells, including lymphoid cells, endothelial cells, and fibroblasts (Heinrich et al., 1990). IL-6 is known to regulate B and T-cell function, it promotes proliferation and differentiation of haematopoietic stem cells (Akira et al., 1990), and has pyrogenic activity (Kluger, 1991). In addition, during the acute phase response, IL-6 is thought to be the main regulator of the production and release of acute phase proteins by the liver (Heinrich et al., 1990). Consequently, stimulation of monocytes and macrophages by LPS has repeatedly been shown to increase IL-6 production (Gauldie et al., 1987; May et al., 1988; Northoff et al., 1987; Ritchie and Fuller, 1983).

Contrary to LPS, one of the most well-established and important effects of glucocorticoids is the inhibition of cytokine production (reviewed in (Wiegers and Reul, 1998). This also includes the production of IL-6 and its effects on target tissues (Chrousos, 1995). One exception in this regards is the effect of IL-6 on the production of acute-phase reactants by the liver, which is potentiated by glucocorticoids (Boumpas et al., 1993; Hirano et al., 1990). Furthermore, glucocorticoids (Rohleder et al., 2003a) and norepinephrine (van der Poll et al., 1994) have repeatedly been shown to suppress IL-6 production e*x vivo* in LPS-stimulated whole blood in a dose-dependent manner. Acute stress in turn has been shown to increase (Gaab et al., 2005) and decrease LPS-stimulated IL-6 production (Rohleder et al., 2003a). This contradictory results may in part be due to the sexual hormone status of subjects investigated, as Rohleder et al. found IL-6 production to decrease in response to acute stress in men (Rohleder et al., 2001) and in women in the luteal phase, while women taking oral contraceptives responded to acute stress with increased IL-6 production (Rohleder et al., 2003b).

Nevertheless, no differences between healthy men and women were found in the present study in the trajectories of LPS-stimulated IL-6 production[6]. Healthy subjects showed a fast response in LPS-stimulated IL-6 production, in that levels decreased immediately after stress. During the subsequent two hours, stimulated IL-6 levels slowly increased and reached levels above baseline at two hours after TSST. This trajectory closely reflected the course of salivary free cortisol levels (see section 4.2): Ten minutes after TSST, cortisol levels reached their maximum and decreased thereafter, with levels close to baseline levels 60 minutes after TSST and levels below baseline 120 minutes after TSST[7]. Consequently, patients with Addison's disease – characterized by a missing cortisol stress response –

[6] 11 females, 5 males; gender: $F_{1,14}$=0.12, p=.73; time: $F_{3,42}$=3.99, p=.014; gender-by-time: $F_{3,42}$=0.35, p=.76.

[7] AD-HC: n=15, HS-NaCl: n=16; salivary free cortisol levels at +10min correlated negatively with LPS-stimulated IL-6 production at +10min (p=.08) and at +60min (p=.097). Data not shown.

showed increases in LPS-stimulated IL-6 levels in response to stress, with little variations in the following two hours. It may be hypothesized that the stress-induced increase in cortisol in healthy subjects is associated with a short-time suppressed immune response to challenge and that such a response is physiologically advantageous, as during stress no energy is wasted on immune system activation. In patients with Addison's disease, stress immediately leads to enhanced immune responsiveness, since no cortisol is secreted. Hence, the immune activation is not protracted to times after stress termination.

Treatment of patients with hydrocortisone in turn resulted in pronouncedly decreased IL-6 production ten minutes after TSST with a return to baseline 1 hour after TSST. These findings clearly point to a suppressive action of glucocorticoids in immune responses to infections. This action seems to be restricted to stress, since no baseline differences in LPS-stimulated IL-6 production could be found between healthy subjects and patients with Addison's disease. From a patients' point-of-view the questions arises, which effects on the immune system have to be expected in regard to intake of glucocorticoid replacement two to three times a day. The pronounced effect of hydrocortisone treatment on LPS-stimulated IL-6 production in this study does not point to a decreased sensitivity of the immune system to glucocorticoid signaling, as may have been expected as an effect of the high doses of glucocorticoid replacement.

In this regard, we also assessed the sensitivity of LPS-stimulated IL-6 production to inhibitory glucocorticoid signals, in that we added dexamethasone in increasing doses to the whole blood cultures. The amount of dexamethasone needed to suppress LPS-stimulation IL-6 production to 50% (IC_{50}) was used as an index of glucocorticoid sensitivity, with higher dexamethasone doses/higher IC_{50} indicating lower sensitivity. The effects of psychosocial stress on glucocorticoid sensitivity of LPS-stimulated IL-6 production have so far been investigated in just a few studies. For example, Miller et al. (Miller et al., 2002) found parents of children suffering from cancer showing a decreased GC sensitivity of LPS-stimulated IL-6 production in whole blood compared to parents of medically healthy children. In regard to acute psychosocial stress, our laboratory did a series of studies and observed glucocorticoid sensitivity to decrease in women and older men, to increase in younger men and women using oral contraceptives, and to not change in women being in the luteal phase of their menstrual cycle (Rohleder et al., 2003a).

Interestingly, independent of gender[8], no differences between healthy subjects and patients with Addison's disease in courses of IC_{50}s were found. Only hydrocortisone treatment resulted in pronounced decreases in glucocorticoid sensitivity, but this effect

[8] AD-NaCl: 10 females, 5 males; HS-NaCl: 11 females, 5 males. Gender: $F_{1,27}$=0.33, p=.57; group: $F_{1,27}$=0.55, p=.47; gender-by-group: $F_{1,27}$=0.20, p=.66; time: $F_{3,81}$=3.01, p=.055; gender-by-time: $F_{3,81}$=2.12, p=.13; group-by-time: $F_{3,81}$=1.18, p=.32; group-by-gender-by-time: $F_{3,81}$=0.29, p=.76.

appeared to be due to LPS-stimulated IL-6 levels being pronouncedly decreased after hydrocortisone-treatment, thereby affecting IC_{50} calculation. Controlling for this LPS-stimulated IL-6 production revealed patients with Addison's disease and healthy subjects to not differ in their glucocorticoid sensitivity trajectories. Patients thereby show this regular responses despite a missing cortisol stress response and taking glucocorticoid replacement in high doses several times per day (see section 4.1).

In summary, a missing cortisol stress response in patients with Addison's disease is associated with increased responsiveness to an infectious stimulus, accompanied by unchanged glucocorticoid sensitivity of IL-6 producing cells. Treatment with hydrocortisone revealed this effect being due to missing cortisol in patients with Addison's disease. If glucocorticoids are important for protection of the organism against an over-reactive immune system, these findings on the one hand point to patients being prone to autoimmune/inflammatory diseases. In parallel to this interpretation, individuals with Addison's disease in fact have been found to be prone to diseases like bronchial asthma (Green and Lim, 1971) and various allergies (Carryer et al., 1960). Surprisingly, patients further responded very sensitive to inhibitory glucocorticoid signals, despite being treated with glucocorticoid replacement in supra-physiological concentrations several times a day over years (see section 4.1). This still existing and pronounced sensitivity to glucocorticoid signaling therefore points on the other hand to an increased risk for infections two to three times a day subsequently to the intake of replacement medication.

4.3.5.3 PHA-Stimulated Cell Proliferation

One of the most common measures of cell-mediated immunity is the nonspecific mitogen-induced lymphocyte proliferation. Therefore, to also receive information on effectiveness of cellular immune responses, especially of T-cells, in the present study PHA-stimulated cell proliferation as well as glucocorticoid sensitivity of cell proliferation were measured.

Despite PHA-stimulated proliferation being a common measure of cell-mediated immunity, research results in this regard are rather contradictory. For example, forced swim tests were found to suppress PHA-stimulated proliferation in peripheral T-cells (Connor et al., 1997) as well as in splenocytes (Ferry et al., 1991) in rats. Contrary, a social stressor was found to facilitate proliferation in rats (Bohus et al., 1993). Also five weeks of isolation and water scheduling enhanced proliferation in rats (Jessop et al., 1987). In humans, reduction in proliferation were found in response to a Stroop test (Bachen et al., 1992). No changes in proliferation were observed in response to a difficult puzzle task (Brosschot et al., 1992) or in response to an electric shock with loud white noise (Weisse et al., 1990). In juvenile squirrel monkeys, 3 hours of separation resulted in suppressed proliferation two days later (Friedman et al., 1991). In these studies not only different types and durations of stressors, but also

different tissues and different PHA concentrations were employed. Therefore, no general conclusion can be drawn on the effects of stress on PHA-stimulated cell proliferation.

A likewise inconsistent picture can be observed in regard to glucocorticoid sensitivity of PHA-stimulated cell proliferation. Only a few studies measured sensitivity and found acute stress to not induce any significant changes in glucocorticoid sensitivity of PHA-stimulated splenocyte and blood lymphocyte proliferation in rats (Bauer et al., 2001), while glucocorticoid sensitivity of PHA-stimulated PBMC proliferation significantly decreased on days with academic examination stress (Sauer et al., 1995). Contrary, our laboratory found acute stress in humans to induce increased glucocorticoid sensitivity (Rohleder et al., 2005). Again, conclusions regarding stress-induced changes in PHA-stimulated cell proliferation are hampered by the investigation of different species, tissues and stressors.

In the present study, only significant time effects were found, indicating stress to result in increases in PHA-stimulated cell proliferation and decreases in glucocorticoid sensitivity. The latter was most pronounced two hours after stress. These findings are in line with data from Rohleder et al. (Rohleder et al., 2005) presented above. This consensus may be explained by type and duration of stressor (i.e., TSST) as well as concentrations of PHA and dexamethasone being identical in both studies. Interestingly, no differences between patients with Addison's disease and healthy subjects and no effect of hydrocortisone treatment in patients were found. These findings lead to the same conclusion as drawn by Keller et al. (Keller et al., 1983). Keller et al. investigated stress-induced changes in lymphocyte stimulation by phytohemagglutinin in isolated lymphocytes and in cultures of whole blood from adrenalectomized rats. In parallel to the missing effects of disease and hydrocortisone treatment, Keller concluded from his data that corticosteroid independent mechanisms participate in stress effects on lymphocyte function.

Taken together, neither PHA-stimulated cell proliferation nor glucocorticoid sensitivity of stimulated T-cells are changed in patients missing a cortisol stress response. Furthermore, both parameters are not sensitive to hydrocortisone treatment. These findings clearly point to stress-induced changes in cell-mediated immunity being mediated by other mechanisms than glucocorticoid signaling.

4.3.5.4 Conclusion and Outlook

Investigation of patients with Addison's disease proved to be a valuable alternative approach to verify in humans stress effects on the immune system found in animal research or *in vitro* approaches. In this regard, three commonly used parameters were investigated: Percentages of leukocyte subtypes in peripheral blood, stimulated IL-6 production and T-cell proliferation. Additionally, sensitivities of the latter two parameters to glucocorticoid signaling were measured. As expected from literature, glucocorticoids seem to play an important role

in leukocyte migration and stimulated cytokine production, while T-cell proliferation appeared to be glucocorticoid-independent.

While treatment of patients with 0.03mg/kg hydrocortisone did restore the early pattern of stimulated IL-6 trajectories, no effects were found in leukocyte trafficking. This may be explained by treatment lasting too shortly for measurably affecting leukocyte trafficking, at least at the time-points used in the present study. Contrary, in LPS-stimulated IL-6 production, incubation of whole blood for 18 hours allowed hydrocortisone treatment to exert its full effects.

From a patient's point-of-view, the findings of the present study may have several implications. The distinctive features in blood cell counts may point to an increased health risk, as the ability of the immune system to respond to infections is attenuated in response to stress. On the other hand, stress responses in cytokine production hint to a hyper-reactive innate immune response due to missing suppressive cortisol signals, which in turn points to patients having an increased risk for autoimmune diseases. This situation may even be exacerbated by the conserved sensitivity to glucocorticoid signaling, seen in the pronounced responses to hydrocortisone treatment in stimulated IL-6 production – especially since patients receive glucocorticoid replacement several times a day, resulting in morning salivary free cortisol levels being up to six fold the levels seen in healthy subjects (see section 4.1).

The findings of the present study should be extended in future studies investigating the effects of missing endocrine stress responses on other parameters of innate and adaptive immunity in these patients, as for example on natural killer cells or T-helper cell 1/T-helper cell 2 balance. Treatment of patients with epinephrine in comparison to or in combination with hydrocortisone may further help to reveal the immune regulatory role of stress mediators in humans. Additional insights into these interactions may be gained by investigating the effects of the daily glucocorticoid replacement regimen on immune functions.

4.4 ADDISON'S DISEASE: PSYCHONEUROIMMUNOLOGY AND THE MEDIATIVE ROLE OF NF-κB

Stress and Addison's Disease: NF-κB as a Mediator of Stress Effects on Immune System?

4.4.1 Abstract

Background: The nuclear transcription factor kappaB (NF-κB) is central to immune system activation. Glucocorticoids in turn are known to suppress most immune functions, an effect thought to be mainly mediated by inhibiting the NF-κB activation cascade. We previously showed psychosocial stress to be associated with increased NF-κB activity in humans. This effect was inducible *in vitro* by norepinephrine. To gain deeper insights into the intracellular processes in humans in regard to stress, we investigated endocrine stress responses in patients with Addison's disease and their association with changes in NF-κB activity.

Methods: 21 patients with Addison's disease and 11 healthy subjects were exposed to a psychosocial stress test. Blood and saliva samples were collected immediately before and repeatedly after stress for analyzing cortisol and catecholamine levels as well as NF-κB-DNA binding activity. Half of the patients were treated with hydrocortisone subsequent to the stress test to mimic normal cortisol stress responses.

Results: In healthy subjects, stress induced significant increases in cortisol and catecholamines. Patients with Addison's disease showed stress-induced increases in norepinephrine only, while cortisol and epinephrine levels were very low and did not vary in response to stress. No significant changes in NF-κB activity were found over the course of investigation, but in healthy subjects, cortisol levels one minute after stress predicted NF-κB decrease from pre-stress to 10 minutes post-stress, while in patients with Addison's disease, this NF-κB decrease was predicted by the decrease in norepinephrine to baseline levels after stress. None of the investigated parameters was found to predict NF-κB variation in hydrocortisone treated patients.

Conclusion: The present study shows for the first time that stress-induced glucocorticoid concentrations inhibit NF-κB activity in humans. Furthermore, findings in patients with Addison's disease suggests that this immune-suppressive action of glucocorticoids is accomplishable also by norepinephrine, if glucocorticoid stress responses are missing in the long term. These results once more emphasize the need for studying complex and interrelated processes also in human whole organism to allow for uncovering compensatory mechanisms.

4.4.2 Introduction

The innate immune response to challenge involves secretion of cytokines and other mediators leading to an inflammatory process as an immediate host defense reaction. The basis of this fast response are phylogenetically conserved signaling mechanisms with cells of innate immunity recognizing pathogen-associated molecular patterns (PAMPs) shared by large groups of pathogens (Medzhitov and Janeway, 1997a; Medzhitov and Janeway, 1997b; Uthaisangsook et al., 2002). Binding of PAMPs to a set of molecules as well as receptors referred to as pattern-recognition molecules (PRMs) and pattern-recognition receptors (PRRs) is the first step in activating functions of innate immunity (Medzhitov and Janeway, 1997a). Centrally to this activation and thus to first-line defense is the nuclear transcription factor kappaB (NF-κB).

NF-κB family members contain a conserved DNA binding and dimerization domain called the Rel homology domain. The mammalian NF-κB/Rel family of proteins consists presently of five members, namely, Rel (c-Rel), p65 (Rel A), Rel B, p50 (NFKB1; precursor: p105), and p52 (NFKB2; precursor: p100). The members p50 and p52 lack transcriptional activation domains and their homodimers are thought to act as repressors. In contrast, p65, Rel B, and c-Rel carry transcriptional activation domains, and with the exception of Rel B, they are able to form homo- and heterodimers with the other members of the protein family. In general, the designation NF-κB refers to the most frequently occurring and ubiquitously expressed heterodimeric complex between the p50 and p65 subunits (Caamano and Hunter, 2002; Wulczyn et al., 1996).

4.4.2.1 NF-κB is Central to Immune System Activation

NF-κB is rapidly activated by a large spectrum of chemically diverse agents and cellular stress conditions, including the component of the cell wall of gram-negative bacteria, namely lipopolysaccharide (LPS; Kitchens, 2000; Uthaisangsook et al., 2002), microbial and viral pathogens, cytokines and growth factors (Baeuerle and Baltimore, 1996). Once activated, NF-κB transcriptionally regulates a wide variety of important immune-related genes by binding to DNA. Among these are growth factors, such as granulocyte/macrophage colony stimulating factor (GM-CSF), pro-inflammatory cytokines, especially IL-1, IL-2, IL-6, IL-8, and TNF-α, cell adhesion molecules, such as inter-cellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM), acute phase proteins (hepatocytes), for example serum amyloid A precursor protein and complement factors B and C4, but also transcriptional regulators including the inhibitory protein IκB-α (McKay and Cidlowski, 1999). In addition to its prominent role in innate immunity, NF-κB exerts important functions in the adaptive immune system, such as lymphocyte activation by controlling proliferation,

immunoglobulin isotype switching and expression of cytokines and their receptors (Attar et al., 1997; Gerondakis et al., 1998). Furthermore, NF-κB serves to protect cells against apoptosis and supports cell cycle progression (Baichwal and Baeuerle, 1997; Foo and Nolan, 1999).

The central paradigm of NF-κB activation involves the removal of inhibitory protein IκB, which forms a cytoplasmic complex with NF-κB, thus retaining NF-κB inactive (Baeuerle and Baltimore, 1988a; Baeuerle and Baltimore, 1988b). The major pathway used by a wide variety of stimuli to activate NF-κB involves the phosphorylation of IκB-α at its regulatory N-terminus on serines 32 and 36 by the cytokine-responsive IκB kinases IKKα and β, an event leading to subsequent conjugation with ubiquitin and proteasome-mediated degradation of the inhibitor (Baldwin, 1996; Verma et al., 1995). As already mentioned, NF-κB itself transcriptionally regulates IκB synthesis. Thus, new synthesis of IκB-α, causing retention of NF-κB in the cytoplasm and attenuation of NF-κB-mediated transcriptional activation, provides a feedback mechanism for modulating the extent and duration of inflammatory responses by a cell (Baldwin, 1996).

4.4.2.2 Glucocorticoid Effects are Mediated by Inhibition of NF-κB Activity

Interestingly, NF-κB and the glucocorticoid receptor appear to exert antagonistic effects regarding the regulation of most immune-related genes. While NF-κB up-regulates most genes, the glucocorticoid receptor is known to repress cytokine genes, for example IL-1, IL-2, IL-6, IL-8, and TNF-α genes, as well as cell adhesion molecule genes, such as ICAM-1 (reviewed in (McKay and Cidlowski, 1999).

However, the glucocorticoid signaling pathway becomes activated by glucocorticoids (GCs) crossing the cell membrane and interacting with the intracellular GR. This ligand binding induces conformational changes in the GR molecule (Bamberger et al., 1996). The GR-GC complex dissociates from a heat shock protein (HSP) complex (Hutchison et al., 1994), the partially phosphorylated receptor protein becomes hyper-phosphorylated (Bodwell et al., 1993), and now unmasked nuclear localization signals (NLS) within the ligand-binding domain of the receptor (Picard and Yamamoto, 1987) cause nuclear translocation of the GR-GC complex (Akner et al., 1994). Within the nucleus, the hormone-activated glucocorticoid receptor can act in two ways, referred to as classic and alternative mechanism of glucocorticoid receptor action.

The classic mechanism of GR action is characterized by GR-binding to specific DNA sequences termed glucocorticoid response elements (GREs), thereby interacting with components of the basic transcription machinery and eventually enhance transcription of glucocorticoid-responsive genes. Furthermore, in some promoters, binding of the activated GR to so-called negative glucocorticoid response elements (nGREs) causes inhibition rather

than enhancement of transcription (Bamberger et al., 1996; McKay and Cidlowski, 1999). Interestingly, the promoters of most genes, which should be repressed by GRs according to the well-known anti-inflammatory actions of glucocorticoids, do not contain nGREs nor do they have any other GR-binding site (Bamberger et al., 1996; Cato and Wade, 1996). But they do carry sites for NF-κB. It has therefore been proposed that immune suppression is mediated by glucocorticoid-induced expression of the inhibitory protein IκB-α, a hypothesis which was confirmed by studies showing that glucocorticoids in fact induce the transcription of the gene encoding IκB-α (Auphan et al., 1995; Scheinman et al., 1995a). The increase in protein synthesis leads to the rapid turnover of IκB-α protein associated with pre-existing NF-κB complexes and in the presence of an activator, such as LPS, newly released NF-κB re-associates with the glucocorticoid induced IκB-α, thus reducing the amount of NF-κB translocating to the nucleus. Additionally, newly snythesized IκB-α may enter the nucleus and inhibit NF-κB-DNA binding. Both pathways eventually down-regulate NF-κB associated gene products (Scheinman et al., 1995a).

The alternative mechanism of glucocorticoid receptor action involves physical interaction of the GR with other transcription factors, such as NF-κB. Accumulating evidence exists that the activated GR specifically interferes with the trans-activation potential of the NF-κB p65 subunit (De Bosscher et al., 1997; De Bosscher et al., 2000b; Ray and Prefontaine, 1994). Another important physical interaction occurs between the GR and AP-1, resulting in reciprocal repression of AP-1 and GR transactivation functions (Hsu et al., 1993; Jonat et al., 1990; Kerppola et al., 1993; Konig et al., 1992; Schule et al., 1990; Yang-Yen et al., 1990). As NF-κB and AP-1 can synergize in the pro-inflammatory pathway, GR repression of AP-1 may be an additional and important indirect mechanism for glucocorticoid suppression of NF-κB-mediated immune responses (McKay and Cidlowski, 1999).

In summary, it appears that the main mechanism by which glucocorticoids exert their anti-inflammatory effects is by inhibition of the NF-κB activation cascade (McKay and Cidlowski, 1999), while the inhibition of Th1 cytokines by glucocorticoids is essentially explained by the action of AP-1 (Refojo et al., 2001). However, it is important to consider that the interaction between NF-κB and GR is one of mutual antagonism: NF-κB represses DNA-binding activity of the GR to the same extent as GRs repress NF-κB activity (Caldenhoven et al., 1995; McKay and Cidlowski, 1998; Ray and Prefontaine, 1994; Scheinman et al., 1995a). Furthermore, NF-κB may repress GR activity by increasing the expression of the non-functional receptor isoform GRβ, thus impairing GR transactivation and eventually causing glucocorticoid resistance (Webster et al., 2001).

4.4.2.3 Catecholamines Interfere with NF-κB Activity

In parallel to glucocorticoids, several lines of evidence exist of catecholamine signal transduction pathways interfering with activity of transcription factors, such as NF-κB and AP-1. Basically, norepinephrine and epinephrine transduce their biological information through stimulation of adrenoceptors, which directly activate G-proteins. Basically, coupling of β-adrenoceptors to G_s protein activates adenylate cyclase (AC), which in turn increases intracellular cAMP. Contrary, coupling of α1-adrenoceptors to G_i proteins inhibits AC and thus subsequently the formation of cAMP from ATP. The α1-adrenoceptor coupling to G_q proteins activates phospholipase C (PLC), which increases inositol triphosphate (IP_3) and diacylglycerol (DAG). Once these second messengers are generated, cAMP activates protein kinase A (PKA), DAG activates protein kinase C (PKC), and IP_3 mobilizes Ca^{2+} from intracellular stores. The latter is further linked to the Ca^{2+}/calmodulin (Ca^{2+}/CaM) pathway, which – like PKA – subsequently transfers signals to the nucleus (Elenkov et al., 2000). For example, activation of β2-adrenoceptors on immune cells has been shown to result in inhibition of TNF-α and IL-12 (Elenkov et al., 1996; Severn et al., 1992; van der Poll et al., 1994) and an increase of IL-10 production (Elenkov et al., 1996; Siegmund et al., 1998; van der Poll et al., 1996; Van der Poll and Lowry, 1997).

All three pathways, i.e. the cAMP/PKA, the PKC, and the Ca^{2+}/CaM pathway, modulate transcription factor activity (Haraguchi et al., 1995). Regarding NF-κB, elevated levels of cAMP are generally known to inhibit its activation, for example by cAMP inhibiting the binding of NF-κB to the NF-κB-DNA site (Chen and Rothenberg, 1994; Neumann et al., 1995; Tsuruta et al., 1995). Furthermore, the cAMP/PKA pathway induces impaired nuclear translocation and DNA binding of p65, probably due to a retarded degradation of IκB-α (Neumann et al., 1995; Paliogianni et al., 1993). Alternatively, this pathway may also inhibit NF-κB transcription by phosphorylating the transcription factor CREB, which then competes with p65 for limited amounts of CREB binding protein (CBP; Parry and Mackman, 1997). Contrary, NF-κB activity can also be stimulated via the Ca^{2+}/CaM pathway enhancing inactivation of the inhibitory protein IκB-α (Frantz et al., 1994).

Accumulating evidence exist, including our own data (Bierhaus et al., 2003) that norepinephrine may also modulate NF-κB (as well as GR activity; Schmidt et al., 2001) by the mitogen-activated protein kinase (MAPK) pathway (Choi and Jeong, 2005). This pathway seems to be induced by the β/γ subunits of G-proteins instead of the α subunit normally regarded as functional subunit and inducing the above described three pathways. In particular, the β/γ subunit from inhibitory G_i proteins have been demonstrated to interact with specific isoforms of phophoinositide-3 kinase (PI3-K) and to stimulate the MAPK pathway (Crespo et al., 1994; Lopez-Ilasaca et al., 1997).

Taken together, catecholamines interfere with NF-κB activity via several pathways. Thereby, pathways involving the α subunit of G-proteins may inhibit (β-adrenoceptor coupling to G_s with subsequent increase in cAMP) or stimulate NF-κB activity (α2-adrenoceptor coupling to G_i with subsequent decrease in cAMP, Ca^{2+}/CaM pathway induced by α1-adrenoceptor coupling to G_q). Additionally, norepinephrine may up-regulate NF-κB activity by activation of the MAPK pathway via β/γ subunits of G-proteins.

4.4.2.4 Aim of the Present Study

The aim of the present study was to test the following assumptions: Based on the previous findings, we propose a sequence of stress-induced events, in which first the increased release of catecholamines increases NF-κB activity, thereby helping to prepare the immune system for potential challenges. With some delay, increased release of cortisol suppresses immune responses by antagonizing the NF-κB activation cascade, thus protecting the organism against an over-reacting immune system.

As almost all of the above cited studies were done in animals or *in vitro*, our focus was explicitly on investigating these processes in humans, since possible compensating mechanisms, such as NF-κB influencing the sensitivity of immune cells to glucocorticoid signaling, may not be detected in *in vitro* approaches or may vary between species. The most promising approach in humans appears to be the investigation of patients with primary adrenal insufficiency (for review of disease see Arlt and Allolio, 2003; Betterle et al., 2002; Oelkers, 1996; Ten et al., 2001).

To accept the assumptions outlined above as true, the missing cortisol stress response in these patients should result in a missing termination of prior catecholamine induced increases in NF-κB activity. Substitution of these patients with hydrocortisone in turn should result in stress-associated NF-κB trajectories comparable to that found in healthy subjects.

4.4.3 Methods

4.4.3.1 Subjects

A total of 27 patients with Addison's disease (AD) and 16 healthy subjects (HS) were investigated. Due to missing data, the final sample consisted of 21 patients with Addison's disease and 10 healthy subjects. 10 age- and gender-matched patients were treated with an injection of 0.03mg/kg hydrocortisone *i.v.* (HC; Sigma, Berlin, Germany) after a psychosocial stress test, while 11 patients and 11 healthy subjects received a placebo injection (saline, NaCl; Sigma, Berlin, Germany). Mean age of subjects was 45.16 years (standard deviation (SD)=7.9) and mean body mass index (BMI) 25.74kg/m^2 (SD=4.0). Distribution of age and

BMI did not differ between the three groups (age: $F_{2,31}$=0.96, p=.40; AD-HC: mean=43.90, SD=8.5; AD-NaCl: mean=43.64, SD=7.3; HS-NaCl: mean=47.82, SD=7.9. BMI: $F_{2,31}$=1.56, p=.23; AD-HC: mean=25.82, SD=4.1; AD-NaCl: mean=24.23, SD=2.7; HS-NaCl: mean=27.19, SD=4.7). The group AD-HC consisted of 6 females and 4 males, the group AD-NaCl of 8 females and 3 males, and the group HS-NaCl of 7 females and 4 males (χ^2=0.41, p=.82).

Of all patients, 13 (61.9%) were diagnosed with autoimmune Addison's disease, based on the existence of autoantibodies (i.e., adrenocortical autoantibodies (ACAs), steroid cell antibodies (StCA); see Peterson et al., 2000) or co-morbidities fulfilling criteria for classification to Autoimmune Polyglandular Syndrom (APS) type 1 or type 2 (Neufeld et al., 1981). Two patient suffered from Addison's disease due to former Cushing's disease (9,5%). Six patients could not provide sufficiently detailed information for differential diagnosis (primary adrenal insufficiency (PAI); 28.6%). Glucocorticoid replacement was hydrocortisone in 17 patients (mean=27.21mg/day, SE=1.5) and cortisone acetate in four patients (mean=41.67mg/day, SE=11.0).

4.4.3.2 Experimental Protocol

Subjects reported to the laboratory at 1p.m. and were examined for past or current health problems by a physician. Patients were asked to postpone their second glucocorticoid replacement dose usually taken around 2a.m. (mean=13:52h, SD=1h54min) to avoid unpredictable and un-physiologically high salivary free cortisol levels (see section 4.1), hampering the comparability of patients an healthy subjects. After catheter insertion and a resting period of 45 minutes, a first blood sample for measure of NF-κB activity was collected (9ml citrate Monovette; Sarstedt, Nümbrecht, Germany). Subsequently, subjects were expose to the psychosocial stress test "Trier Social Stress Test" (TSST), which consists of a three minute preparation period, a five minute free speech and a five minute mental arithmetic task in front of an audience (Kirschbaum et al., 1993). Additional blood samples were collected 10, 60, and 120 minutes after stress exposure. For measure of salivary free cortisol and catecholamine levels, additionally saliva samples (Salivettes, Sarstedt, Nümbrecht, Germany) and blood samples (9ml ethylenediamine tetraacetic acid (EDTA) Monovette, Sarstedt, Nümbrecht, Germany) were collected before (-1 minute) and 1, 10, 20, 30, 45, 60, 90, and 120 minutes after TSST, respectively. Immediately after TSST, HC or saline were injected. This time-point was chosen since the stress test has a duration of 13 minutes and the maximum cortisol stress response is to be expected 15-20 minutes after stress onset. The study protocol was approved by the ethics committee of the University of Düsseldorf, and written informed consent was obtained from all subjects.

4.4.3.3 Biochemical Analyses

4.4.3.3.1 Hormone analyses

Saliva samples: Free cortisol levels in saliva were measured using a commercially available chemiluminescence assay (IBL, Hamburg, Germany). Samples were measured in duplicates and averaged for subsequent statistical analyses.

Blood samples: Plasma was separated at 4°C and stored at -80°C. Plasma concentrations of norepinephrine and epinephrine were determined by high-performance liquid chromatography with electrochemical detection (Smedes et al., 1982). Epinephrine levels under detection limit were labeled 5pg/ml representing half of the lowest standard.

4.4.3.3.2 NF-κB-DNA binding activity

Preparation of nuclear proteins: Immediately after collection peripheral blood mononuclear cells (PBMCs) were separated. 10ml whole blood anticoagulated with sodium citrate were loaded onto 10ml Ficoll (Biochrom, Berlin, Germany) and centrifuged for 30 minutes at 500g. The PBMC containing band was aspirated, cells were washed three times in PBS (PAA, Coelbe, Germany), and counted on a coulter AcTdiff cell counter (Beckman-Coulter, Krefeld, Germany). Three million cells were separated for nuclear protein preparation and assayed for transcription factor-binding activity by using NF-κB consensus oligonucleotides (Promega, Mannheim, Germany) as described (Bierhaus et al., 2001; Hofmann et al., 1999). In brief, $3x10^6$ PMBCs were lysed in 300μl ice-cold butter A (10mmol/l HEPES-KOH, pH 7.9 at 4°C, 1.5mmol/l $MgCl_2$, 10mmol/lKCl, 0.5mmol/l DTT, 0.2mmol/l PMSF), incubated on ice and centrifuged for 30 seconds at 4°C and 15.000rpm. Supernatant was discarded and the nuclear pellet was resuspended in 100μl ice-cold buffer B (20mmol/l HEPES-KOH, pH7.9 at 4°C, 25% glycerol, 1.5mmol/l $MgCl_2$. 420mmol/l NaCl, 0.2mmol/l EDTA, 0.5mmol/l DTT, 0.2mmol/l PMSF), incubated on ice for 20 minutes and centrifuged for 2 minutes at 4°C and 15.000rpm. The supernatant containing nuclear proteins was immediately quickfrozen at -80°C. Protein concentration was determined according to Bradford using a BCA assay (Pierce, Ill. USA).

Electrophoretic mobility shift assay (EMSA): For EMSA 10μg of nuclear proteins were used. Binding of NF-κB to 1ng of 5'-radiolabelled NF-κB consensus oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C-3') was done for 20 minutes at room temperature in 10mmol/l HEPES, pH7.5, 0.5mmol/l EDTA, 100mmol/l KCl, 2mmol/l DTT, 2% glycerol, 4% Ficoll, 0.25% NP-40, 1mg/ml BSA, and 0.1μg/μl poly d/dC by Pahl's method (Pahl and Baeuerle, 1995). Protein-DNA complexes were separated from unbound DNA probe by electrophoresis through 5% native polyacrylamide gels containing 2.5% glycerol and 0.5xTris-Borat-EDTA (TBE). Gels were dried under vacuum and exposed for 48 to 64 hours

to Amersham Hyperfilms (Amersham, Braunschweig, Germany) at -80°C with intensifying screens. Specificity of binding was ascertained by competition with a 160-fold molar excess of unlabelled consensus oligonucleotides and by characterization using specific antibodies for the NF-κB subunits p65, p50, c-Rel and Rel B (Santa Cruz Biotechnology, Heidelberg, Germany).

Semi-quantitative determination of NF-kBp65 binding activity: To quantify the NF-κB signal obtained from isolated PBMCs, 2.5, 5.0, and 7.5μg recombinant NF-κBp65 (rNF-κBp65) containing lysates were included in each EMSA on each gel. The resulting NF-κB signals were quantified by densitometry (Bio-Rad, München, Germany) and used to establish a standard curve for each gel (Hofmann et al., 1998). The densitometric value for the NF-κB signal of a given subject was converted into rNF-κBp65 equivalents by using the internal standard curve for rNF-κBp65 (Hofmann et al., 1998).

4.4.3.4 Statistical Analysis

Data were analyzed using the Statistical Package for the Social Science Version 11.0.2 (SPSS Institute, Chicago, IL). All data were tested for normality prior to statistical analysis using the Kolmogorov-Smirnov test and Greenhouse-Geisser corrections for repeated measures were calculated where appropriate. Values of $p<0.05$ were considered significant.

Various parameters, such as gender and age, are known to modulate endocrine stress responses (Barnes et al., 1982; Kirschbaum et al., 1992) and NF-κB binding activity (Trebilcock and Ponnappan, 1996b) and were therefore initially included as covariates or predictors. As none of these variables changed results, they were no longer considered in the present analyses (section 4.4.4).

4.4.3.4.1 Effects of disease, stress, and treatment on endocrine parameters

Student's *t* tests were applied for comparing baseline measures between patients and healthy subjects (AD vs. HS). To test for effects of stress and hydrocortisone injection ('treatment') on salivary cortisol levels, a repeated measures ANOVA was calculated. Additionally, maximum cortisol levels as well as increases in cortisol from pre-TSST to maximum levels were compared by univariate ANOVAs and subsequent Scheffé tests to confirm adequacy of hydrocortisone dose of 0.03mg/kg to mimic cortisol stress responses in patients with Addison's disease. Repeated measures ANOVAs were also calculated for norepinephrine and epinephrine levels comparing the courses of healthy subjects and patients with Addison's disease. No effects of hydrocortisone treatment were expected in these two parameters (Bornstein et al., 1995).

4.4.3.4.2 Effects of stress, disease, and treatment on NF-κB binding activity

To test for effects of Addison's disease and hydrocortisone treatment on NF-κB binding activity, a set of three repeated measures ANOVAs was calculated: To test for effects of disease, first two between-group levels were included representing the groups AD-NaCl and HS-NaCl ('disease'). To test for effects of hydrocortisone treatment in patients with Addison's disease, next the two groups AD-HC and AD-NaCl were included ('treatment'). The third repeated measure ANOVA included all three groups (AD-HC, AD-NaCl, and HS-NaCl) as between-subject factors. Since rather changes in NF-κB binding activity than the degree of NF-κB binding activity per se seem to be of clinical relevance (Bohrer et al., 1997), this set of ANOVAs was repeated with signal intensity of NF-κB binding activity being transferred to percent change to baseline.

4.4.3.4.3 Endocrine variables: indices of variation

For later regression analyses, indices of variations in hormone levels (i.e. stress-induced increases and subsequent decreases) were computed. Generally lower values were subtracted from higher values, i.e., for decreases the second value was subtracted from the first, while for increases the first values was subtracted from the second. In detail, increases in cortisol from pre-stress to maximum were calculated by subtraction of baseline levels from maximum cortisol levels (cort_incr1>3). Two decrease indices were calculated by subtraction of cortisol levels at 60 and 120 min after TSST from maximum cortisol levels (cort_decr3>7 and cort_decr3>9, respectively). One increase (baseline subtracted from maximum) and one decrease index (maximum-levels at +10min) for each norepinephrine (NE_incr1>2, NE_decr2>3, respectively) and epinephrine were calculated (E_incr1>2, E_decr2>3, respectively). Univariate ANOVAs with subsequent Scheffè test were computed for comparison of cortisol indices between the three groups. Since no effects of hydrocortisone treatment are to be expected, for comparison of catecholamine indices between patients with Addison's disease and healthy subjects Student's *t* tests were computed.

4.4.3.4.4 Predictors of NF-κB binding activity variations

In parallel to endocrine indices of variation, three indices of NF-κB binding activity variations were computed (NF-κB1>2: changes from pre-TSST to 10min post TSST; NF-κB2>3: changes from +10min to +60min; NF-κB3>4: changes from +60min to +120 min). Next, each of the three indices were subjected to a stepwise regression analysis as dependent variable. As independent variables, the following parameters were included as predictors of NF-κB1>2: cortisol, norepinephrine, and epinephrine levels at time-points -1min, +1min, and +10min, cort_incr1>3, NE_incr1>2, NE_decr2>3, E_incr1>2, and E_decr2>3. For NF-κB2>3, additionally cortisol levels at time-points +20min, +30min, +45min, and +60min as

well as the variable cort_decr3>7 were included. No further norepinephrine and epinephrine concentrations were considered, as no variations in catecholamine levels are to be expected after time-point +10min. For NF-κB3>4, also cortisol levels at +90min and +120min and the variable cort_decr3>9 were included as predictors.

First, regression analyses were computed regarding all subjects. In these cases, the variable 'group' was included as an additional predictor. Next, regression analyses were calculated for each group separately, since in patients with Addison's disease the altered endocrine status may cause other variables to influence NF-κB binding activity trajectories than in healthy subjects. Due to the small numbers of subjects, these regression analyses are considered preliminary. Hence only regression analyses with an ANOVA of the respective model yielding a significant result ($p<.05$) will be presented. Adjusted R squares (R^2) and standardized betas (β) will be given as indicators of model quality.

4.4.4 Results

4.4.4.1 Baseline Differences

NF-κB binding activity ($t_{30}=-0.90$, $p=.38$) and norepinephrine levels ($t_{30}=0.99$, $p=.33$) did not differ significantly between patients with Addison's disease and healthy subjects, while in patients significantly lower free cortisol ($t_{30}=-5.04$, $p<.001$) and epinephrine ($t_{30}=-3.65$, $p=.001$) levels were found compared to healthy subjects (see chapter 4.2).

4.4.4.2 Salivary Free Cortisol Levels: Effects of Stress and Treatment

In parallel to former results (see section 4.2), healthy subjects in the present subset showed significant increases in free cortisol levels in response to stress, while in patients with Addison's disease, very low cortisol levels and no stress response was observed. Treatment of patients with hydrocortisone resulted in cortisol trajectories comparable to healthy subjects (repeated measures ANOVA: group: $F_{2,29}=19.92$, $p<.001$; time: $F_{8,232}=56.32$, $p<.001$; group-by-time: $F_{16,232}=16.15$, $p<.001$). This was confirmed by univariate ANOVAs comparing free cortisol levels at time-point +10 and increases in free cortisol levels from time-point -1 to +10 between the three groups (*cort+10:* $F_{2,31}=27.30$, $p<.001$; Scheffé tests: AD-HC vs. AD-NaCl: $p<.001$; AD-HC vs. HS-NaCl: $p=.072$; AD-NaCl vs. HS-NaCl: $p<.001$ – *incr_cort1>3:* $F_{2,31}=24.68$, $p<.001$; Scheffé tests: AD-HC vs. AD-NaCl: $p<.001$; AD-HC vs. HS-NaCl: $p=.99$; AD-NaCl vs. HS-NaCl: $p<.001$).

4.4.4.3 Catecholamine Levels: Effects of Stress

In healthy subjects, stress additionally induced significant increases in norepinephrine and epinephrine. Patients with Addison's disease showed norepinephrine levels comparable

to healthy subjects throughout the investigation, but significantly lower epinephrine levels and no epinephrine stress response (see section 4.2; repeated measures ANOVAs – norepinephrine: group: $F_{1,30}$=0.82, p=.37; time: $F_{8,240}$=9.59, p<.001; group-by-time: $F_{8,240}$=0.88, p=.49; epinephrine: group $F_{1,30}$=24.27, p<.001; time: $F_{8,240}$=11.13, p<.001; group-by-time: $F_{8,240}$=6.35, p<.001).

4.4.4.4 NF-κB Binding Activity: Effects of Stress, Disease, and Treatment

No group differences were found in NF-κB binding activity trajectories: Neither did patients with Addison's disease differ from healthy subjects, nor did hydrocortisone treatment have significant effects. Additionally, missing time effects indicate stress to not induce significant changes in NF-κB binding activity. Table 4-9 summarizes the statistical results for both NF-κB signal intensity and its transformation into percent change to baseline, while figures 4-17 and 4-18 depict the respective trajectories.

Tab. 4-9: Results of repeated measures ANOVAs for NF-κB signal intensity (1st row) and NF-κB activity in percent change to baseline (2nd row) with two between-group levels testing for group (column **A**) and treatment effects (column **B**), and with three levels comparing changes over time between all three groups (column **C**).

		A: DISEASE AD-NaCl (n=11) / HS-NaCl (n=11)	**B:** TREATMENT AD-HC (n=10) / AD-NaCl (n=11)	**C:** DIS. x TREAT. AD-HC / AD-NaCl / HS-NaCl
NF-κB signal	group:	$F_{1,20}$=0.57, p=.46	$F_{1,19}$=0.01, p=.92	$F_{2,29}$=0.53, p=.59
	time:	$F_{3,60}$=0.98, p=.40	$F_{3,57}$=0.49, p=.65	$F_{3,87}$=0.81, p=.48
	group x time:	$F_{3,60}$=2.19, p=.12	$F_{3,57}$=0.64, p=.56	$F_{6,87}$=0.79, p=.57
NF-κB % change	group:	$F_{1,20}$=0.16, p=.69	$F_{1,19}$=0.14, p=.72	$F_{2,29}$=0.09, p=.92
	time:	$F_{3,60}$=0.61, p=.56	$F_{3,57}$=0.17, p=.86	$F_{3,87}$=0.50, p=.64
	group x time:	$F_{3,60}$=1.45, p=.25	$F_{3,57}$=0.74, p=.50	$F_{6,87}$=0.63, p=.67

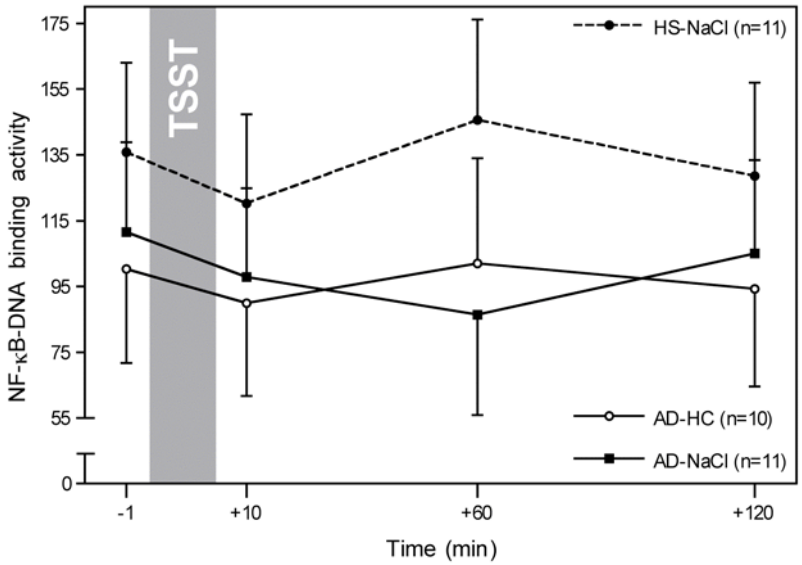

Fig. 4-17: NF-κB-DNA binding activity (signal intensity) before and repeatedly after stress in patients (with and without hydrocortisone treatment) and healthy subjects.

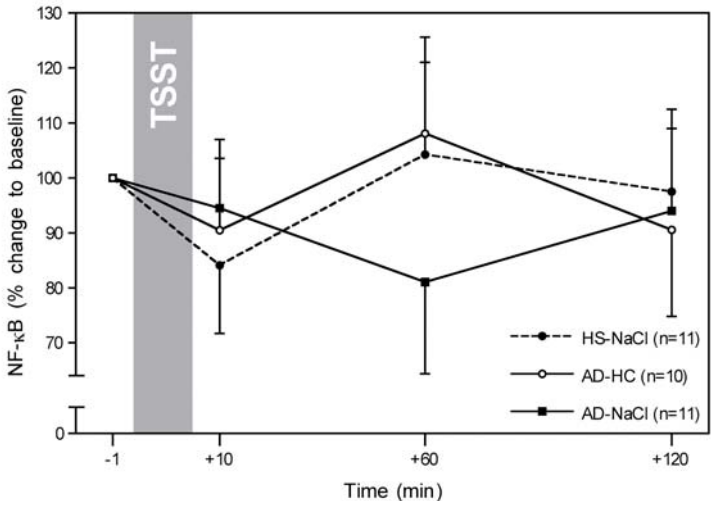

Fig. 4-18: NF-κB-DNA binding activity (% change to baseline) before and repeatedly after stress in patients (with and without hydrocortisone treatment) and healthy subjects.

4.4.4.5 Endocrine Variables: Indices of Variation

As expected, overall the groups did differ significantly in the degree cortisol levels increased in response to TSST ($F_{2,31}$=24.68, p<.001). Thereby, patients showed no stress response and hence cortisol increases differed significantly from healthy controls (Scheffé test: p<.001). Hydrocortisone treatment resulted in cortisol increases comparable to increases in healthy subjects (Scheffé test: p=.99) and significantly different from untreated patients (Scheffé test: p<.001). In the subsequent decreases in cortisol levels, again significant group differences were found (*cort_decr3>7:* $F_{2,31}$=19.71, p<.001; *cort_decr3>9:* $F_{2,31}$=27.20, p<.001). Patients treated with hydrocortisone showed decreases comparable to healthy subjects (Scheffé tests; *cort_decr3>7:* p=.99; c*ort_decr3>9:* p=.38), while decreases in cortisol levels differed significantly in both treated patients and healthy subjects from untreated patients (Scheffé tests; *cort_decr3>7:* AD-HC vs. AD-NaCl: p<.001; NaCl-AD vs. HS-NaCl: p<.001 – *cort_decr3>9:* AD-HC vs. AD-NaCl: p<.001; NaCl-AD vs. HS-NaCl: p<.001). Figures 4-19 depicts means and standard errors of changes in cortisol levels for each of the three groups separately.

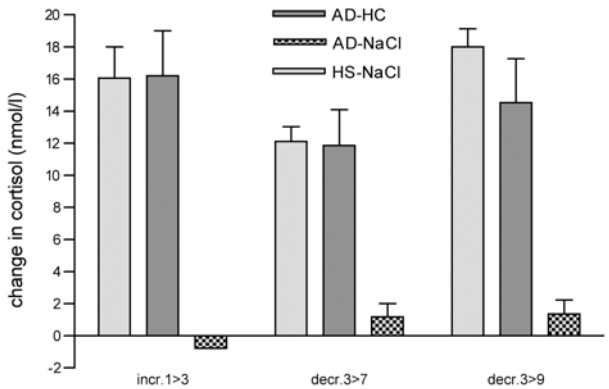

Fig. 4-19: Means and standard errors of stress-induced increases in cortisol levels from pre-TSST to +10min (incr. 1>3), decreases from +10min to +60min (decr. 3>7), and decreases from +10min to +120min (decr. 3>9) in healthy subjects and patients with Addison's disease with and without hydrocortisone treatment.

Regarding stress-induced norepinephrine changes, no significant differences between patients with Addison's disease and healthy subjects were found (NE_incr1>2: t_{30}=-1.20, p=.24; NE_decr2>3: t_{30}=-1.99, p=.056). Contrary, as patients with Addison's disease showed very low epinephrine levels and no changes throughout the investigation, their changes in epinephrine levels differed significantly from healthy subjects (E_incr1>2: t_{30}=-4.17, p<.001; E_decr2>3: t_{30}=-3.04, p=.005). Figure 4-20 depicts means and standard errors of changes in norepinephrine and epinephrine levels for patients with Addison's disease and healthy subjects.

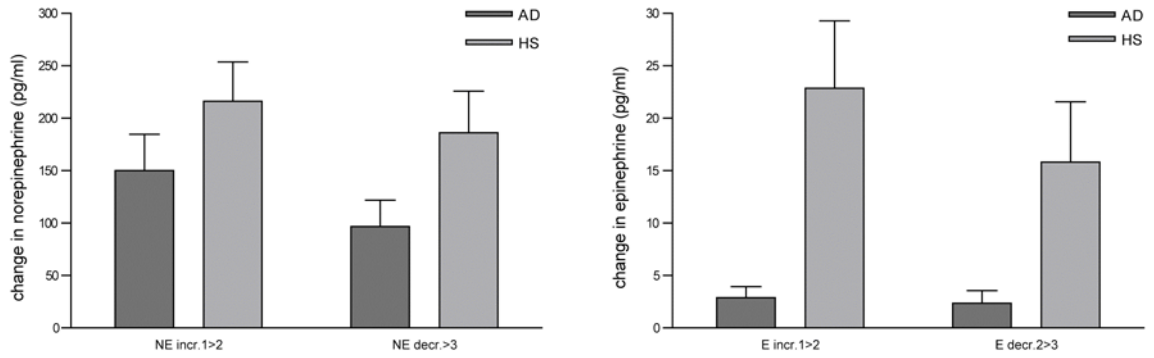

Fig. 4-20: Means and standard errors of stress-induced changes in norepinephrine (NE, left) and epinephrine (E, right) levels from pre-TSST to +1min (incr. 1>2) and from +1min to +10min (decr. 2>3) in patients with Addison's disease and healthy subjects.

4.4.4.6 Regression Analyses: Predictors of NF-κB Binding Activity Variations

Based on the given NF-κB trajectories (see fig. 4-18), the following indices of variations were calculated and included in regression analyses as dependent variable: decr.1>2 (=NF-κB1–NF-κB2), incr2>3 (=NF-κB3–NF-κB2), decr3>4 (=NF-κB3–NF-κB4).

Out of 12 regression analyses with NF-κB binding activity variations as dependent variables, only three yielded significant results. The free cortisol concentration immediately after TSST (+1min) was the only predictor of decreases in NF-κB activity from pre-TSST to +10min after TSST (NF-kB_decr1>2) in healthy subjects, in that higher cortisol levels predicted more pronounced decreases in NF-κB binding activity (R^2=0.39, adjusted R^2= 0.32, $F_{1,10}$=5.66, p=.041, β=0.62). Contrary, in patients with Addison's disease more pronounced decreases in norepinephrine after TSST predicted attenuated decreases in NF-κB binding activity (R^2=0.41, adjusted R^2= 0.35, $F_{1,10}$=6.27, p=.034, β=-0.64). In patients treated with hydrocortisone, steeper increases in cortisol levels in turn predicted less pronounced increases in NF-κB binding activity from time-point +10min to +60min after TSST (R^2=0.45, adjusted R^2= 0.38, $F_{1,9}$=6.57, p=.033, β=-0.67). Scatterplots of these results (see figure 4-21) indicated that the third significant result is most probably caused by only one subject. Therefore this regression analysis was repeated excluding the respective patient and the regression no longer remained significant.

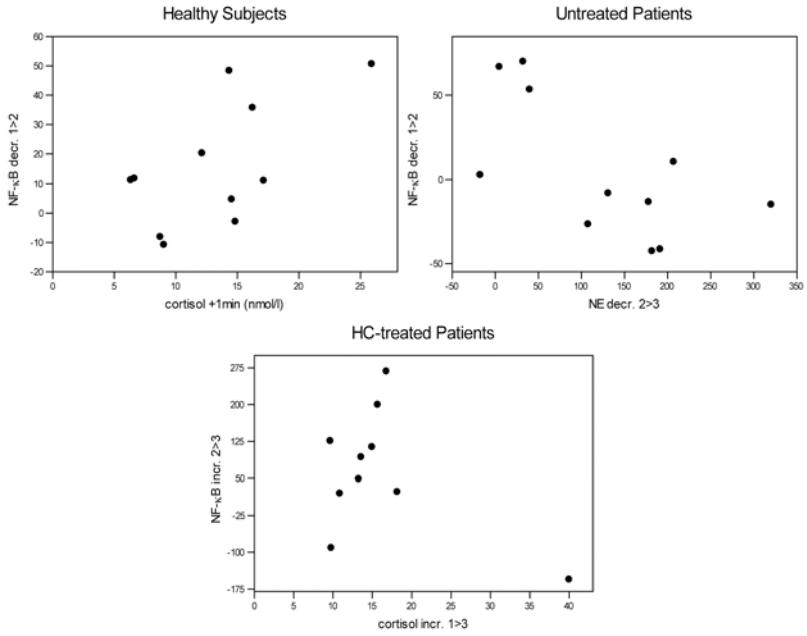

Fig. 2-21: Scatterplots summarizing the significant regression models regarding variations in NF-κB binding activity.

4.4.5 Conclusion

The present findings are in line with previously reported endocrine stress responses in healthy subjects and patients with Addison's disease (see section 4.2): While healthy subjects showed the expected significant increases in all endocrine parameters, i.e. free cortisol, norepinephrine, and epinephrine, patients with Addison's disease responded to stress only with increases in norepinephrine. Furthermore, hydrocortisone treatment of patients with Addison's disease resulted in post-TSST free cortisol levels as well as increases from pre- to post-TSST levels comparable to that found in healthy subjects. These findings provide the necessary basis for investigation of the role of glucocorticoids in NF-κB activity with regard to stress by fulfilling the criteria of hormone subtraction (Addison's disease) and replacement (hydrocortisone treatment).

Interestingly, in the present study NF-κB activity *per se* did statistically neither change in response to stress nor to hydrocortisone treatment and did not vary between patients with Addison's disease and healthy subjects. This is in clear contrast to own previous findings of stress-associated increases in NF-κB activity (Bierhaus et al., 2003). This contradictory findings may be explained, at least in part, by subjects in the present study being approximately 20 years older than in the previous. Nevertheless, considering age statistically did not change results and age did not reveal to be an important factor on its own (see above), but variation of age may also have been to low in the present study. Own preliminary data from a greater sample of 44 healthy subjects suggested that NF-κB activity increases in response to stress only in younger subjects, while NF-κB activity decreases in subjects older than 30 years (Wolf et al., 2005). In the present study just two 32 years old subjects came

close to this cut-off of 30 years. Consequently, the present NF-κB trajectories, though not varying significantly in response to stress, mimicked more closely the courses found in older subjects. In parallel to these stress-induced decreases in NF-κB activity, *in vitro* studies repeatedly found activity of NF-κB in T-cells to decrease during aging (Trebilcock and Ponnappan, 1996a; Trebilcock and Ponnappan, 1996b), an effect most probably due to failure of full degradation of IκB-α and interpreted in the context of immunosenescence (Ponnappan et al., 2004; Ponnappan et al., 1999a; Ponnappan et al., 1999b; Trebilcock and Ponnappan, 1998). Contrary, in brains of aged mice and rats, DNA-binding activity of NF-κB was found to be elevated (Korhonen et al., 1997; Toliver-Kinsky et al., 1997; Ye and Johnson, 2001). This is also true for nuclear extracts from other tissues including liver (Supakar et al., 1995; Walter and Sierra, 1998), heart (Helenius et al., 1996), and spleen (Poynter and Daynes, 1998). This points to tissue-specific age-related processes altering NF-κB activity. It is therefore conceivable that age may also influence stress-associated changes in NF-κB activity and that just the low number of subjects in the present study prevented its discovering.

Furthermore, although no statistical significant differences in NF-κB trajectories between healthy subjects and patients with and without hydrocortisone treatment were found, regression analyses revealed distinguishable influences of free cortisol and norepinephrine. In healthy subjects, free cortisol levels immediately after completion of TSST predicted decreases in NF-κB activity from pre- to post-stress: With higher cortisol levels, this decrease in NF-κB activity became more pronounced. This is in line with findings of glucocorticoids suppressing NF-κB activity by interfering with its activation cascade, both via increasing IκB-α and via physical interaction between NF-κB- and GR-DNA binding (Auphan et al., 1995; De Bosscher et al., 1997; De Bosscher et al., 2000b; Ray and Prefontaine, 1994; Scheinman et al., 1995a). The fact that not maximum cortisol levels predicted NF-κB decreases but the sample 10 minutes earlier suggests that intracellular cortisol signaling requires a certain time to cause changes in NF-κB activity. In parallel to this finding, the same time lag of 10 minutes was found in NF-κB response to infection (Naumann, 2000). It may be hypothesized that maximum stress-induced cortisol levels exert even more pronounced effects on NF-κB activity at later time-points (i.e., 20 to 30 minutes after TSST) not considered in the present study. However, to our knowledge this is the first study showing glucocorticoids to inhibit NF-κB activity in humans and also with regard to stress. These findings are further substantiated by data of patients with Addison's disease. As patients with Addison's disease can not respond to stress with increases in free cortisol levels, their NF-κB trajectories were consequently not predicted from cortisol levels. Instead, associations between norepinephrine, the only hormone showing normal concentrations and stress responses in Addison's disease, and NF-κB activity were found. This finding points to

stress-induced norepinephrine also being an inhibitor of NF-κB activity, as faster clearance of norepinephrine levels after stress predicted attenuated decreases or more pronounced increases in NF-κB activity from pre- to post TSST. That norepinephrine inhibited NF-κB activity suggests that in patients with Addison's disease the cAMP/PKA pathway of catecholamine signal transduction may be involved, since as outline above, elevated levels of cAMP have repeatedly been shown to inhibit NF-κB (Chen and Rothenberg, 1994; Neumann et al., 1995; Paliogianni et al., 1993; Parry and Mackman, 1997; Tsuruta et al., 1995). Interestingly, former results in healthy subjects showed psychosocial stress to increase NF-κB activity most probably by norepinephrine inducing the MAPK pathway (Bierhaus et al., 2003). But since patients with Addison's disease do not secrete cortisol in response to stress, it may be hypothesized that norepinephrine inhibiting NF-κB activity functions as a compensation of the missing inhibitory cortisol actions.

Based on these results, glucocorticoid stress responses can not be regarded as necessary (and hence also not as sufficient) for suppressing NF-κB activity. This conclusion is supported by free cortisol levels and stress-induced variations not predicting changes in NF-κB activity in patients treated with hydrocortisone. However, norepinephrine as well did not predict changes in NF-κB activity. It may be hypothesized that treatment with hydrocortisone represents an unusual situation in patients with Addison's disease resulting in conflicting intracellular processes regarding (usual) catecholamine and (unusual) glucocorticoid signaling pathways interfering with the NF-κB activation cascade. Nevertheless, treatment of patients resulted in NF-κB trajectories phenomenologically similar to that found in healthy subjects. This especially concerns changes in NF-κB activity from ten minutes to one hour after stress. Within this period, both healthy subjects and hydrocortisone treated patients showed increases in NF-κB activity, while NF-κB activity in untreated patients further declined (see figure 4.18). Unfortunately, none of the endocrine parameters appeared to be associated or to predict these later variations in NF-κB activity. But as suppressed NFκB activity implicates attenuated immune reactivity, prolonged decreased NF-κB activity subsequently to stress may put patients with Addison's disease at a higher risk for infections during this period. However, studies with higher numbers of subjects will be needed to approve this hypothesis.

In summary, the present study showed for the first time that stress-induced glucocorticoid concentrations inhibit NF-κB activity in humans. Furthermore, findings in patients with Addison's disease suggests that this immune-suppressive action of glucocorticoids is accomplishable also by norepinephrine, if glucocorticoid stress responses are missing in the long term. These results once more emphasize the need for studying complex and interrelated processes also in the human whole organism to allow for uncovering compensatory mechanisms.

5 SUMMARY, GENERAL DISCUSSION, AND OUTLOOK

In the following, all results of the former sections will be briefly summarized and placed in perspective to the present literature (section 5.1) in order to discuss them subsequently in a greater context (section 5.2). Finally, an outlook regarding the role of Addison's disease in PNI as well as the relevance of the findings for patients will be given.

5.1 SUMMARY OF RESULTS

The general aim of the present doctoral thesis was to scrutinize the method Addison's disease with respect to its value as a complemental approach to decide on the relevance of psycho-neuro-immunological findings for human health and disease. In this regard, four specific problem formulations were investigated empirically, with two aiming at clarifying the endocrinological initial conditions of Addison's disease and two representing applications of the method with respect to the immune system. The four problem formulations were:

1) What are the initial basal endocrinological conditions in Addison's disease?
2) How are endocrine stress responses in Addison's disease characterized?
3) Does the immune system show influences from long-term altered endocrine stress responses?
4) Does compensation of long-term altered endocrine stress responses occur intra-cellularly at the level of NF-κB?

5.1.1 Initial Endocrinological Conditions in Addison's Disease

The first study shows salivary cortisol levels in patients with Addison's disease to be notably elevated compared to standard values, which points to an over-replacement of patients already repeatedly reported by others (al-Shoumer et al., 1995; Florkowski et al., 1994; Howlett, 1997; Lovas and Husebye, 2003; Peacey et al., 1997; Zelissen et al., 1994). Furthermore, on average 7 hours after the first replacement dose, free cortisol levels of patients with Addison's disease fell below levels of healthy subjects, which is in line with data on pharmacokinetics and pharmacodynamics of cortisol (Howlett, 1997) and endorse recommendations of replacement regimens which allow for lower but more frequent doses (Howlett, 1997; Peacey et al., 1997). As in plasma cortisol, free cortisol trajectories of patients taking cortisone acetate are blunted and delayed compared to that of hydrocortisone (Feek et al., 1981). The missing linear association between hydrocortisone doses and resulting salivary cortisol levels supports findings showing no beneficial effects of hydrocortisone doses greater than 20mg a day (Wichers et al., 1999).

The second study revealed elevated general and physical fatigue scores in patients with Addison's disease compared to healthy subjects, which is in accordance with former reports (Lovas et al., 2002). Contrary to subjective self-reports, no differences in mental fatigue, activity and motivation were found. Beside psychometric investigation of patients with Addison's disease, the second study clearly focused on endocrine and cardiovascular stress responses. These stress-related endocrine as well as blood pressure findings are summarized in figure 5-1.

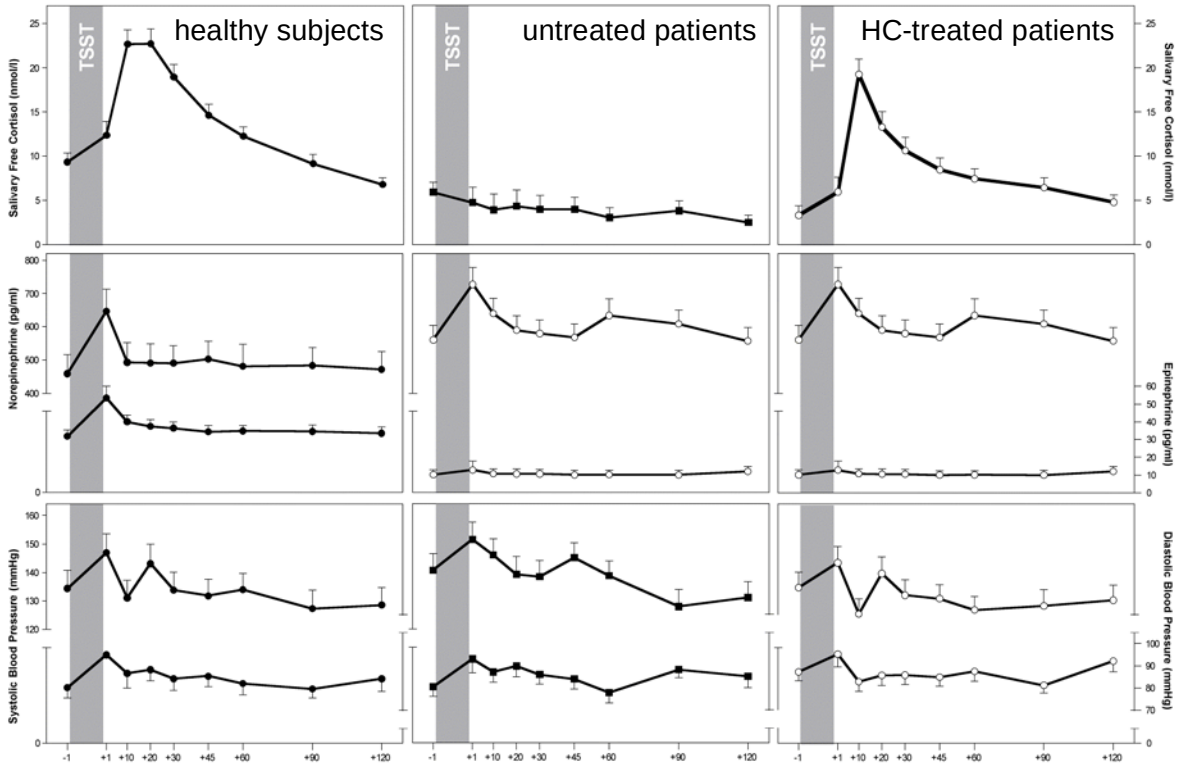

Fig. 5-1: Summary of endocrine (cortisol: top row, norepinephrine and epinephrine: middle row) and blood pressure (bottom row) results. Data from healthy subjects are depicted in the left column, from patients with Addison's disease in the middle column, and from patients treated with hydrocortisone in the right column. Please note that catecholamine responses in patients were not divided according to treatment vs. no treatment.

Patients with Addison's disease did not show stress-responses in free cortisol. Treatment of patients with 0.03mg/kg hydrocortisone resulted in stress-induced cortisol increases and maximum salivary cortisol levels mimicking those of healthy subjects. However, patients showed a more pronounced decrease in salivary cortisol levels thereafter. The findings of very low basal epinephrine levels accompanied by a trend to elevated basal norepinephrine levels are in line with prior findings, such as in patients with Addison's disease (Bornstein et al., 1995), in experimental animals with reduced glucocorticoid synthesis (Wurtman, 1966), and after adrenalectomy (Eisenhofer et al., 1995; Merke et al., 2000). The present minimal stress responses in epinephrine and slightly higher norepinephrine responses compared to healthy subjects in turn parallel findings in patients with isolated glucocorticoid deficiency (Zuckerman-Levin et al., 2001). As expected from studies showing glucocorticoid and mineralocorticoid replacement therapy to restore cardiac

functions (Fallo et al., 1999; Fallo et al., 1994), no significant differences in resting blood pressure or heart rate in patients with Addison's disease compared to healthy subjects were observed. Additionally, hydrocortisone treatment did not influence the trajectories of both cardiovascular parameters suggesting permissive actions of cortisol (i.e., basal cortisol levels induced by replacement therapy) being sufficient for catecholamine stress effects on blood pressure and heart rate (Allolio et al., 1994).

5.1.2 Effects of Altered Endocrine Stress Responses on the Immune System

To investigate the effects of altered endocrine stress responses on related immune functions, percentages of blood cells, stimulated IL-6 production, stimulated cell proliferation, and NF-κB activity were measured repeatedly over the course of the experiment. The main results are summarized in figure 5-2.

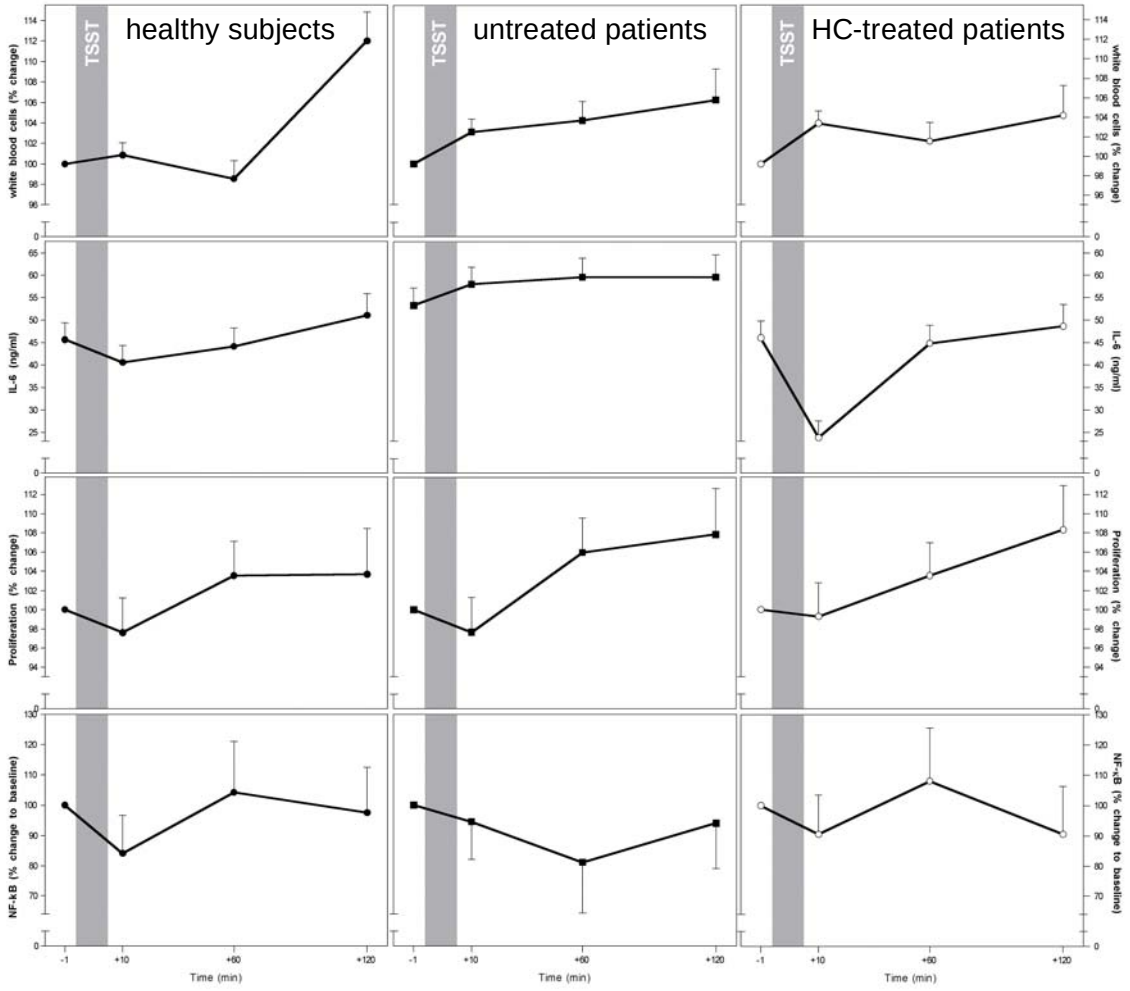

Fig. 5-2: Summary of immune (white blood cells: top row, LPS-stimulated IL-6 production: 2nd row, PHA-stimulated cell proliferation: 3rd row) and NF-κB (bottom row) results. Data from healthy subjects are depicted in the left column, from patients with Addison's disease in the middle column, and from patients treated with hydrocortisone in the right column.

5.1.2.1 Blood Cell Counts

In detail, patients with Addison's disease showed slow increases in numbers of white blood cells, while healthy subjects showed most pronounced increases between one and two hours after stress. This is due to the various changes in percentages in cell subtypes. While immediate stress responses in lymphocytes and granulocytes were comparable in patients and healthy controls, at one and two hours after stress differences in trajectories were found with patients showing a continuation of prior trends, i.e. lymphocytes continued increasing and granulocytes continued decreasing. These findings reflect the two phases typically found after catecholamine administration and emphasize the interpretation of early events being mediated by catecholamines (Benschop et al., 1996) and later time-points being influenced by glucocorticoids (Dhabhar et al., 1996). Nevertheless, hydrocortisone treatment of patients did not restore these patterns, as would have been suggested.

5.1.2.2 LPS-Stimulated IL-6 Production and its Glucocorticoid Sensitivity

In LPS-stimulated IL-6 production, no baseline differences could be found between healthy subjects and patients with Addison's disease. However, stress-related trajectories of LPS-stimulated IL-6 production did vary: While healthy subjects showed decreases immediately after stress and slow increases during the following two hours, reaching levels above baseline at two hours after TSST, patients with Addison's disease showed increases in LPS-stimulated IL-6 levels immediately after stress with little variations thereafter. Treatment of patients with hydrocortisone resulted in pronounced decreases in IL-6 production ten minutes after TSST with a return to baseline 1 hour after TSST, thereby mimicking more closely the trajectories found in healthy subjects than in untreated patients. Unfortunately, just a few studies are available measuring stress-related LPS stimulated IL-6 production in humans and in whole blood assays and these studies additionally report contradictory results. Thus, findings of Rohleder et al. exposing men (Rohleder et al., 2001) and young women in the luteal phase (Rohleder et al., 2003b) to the TSST and of Smits et al. investigating the effects of exercise in oarsmen (Smits et al., 1998) are in parallel with the present findings in healthy subjects. Contrary, the trajectories of LPS-stimulated IL-6 production in patients with Addison's disease reflect those found by Goebel et al. in healthy volunteers during an acute speech stress (Goebel et al., 2000), by Maes et al. investigating academic examination stress (Maes et al., 1998), and by Rohleder et al. exposing young women taking oral contraceptives to the TSST (Rohleder et al., 2003b). Studies in adrenalectomized animals are rare as well and procedures additionally differ too much from procedures in the present studies to compare results. For example, Meltzer et al. not only used *iv* LPS injection in adrenalectomized and intact rats, but also investigated the effects of footshock on splenic cytokine production (Meltzer et al., 2004), with spleen cells being known

to respond differently to glucocorticoids due to different expression levels of glucocorticoid receptors (Miller et al., 1998). The findings in humans suggest stress-related IL-6 production to be influenced by gender or sex hormones. However, no influence at least from gender was found in the present study.

Regarding glucocorticoid sensitivity of IL-6 producing cells, no differences between healthy subjects and patients with Addison's disease were found. Only hydrocortisone treatment resulted in pronounced decreases in glucocorticoid sensitivity, which appeared to be due to LPS-stimulated IL-6 levels being pronouncedly decreased after hydrocortisone-treatment, thereby affecting IC_{50} calculation. Overall, the course of IL-6 IC_{50} slightly increased in response to stress, indicating attenuated glucocorticoid sensitivity immediately after stress. Own former studies suggest glucocorticoid sensitivity also to be influenced by gender or sex hormones: glucocorticoid sensitivity decreased in women and older men, increased in younger men and women using oral contraceptives, and did not change in women being in the luteal phase of their menstrual cycle (Rohleder et al., 2003a). But again, in the present study no influence of gender could be observed.

5.1.2.3 PHA-Stimulated Cell Proliferation and its Glucocorticoid Sensitivity

In PHA-stimulated cell proliferation, only significant time effects were found, indicating stress to result in increases in PHA-stimulated cell proliferation and decreases in glucocorticoid sensitivity. The latter was most pronounced two hours after stress. The finding of stress-induced increases in PHA-stimulated cell proliferation is in line with other data reporting of enhanced proliferation in response to a social stressor (Bohus et al., 1993) and five weeks of isolation and water scheduling in rats (Jessop et al., 1987). Contrary, suppressed PHA-stimulated proliferation was reported in humans in response to a Stroop test (Bachen et al., 1992), in peripheral T-cells (Connor et al., 1997) as well as in splenocytes (Ferry et al., 1991) in rats in response to a forced swim tests, and in juvenile squirrel monkeys two days after 3 hours of separation (Friedman et al., 1991). No changes in proliferation were observed in response to a difficult puzzle task (Brosschot et al., 1992) or in response to an electric shock with loud white noise (Weisse et al., 1990). Only a few studies measured sensitivity and again the results are contradictory. Comparable to the present study, decreases in glucocorticoid sensitivity in peripheral blood mononuclear cells (PBMCs) were found on days with academic examination stress (Sauer et al., 1995). On the other hand, acute stress did not induce any significant changes in glucocorticoid sensitivity of PHA-stimulated splenocyte and blood lymphocyte proliferation in rats (Bauer et al., 2001), and our own laboratory found acute stress in healthy subjects to be associated with increased glucocorticoid sensitivity (Rohleder et al., 2005). However, in all the cited studies not only different types and durations of stressors, but also different tissues and PHA concentrations were employed, which hampers an integration of the present findings considerably.

Furthermore, no differences between patients with Addison's disease and healthy subjects and no effect of hydrocortisone treatment in patients were found. These findings lead to the same conclusion as drawn by Keller et al. (Keller et al., 1983) that stress effects on lymphocyte function are glucocorticoid independent.

5.1.2.4 NF-κB Activity

The fourth problem formulation concerned the role of the transcription factor NF-κB as mediator of immune-suppressive glucocorticoid actions. In the present study, NF-κB activity did neither change in response to stress nor to hydrocortisone treatment, and did further not vary between patients with Addison's disease and healthy subjects. This is on the one hand in clear contrast to own previous findings of stress-associated increases in NF-κB activity (Bierhaus et al., 2003). However, age differs in the two studies and is known to influence NF-κB activity (Ponnappan et al., 2004; Trebilcock and Ponnappan, 1998). Thus, on the other hand the present NF-κB trajectories, though not varying significantly in response to stress, mimicked more closely the courses previously found in older subjects (Wolf et al., 2005). Contrary, in brain of aged mice and rats, DNA-binding activity of NF-κB was found to be elevated (Korhonen et al., 1997; Toliver-Kinsky et al., 1997; Ye and Johnson, 2001) as it was the case in other tissues, such as liver (Supakar et al., 1995; Walter and Sierra, 1998), heart (Helenius et al., 1996), and spleen (Poynter and Daynes, 1998). This points to NF-κB activity being not only age-dependent but also tissue specific. However, treatment of patients resulted in NF-κB trajectories phenomenologically similar to that found in healthy subjects, with both healthy subjects and hydrocortisone treated patients showing increases in NF-κB activity from ten minutes to one hour after stress, while NF-κB activity in untreated patients further declined.

Regression analyses revealed distinguishable influences of free cortisol and norepinephrine. In healthy subjects, higher cortisol levels immediately after completion of TSST predicted more pronounced decreases from pre- to post-stress in NF-κB activity. This is in line with findings of glucocorticoids suppressing NF-κB activity by interfering with its activation cascade (Auphan et al., 1995; De Bosscher et al., 1997; De Bosscher et al., 2000b; Ray and Prefontaine, 1994; Scheinman et al., 1995a). In patients with Addison's disease, faster clearance of norepinephrine subsequent to stress predicted attenuated decreases or more pronounced increases in NF-κB activity from pre- to post TSST. This indirect finding of norepinephrine inhibiting NF-κB activity is in accordance with studies showing elevated levels of cAMP to inhibit NF-κB (Chen and Rothenberg, 1994; Neumann et al., 1995; Paliogianni et al., 1993; Parry and Mackman, 1997; Tsuruta et al., 1995). No predictors of NF-κB variations were found for patients treated with hydrocortisone.

5.2 GENERAL DISCUSSION

The first two of four problem formulations concerned the initial endocrinological conditions, which have to be taken into consideration when studying patients with Addison's disease in order to decide on the relevance of psychoneuroimmunological findings for human health and disease. Thus, in the following the respective implications of these findings will be discussed to provide a framework for interpretation of the subsequent immunological findings.

5.2.1 Initial Endocrinological Conditions

5.2.1.1 Initial Basal Conditions

Generally, measurement of salivary free cortisol in Addison's patients has been shown to be a reliable method also applicable in further studies. Beyond that, the present results regarding the replacement of basal glucocorticoid levels in Addison's disease clearly show patients to be over-treated. This questions the comparability of the basal hormonal situation in patients with Addison's disease and healthy persons and hence also has implications for Addison's disease as an alternative method in stress research. As outlined above (section 2.1.4), basal glucocorticoid levels exert permissive functions in stress responses, in that their cardiovascular, immunological, and cognitive effects as well as their effects on metabolism permissively help mediating the pending or ongoing stress-response (Sapolsky et al., 2000). This implies consequences to occur whether or not there is a stress-induced increase in glucocorticoid concentrations (Ingle, 1952), but also that normal basal glucocorticoid levels have to be regarded as mandatory. Furthermore, in parallel to the concept of allostasis (i.e., ability to achieve stability through change) and allostatic load in stress research suggested by McEwen et al. (McEwen, 1998a), it can be argued that glucocorticoid replacement in patients with Addison's disease produces pronounced activations of allostatic systems every day. This represents a challenge to allostatic systems of the organism and this wear and tear has a price, which McEwen et al. termed allostatic load. Allostatic load in turn was repeatedly associated with increased health risks, like elevated ambulatory blood pressure (Schnall et al., 1992), increased progression of atherosclerosis (Everson et al., 1997), atrophy of dendrites of pyramidal neurons in the hippocampus (McEwen et al., 1995; Uno et al., 1989), and changes in immune system responses (Cohen et al., 1991; Dhabhar and McEwen, 1997; Hadid et al., 1996). So far, it is largely unknown, which mechanisms in Addison's disease are present to compensate the allostatic load produced by replacement therapy and on what long-term costs for health. Therefore, when investigating the effects of stress on immune parameter in patients with Addison's disease, results have to be interpreted with caution. Distinctive features in patients

with Addison's disease may not be ascribable solely to the missing cortisol stress response but rather to glucocorticoid (over-)treatment, which results in non-physiological high 'basal' glucocorticoid levels and evokes compensatory mechanisms in other bodily systems.

The straightest implication of the present results for further investigations concerns the study design. For studying stress effects in patients with Addison's disease in comparison to healthy controls, patients should avoid ingestion of glucocorticoid replacement several hours prior to study. Otherwise patients will already at baseline show cortisol levels far higher than those healthy subjects will show in response to a laboratory stressor. Again, this will hamper a comparison of patients and healthy subjects as well as the interpretation of results. For this reason, subjects in the second study were asked to postpone the second dose of glucocorticoid replacement at the day of investigation. Since in all patients the first saliva sample was collected at about 2p.m., this requirement resulted in baseline free cortisol levels even lower than those of healthy subjects. Given the free cortisol trajectories found in the first study, this actually was to be expected. Nevertheless, it raises a general methodological question: For satisfying the criterion of hormone subtraction in stress research, may a missing cortisol stress response or no cortisol at all be considered necessary? Basically, this concerns which actions of glucocorticoids one would like to differentiate. According to Sapolsky et al., elimination of stress-induced glucocorticoid levels is one criterion which allows for differentiation between stimulatory, suppressive, and preparative actions of glucocorticoids. Lack of glucocorticoids for several days before a stressor allows to decide whether permissive glucocorticoid actions are required (Sapolsky et al., 2000). As it is not tenable to ask patients for discontinuing of glucocorticoid replacement therapy for several days, we decided in favor of the outlined procedure. However, the rationale for the details were on the one hand related to the circadian rhythm of cortisol. Due to these variations, it is preferable to schedule investigations including measures of cortisol in the afternoon, at the time when cortisol levels show less variations (Kirschbaum and Hellhammer, 1989). On the other hand, patients with Addison's disease take different doses of glucocorticoid replacement as well as different types of medication. Thus, free cortisol levels in patients would have been unpredictable starting the experiment at an earlier hour. The resulting variations in free cortisol levels between patients would have hampered not only the comparability between patients, but also rendered it impossible to utilize a defined dose of hydrocortisone for mimicking cortisol stress responses of healthy subjects. Nevertheless, it is important to keep in mind that investigation of patients with Addison's disease may not be instrumental in deciding on permissive glucocorticoid actions, since other criteria (e.g., time-course, homeostasis) for determining whether a particular glucocorticoid action is permissive require basal levels of glucocorticoids. Which in turn raises an issue already discussed (see above).

5.2.1.2 Initial Stress-Related Conditions

5.2.1.2.1 Stress-related free cortisol levels and hydrocortisone substitution

Beside these considerations regarding basal cortisol levels, the chosen study design proved to be effectually concerning the investigation of stress-related distinctive features in Addison's disease. The present study showed for the first time also empirically that no free cortisol stress response is to be observed in patients with Addison's disease. Thereby, the method Addison's disease fulfills the criterion of hormone subtraction. Furthermore, the observed low baseline cortisol levels in patients allow additionally for realization of the criterion of substitution by hydrocortisone injection. Because as outlined above, findings of an effect being associated with missing glucocorticoid stress responses only provide evidence of glucocorticoids being necessary for this effect. To establish that changes result specifically from lack of glucocorticoid activity, it has to be demonstrated that appropriate administration of exogenous glucocorticoids reverses these changes (Sapolsky et al., 2000). Therefore, we first determined a dose of 0.03mg/kg hydrocortisone *iv* in healthy subjects being appropriate in inducing stress-like free cortisol levels. Subsequently, half of the patients investigated in the second study were treated with this dose of hydrocortisone. The resulting free cortisol levels closely mimicked stress-induced increases and maximum concentrations of healthy subjects. Thus, for these early time-points the chosen study design turned out to be effective. Then again, for later time-points, a faster clearance of free cortisol in patients with Addison's disease was observed. Hence, treatment of patients with a bolus injection is not appropriate to closely mimic the entire free cortisol trajectory found in healthy subjects. In healthy subjects, HPA-reactivity is adjusted to stress duration and this context-dependent HPA-reactivity is controlled by a negative feedback circuit (Chrousos, 1998b; Dallman, 2000). To mimic these cortisol dynamics more closely, it might have been advantageous to administer hydrocortisone as an infusion rather than a bolus injection. Additionally, an infusion could have been applied already shortly before the onset of the stressor and thereby substitute also the increasing cortisol levels as the stressor continues. In the present study, such a procedure would have been not only unpleasant for the patients, but also it was practically not feasible without the disadvantages resulting from adjustments in the study protocol outweighing the disadvantages of a bolus injection. Nevertheless, the resulting differences in free cortisol trajectories between patients treated with hydrocortisone and healthy subjects is an issue to be taken into consideration regarding the interpretation of immune-related results.

5.2.1.2.2 Stress-related catecholamine levels

Regarding catecholamine stress responses, the findings of very low to absent epinephrine levels accompanied by slightly elevated norepinephrine levels are again in accordance with former findings (Bornstein et al., 1995; Eisenhofer et al., 1995; Merke et al.,

2000; Wurtman, 1966), albeit to our knowledge this has not been demonstrated so far during stress and in humans contemporaneously. However, to which extent norepinephrine compensates nearly nonexistent epinephrine levels is debatably. Basically, norepinephrine and epinephrine differ in their binding affinity to adrenoceptors: While norepinephrine predominantly activates α- and β1-adrenoceptors and is a weak stimulator of β2-adrenoceptors, epinephrine is a strong stimulator of β-adrenoceptors (Motulsky and Insel, 1982). Adrenoceptors in turn are not uniformly distributed. For example, β2-adrenoceptors are not expressed on Th2 cells (Sanders et al., 1997). Theoretically, this might raise the question, if in cases of lacking high-affinity ligands (i.e. epinephrine) binding of a ligand with lower affinity (i.e. norepinephrine) to the respective receptor will in all circumstances induce the identical signal. Generally, if a ligand with higher receptor affinity is lacking, the ligand with the next lower affinity will bind preferentially to this respective receptor, thereby inducing the same signal as the high-affinity ligand. Adjustment of receptor expression and sensitivity may support this compensation (Elenkov et al., 2000). Further, since epinephrine is synthesized in the adrenal medulla by conversion of norepinephrine (Bornstein et al., 1995), it can be argued that without such conversion norepinephrine levels are slightly elevated, because they include the fraction to be converted to epinephrine. Thus, it is conceivable to assume that norepinephrine compensates lacking epinephrine not only at the receptor level with regard to signal induction, but also with regard to circulating levels. Hence, in the present thesis Addison's disease will be primarily understood as a model for missing glucocorticoid stress responses. Nevertheless, regarding interpretation of immune-related results, lacking epinephrine levels will be considered where appropriate.

5.2.1.2.3 Cardiovascular stress-response

The present findings regarding cardiovascular parameters encourage the assumption of norepinephrine compensating lacking epinephrine levels, as no differences in blood pressure and heart rate trajectories were found between patients with Addison's disease and healthy subjects and additionally hydrocortisone treatment revealed to be without any effect. Certainly the missing effect of hydrocortisone treatment may also be attributable to dosage and/or frequency being too low to exert effects. But the hydrocortisone dose utilized in the present study was more in a physiological range of cortisol levels than the doses used in studies observing hydrocortisone-induced increases in blood pressure (cf. Kelly et al., 1998; Whitworth et al., 1995). This points to glucocorticoids at physiological concentrations rather exerting permissive than stimulatory actions, an interpretation supported by findings of Allolio et al. demonstrating the importance of normal glucocorticoid levels for catecholamine responsiveness of β2-adrenoceptor in patients with Addison's disease (Allolio et al., 1994). And in this regard, glucocorticoid and mineralocorticoid replacement therapy appears to be

successful, since it was previously shown to restore cardiac functions in patients with Addison's disease (Fallo et al., 1999; Fallo et al., 1994). This is also suggested by the finding of missing differences in resting blood pressure and heart rate between patients with Addison's disease and healthy subjects in the present study.

5.2.1.2.4 Psychometric data

In addition to endocrine and cardiovascular data, also psychometric data were collected in the present studies. These included stress-related questionnaires measuring perceived stress, chronic stress, and stress resistance, but also questionnaires measuring mood, fatigue, depressive symptoms, self-esteem, and anxiety. Interestingly, with the one exception of patients reporting more general and physical fatigue (Multidimensional Fatigue Inventory; Smets et al., 1995), patients with Addison's disease did not differ in any of the questionnaires from healthy subjects and none of the questionnaires were related to any of the endocrine data (data not shown). Whereas the former is in clear contrast to oral information of, for example, reduced physical resilience or reduced stress resistance, the latter is a quite common phenomenon in stress research. In both cases it may only be speculated as for what reason data do not correspond. However, from a methodological point of view, missing distinctive features in Addison's disease other than an altered endocrine state have to be regarded as advantageous for a stringent interpretation of results.

5.2.1.2.5 Altered endocrine state and diseases

Taken together, patients with Addison's disease are characterized by elevated basal free cortisol levels at most times throughout the day accompanied by a lacking cortisol stress response. Beside understanding Addison's disease as a method, this endocrine situation may as well be translated into basal hypercortisolism accompanied by stress-related HPA-hyporeactivity, which from a patient's point of view raises the question, what consequences for health and disease this combination implicates. To predict such consequences, first it would be necessary to determine which diseases are associated with basal and which with stress-related hypo- or hyperactivity and further, which diseases are characterized by a combination of these. For example in diabetes, basal hypersecretion has been found to be associated with impaired cortisol stress responsiveness (Chan et al., 2003). Next, cause and consequence have to be distinguished, but often processes are interrelated and no clear-cut distinction can be made, such as HPA dysregulation in diabetes involving impaired glucocorticoid negative feedback sensitivity and hypoinsulinemia, hyperglycemia and/or hypoleptinemia increasing central drive of the HPA axis (Chan et al., 2003). The picture becomes even more complicated by including short- vs. long-term effects: While hyperthyroidism is primarily associated with hypercortisolism, long-term experimentally-induced hyperthyroidism is associated with diminished adrenal functional reserve (Johnson

et al., 2005). Furthermore, glucocorticoid concentrations are not directly related to glucocorticoid actions, as a wide range of factors (see section 2.3.4.2) modulate glucocorticoid sensitivities of target tissues (Bamberger et al., 1996). Unfortunately, this leads to the conclusion that not only in patients with Addison's disease virtually every pathophysiological state may be predicted from or explained by the given endocrine situation, depending on which and how many factors will be considered or discounted. Wherewith one arrives at the starting point of the present thesis, which is to improve exactly this situation.

Nevertheless, specifically in autoimmune Addison's disease the components of autoimmune polyendocrine syndromes 1 (APS-1) and APS-2 tend to develop in a specific sequence. In APS-1, chronic mucocutaneous candidiasis is generally the first disease to appear, followed by hypoparathyroidism, which usually precedes Addison's disease (Betterle et al., 1998). In APS-2, type 1 diabetes mellitus precedes Addison's disease, with in turn mostly precedes chronic thyroiditis, whereas Graves' disease/hyperthyroidism usually precedes and Hashimoto's thyroiditis/hypothyroidism follows Addison's disease (Betterle et al., 2004). However, both APS-1 and APS-2 are associated with further, more or less frequently occurring so-called minor diseases. Beside every component of APS-1 potentially being found as co-morbidity in APS-2 and the other way around, minor diseases are gastrointestinal diseases, such as chronic atrophic gastritis, pernicious anemia, and malabsorption, chronic active hepatitis, or autoimmune skin diseases, such as vitiligo and alopecia (Betterle et al., 1998; Betterle et al., 2004). Given the etiologies and immunogenetics of Addison's disease (Betterle and Zanchetta, 2003; Peterson et al., 2000), it is certainly not assumed that preceding diseases *cause* later appearing diseases. But from a psychoneuroendocrinological and -immunological point of view it would be most interesting to longitudinally survey patients with Addison's disease in order to reveal the impact of lacking glucocorticoids and various replacement regimens on the developmental pace and progress of following minor diseases not sharing a common immunogenetic cause with Addison's disease. However, maybe due to the small number of patients and/or the cross-sectional design, no associations between etiologies, disease durations, replacement regimens, time intervals between disease onsets, and number or types of co-morbidities were found in the present studies (data not shown).

5.2.1.3 Summary

In summary, patients with Addison's disease show no cortisol stress response but elevated basal free cortisol levels at most times throughout the day. The lacking cortisol stress response thereby constitutes the primary requirement for the present thesis, with the findings regarding free cortisol levels resulting from glucocorticoid replacement therapy setting the specifics for study design. The almost inexistent epinephrine levels and stress

responses are regarded as unproblematic since norepinephrine is to be expected to compensate lacking epinephrine actions. No distinctive features in patients with Addison's disease were found with respect to psychometric and cardiovascular data. Thus, they do not represent influencing factors mandatory to consider for further investigations and interpretation of subsequent results. Based on these findings, investigation of patients with Addison's disease can be regarded as a valuable approach helping to reveal the relevance of a long-term altered endocrine stress response for health and disease.

5.2.2 Effects of Altered Endocrine Stress Responses on the Immune System

Beside empirically establishing the initial endocrinological conditions in Addison's disease, the present thesis also aimed at applying this approach to psychoneuroimmunological problem formulations. In this regard, several immune parameters were measured before and repeatedly after stress: Numbers and percentages of blood cell subtypes, LPS-stimulated IL-6 production, PHA-stimulated cell proliferation, glucocorticoid sensitivities of the latter two, and NF-κB activity. These parameters were chosen on behalf of their widespread use in PNI and/or the central role these parameters are playing in the immune system (see section 4.2 und 4.3).

5.2.2.1 Baseline Differences

Interestingly, in none of these parameters differences between patients with Addison's disease and healthy subjects were found *before* stress onset. Considering the high free cortisol levels induced by replacement therapy, it certainly would have been reasonable to expect at least some distinctive features in patients with Addison's disease. Especially, since the measure of glucocorticoid sensitivity of IL-6 production and cell proliferation not only involved stimulation of immune cells, but also inhibition by dexamethasone, and thus processes compensating for almost permanently elevated free cortisol levels – at least those not involving increased production of corticosteroid-binding globulin or 11β-HSD2 – should have become apparent. But the dose-response curves in both parameters were virtually identically. Alternatively, it may have been argued that patients were asked to skip their second replacement dose and thus their free cortisol levels were atypical low. But again, immune parameters turned out to be unaffected by this altered endocrine state. Although these findings are rather surprising, from a patient's point of view they may be regarded as advantageous, as well as from a methodological point of view, as they allow to focus on stress-related *re*activity. Conversely, these results suggest that basal glucocorticoid *activity* per se does not alter immune states, but this assumption certainly has to be proven by future studies investigating additional immune parameters and functions.

5.2.2.2 Stress-Related Differences

The measure of number and proportion of immune cells in the circulation can be regarded as the easiest way to gain insight into stress effects on immune system activation. Thereby, initially increasing cell numbers point to immune activation due to recruitment of cells, while subsequently decreasing numbers of cells point to redistribution of cells to inflammatory sites. The most pronounced differences in this stress-related immune cell trafficking between patients with Addison's disease and healthy subjects were observed in the lymphocyte proportion. Comparing numbers and proportion of cell subtypes, changes in white blood cell numbers in healthy patients appear to be due to lymphocyte recruitment immediately after stress, followed by their redistribution one hour after stress and a recruitment of granulocytes between one and two hours after stress. In patients with Addison's disease, the slow increase in white blood cell numbers over the course of the investigation is attributable solely to recruitment of lymphocytes. Both findings closely reflect previous findings in healthy subjects (Benschop et al., 1996) and adrenalectomized rats (Dhabhar et al., 1996), which additionally suggest catecholamines to be responsible for recruitment and glucocorticoids for redistribution. The present finding in patients with Addison's disease supports this interpretation of glucocorticoids being important for lymphocyte redistribution. But while the processes in healthy subjects do make sense physiologically (see section 4.3.5), a recruitment of lymphocytes to peripheral blood without their subsequent migration to inflammatory sites cannot be regarded as immunological advantageous, since it attenuates the ability of the immune system to efficiently respond to local infections. Stress thus increases for several hours the risk of infections in patients with Addison's disease. Interestingly, treatment of patients with hydrocortisone did not restore cell number trajectories. This may be explained on the one hand by the faster clearance of free cortisol levels in patients resulting in cortisol levels at later time-points being to low to provide for redistribution. Alternatively, over-treatment of patients with glucocorticoid replacement may have decreased the sensitivity of cells to regulatory actions of glucocorticoids regarding the expression of cell adhesion molecules (Cronstein et al., 1992; Pitzalis et al., 2002).

However, findings in LPS-stimulated IL-6 production rather argue for the former interpretation, as patients do not show – at least in general – a reduced sensitivity of immune cells to glucocorticoid signaling. In fact, patients in the present study showed a very pronounced decrease in LPS-stimulated IL-6 production after hydrocortisone treatment suggesting a preserved sensitivity of peripheral blood mononuclear cells to inhibiting glucocorticoid action. The assumption of glucocorticoids suppressing IL-6 production is also supported by findings in healthy subjects, in which higher free cortisol levels were associated with lower LPS-stimulated IL-6 production and vice versa. Furthermore, untreated patients characterized by very low free cortisol levels showed increases in LPS-stimulated IL-6 levels

in response to stress without further variations in the subsequent two hours. Taken together, all these data clearly point to glucocorticoids suppressing IL-6 production.

What causes the increase in LPS-stimulated IL-6 production after stress seen in patients with Addison's disease? One self-evident candidate is norepinephrine, which is known to up-regulate IL-6 production (Maimone et al., 1993; Norris and Benveniste, 1993). Own previous findings support this assumption, as norepinephrine was shown to increase NF-κB activity (Bierhaus et al., 2003) and increased NF-κB activity in turn will result in increased cytokine production (McKay and Cidlowski, 1999). Unfortunately, in the present study NF-κB activity in untreated patients decreased instead of increased in response to stress and again norepinephrine appeared to mediate this effect. This points to different signaling pathways by which norepinephrine may influence cytokine production. Thus, while suppressing NF-κB activity for example by activating the cAMP/PKA pathway via β-adrenoceptor coupling to G_s-protein (Elenkov et al., 2000), norepinephrine may directly or indirectly increase cytokine production by another pathway. However initiated, increased IL-6 production puts patients with Addison's disease on risk of enhanced inflammatory responses during and several hours after stress.

Another already mentioned conclusion to be drawn from these data is that peripheral blood mononuclear cells remain sensitive to glucocorticoid signaling despite long-term replacement therapy and despite long-term lacking glucocorticoid stress responses. Importantly, this conclusion is based on the trajectories of LPS-stimulated IL-6 production, not on the findings regarding glucocorticoid sensitivity measured by dexamethasone dose-response curves. In these actual glucocorticoid sensitivity measures, no differences between healthy subjects and patients were found, just a slight decrease in sensitivity immediately and one hour after stress, respectively. Although glucocorticoid sensitivity in patients showed very pronounced decreases in response to hydrocortisone treatment, this effect was solely mediated by the pronounced effects of hydrocortisone on LPS-stimulated IL-6 production. Hence, it has to be concluded that the presently utilized assay was ill-suited to reflect glucocorticoid sensitivity properly due to a ceiling effect induced by hydrocortisone. If it would not have been for this ceiling effect, surely the use of dexamethasone as suppressant in the whole blood assay instead of the more obvious use of hydrocortisone would have to be discussed. But in the given situation, the use of dexamethasone – theoretically – even increased the change to detect differences in glucocorticoid sensitivity due to its higher receptor binding affinity and independence of CBG and 11β-HSD2 levels. Nevertheless, in future studies it might be interesting to use both dexamethasone and hydrocortisone in parallel and compare their effects. However, beside these methodological considerations, the fact remains that patients with Addison's disease respond surprisingly sensitive to glucocorticoid signaling. Since at most times throughout the day patients show pronouncedly

elevated instead of almost undetectable free cortisol levels, it may be hypothesized that this conserved glucocorticoid sensitivity will result in attenuated immune responses to challenge. This leaves patients not only with a higher risk for infections during times of stress (see above), but also with an impaired inflammatory response at most times of the day.

Although not in the focus of the present thesis, the finding of free cortisol levels predicting NF-κB activity in healthy subjects is worth to mention. It is well known that glucocorticoids repress NF-κB activity und by this mechanism exert many of its suppressive actions (De Bosscher et al., 1997; De Bosscher et al., 2000b; Ray and Prefontaine, 1994; Scheinman et al., 1995a). But to the best of our knowledge, this is the first time that this was also shown in humans and for stress-related glucocorticoid levels.

The last two parameters not addressed so far are PHA-stimulated cell proliferation and glucocorticoid sensitivity of PHA-stimulated cell proliferation. Here, neither untreated nor hydrocortisone treated patients show any differences compared to healthy subjects. Therefore it has to be concluded that cell proliferation appears to be uninfluenced by glucocorticoids. It may further be concluded that as soon as an immune response is initiated and the third line of immune defense, i.e. adaptive immunity comes into operation, glucocorticoids no longer interfere with these processes. But although proliferation of T-cells (which are predominantly stimulated by PHA) is centrally to an effective adaptive immune response, it reveals little about the functional capabilities of the effector cells T-cell differentiate into. These must be assessed by functional assays, such as cytotoxicity assays in the case of CTLs or assays measuring cytokines produced by Th1 and Th2 cells. Unfortunately, in the present study IL-4 and IFN-γ levels were not detectable in too many subjects to be statistically evaluated. However, glucocorticoids not only do influence immune responses at these later stages in causing a shift from cellular to humoral immunity (Daynes and Araneo, 1989; Elenkov, 2004; Tuckermann et al., 2005), but already interfered with these processes at very early stages by modulating the differentiation, maturation and function of antigen-presenting cells (Kitajima et al., 1996; Tuckermann et al., 2005). Therefore, physiologically it would not make sense to first direct an immune response and next to interfere with such a central step as proliferation of rare antigen-specific lymphocytes.

Beside missing effects of disease and treatment, one to two hours after stress all subjects showed an increase in PHA-stimulated cell proliferation accompanied by a decrease in its glucocorticoid sensitivity. While the latter effect appeared to be mainly a consequence of the former (data not shown), the question arises what causes cell proliferation to increase. Despite stress-induced increases in norepinephrine being responsible for this rather late effect is unlikely, PHA-stimulated T-cell proliferation has repeatedly been shown to be inhibited and not stimulated by catecholamines or β-adrenoceptor agonists (Chambers et al., 1993; Hadden et al., 1970). The picture becomes even more complicated by findings of a

meta-analysis revealing acute laboratory stressors to decrease lymphocyte proliferation (Segerstrom and Miller, 2004). And since patients with Addison's disease are lacking cortisol, findings of circadian variations in cortisol levels negatively correlating with lymphocyte proliferation (to tetanus toxoid) can not be considered as explanation (Hiemke et al., 1995). Hence, further studies are needed to confirm or disprove the present findings and – in the former case – to determine the mediators and mechanisms.

5.2.3 Summary of General Discussion

Taken together, the findings of the empirical studies presented in this thesis reveal investigation of patients with Addison's disease to be a valuable approach for psychoneuroendocrinology and -immunology. As patients appear to be over-treated resulting in pronouncedly elevated free cortisol levels, it has to be recommended to investigate patients some hours after the last replacement intake to avoid high free cortisol levels interfering with parameters of interest. Furthermore, stress elicited only norepinephrine responses, while no stress response in free cortisol or epinephrine was found. As norepinephrine is expected to compensate lacking epinephrine, an assumption supported by the present cardiovascular data, missing cortisol stress responses remain the main characteristic of Addison's disease, thus fulfilling the criteria of hormone subtraction. Bolus injection of 0.03mg/kg hydrocortisone revealed to be only to a limited degree appropriate to restore normal cortisol stress responses. While it was sufficient to produce pronounced effects in LPS-stimulated IL-6 production, regarding cell trafficking it is suggested that the faster clearance prevented its regulation of lymphocyte redistribution. Surprisingly, the immune system appeared to be rather unaffected by the endocrine state in Addison's disease, as glucocorticoid replacement therapy did not induce generally altered immune functions, including the suggested decreased glucocorticoid sensitivity of immune cells. Quite the contrary was found, LPS-stimulated IL-6 production revealed to be distinctively sensitive to inhibitory glucocorticoid signals. Furthermore, immune-related findings in untreated patients suggest them being at an increased risk for infections during and after stress accompanied by an exacerbated inflammatory response once the immune system is activated. However, this situation may be reversed if findings are transferred to the daily free cortisol situation induced by glucocorticoid replacement therapy. Thus, without further investigations of the effects of daily replacement therapy on immune processes, additional glucocorticoid replacement of patients during times of stress cannot be recommended. The role of NF-κB as mediator of immune-inhibitory glucocorticoid actions also regards further investigation. From the present data it can only be hypothesized that during stress norepinephrine may compensate missing glucocorticoid signaling by itself repressing NF-κB activity. But such an interpretation is hampered by the missing association between

endocrine parameters and NF-κB activity in hydrocortisone treated patients and the low overall numbers of subjects investigated in the present study. Nevertheless, the present – for the most part surprising – results clearly emphasize the need for studying complex and interrelated processes also in human whole organism to allow for uncovering compensatory mechanisms.

5.3 OUTLOOK

Although a large number of studies have investigated HPA axis function and abnormalities in human diseases, clear-cut evidence for the involvement of glucocorticoids in predisposing individuals to the development or exacerbations of specific diseases have so far predominantly been derived from animal studies or have been deduced from *in vitro* findings. This work introduced an additional approach, namely investigation of patients with Addison's disease, to measure the effects of context-independently long-term altered glucocorticoid stress responses on the immune system. Studying such effects in patients with Addison's disease may provide valuable insights in the interplay of endocrine stress systems in human whole organism, the extend to which an organisms is able to compensate dysregulations in these systems, and the clinical relevance of psychoneuroendocrinological and -immunological findings for health and disease.

Based on the present findings, future studies certainly should extend the assay repertoire and investigate effects of missing endocrine stress responses also on other parameters of innate and adaptive immunity, such as natural killer cells or Th1/Th2 balance. Furthermore, it would be interesting to evaluate plasma cytokine levels instead of the stimulated levels measured in the present study. This would allow to directly get a picture of what *is* going on instead of what *could* happen. Additional valuable insights into glucocorticoid-immune interactions may be gained by investigating patients with Addison's disease for influences of daily glucocorticoid replacement therapy on immune functions, disease progression, and co-morbidity development. Another interesting problem formulation for future studies concerns the immune-related role of augmented and sustained ACTH stress responses repeatedly found in association with adrenalectomy (Akana et al., 1988; Jacobson and Sapolsky, 1993). Treatment of patients with epinephrine in contrast to or in combination with hydrocortisone may further help to decide on the specificity of this stress mediator with regard to immune regulation in humans. It may further be advantageously to find a way to decelerate the clearance of hydrocortisone, so that free cortisol trajectories in patients with Addison's disease more closely resemble stress-induced cortisol trajectories found in healthy subjects.

Further efforts should also be undertaken to uncover the role of variables potentially mediating or moderating associations found between stress/glucocorticoids and immune

functions, such as gender or age. In this regard, the interplay between stress, age, NF-κB activity, and stress-related immune alterations might be of special interest in relation to the well-known phenomenon of immune senescence. Furthermore, it will be of particular importance to evaluate in future studies to what extent serologic and immunogenetic states characterizing different etiologies of Addison's disease (see section 2.2.6) influence stress-related endocrine-immune associations.

In summary, the results presented in this work show that investigation of patients with Addison's disease is a promising approach to further elaborate in humans the role of glucocorticoid stress responses for health and disease. Employment of this approach in future studies will certainly contribute to clarify the clinical relevance of PNE and PNI findings.

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(Jutta M. Wolf)