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Effects of Stress Mechanisms on Pain Processing

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General Abstract

Stress and pain are common experiences in human lives. Both, the stress and the pain system have adaptive functions and try to protect the organism in case of harm and danger. However, stress and pain are two of the most challenging problems for the society and the health system. Chronic stress, as often seen in modern societies, has much impact on health and can lead to chronic stress disorders. These disorders also include a number of chronic pain syndromes. However, pain can also be regarded as a stressor itself, especially when we consider how much patients suffer from long-lasting pain and the impact of pain on life quality. In this way, the effects of stress on pain can be fostered. For the generation and manifestation of chronic pain symptoms also learning processes such as classical conditioning play an important role. Processes of classical conditioning can also be influenced by stress. These facts illustrate the complex and various interactions between the pain and the stress systems. Both systems communicate permanently with each other and help to protect the organism and to keep a homeostatic state. They have various ways of communication, for example mechanisms related to endogenous opioids, immune parameters, glucocorticoids and baroreflexes. But an overactivation of the systems, for example caused by ongoing stress, can lead to severe health problems. Therefore, it is of great importance to understand these interactions and their underlying mechanisms.

The present work deals with the relationship of stress and pain. A special focus is put on stress related hypocortisolism and pain processing, stress induced hypoalgesia via baroreceptor related mechanisms and stress related cortisol effects on aversive conditioning (as a model of pain learning). This work is a contribution to the wide field of research that tries to understand the complex interactions of stress and pain. To demonstrate the variety, the selected studies highlight different aspects of these interactions. In the first chapter I will give a short introduction on the pain and the stress systems and their ways of interaction. Furthermore, I will give a short summary of the studies presented in Chapter II to V and their background. The results and their meaning for future research will be discussed in the last part of the first chapter.

Chronic pain syndromes have been associated with chronic stress and alterations of the HPA axis resulting in chronic hypocortisolism. But if these alterations may play a causal role in the pathophysiology of chronic pain remains unclear. Thus, the study described in Chapter II investigated the effects of pharmacological induced hypocortisolism on pain perception.

Both, the stress and the pain system are related to the cardiovascular system. Increase of blood pressure is part of the stress reaction and leads to reduced pain perception. Therefore, it is important for the usage of pain tests to keep in mind potential interferences from activation of the cardiovascular system, especially when pain inhibitory processes are investigated. For this reason we compared two commonly and interchangeably used pain tests with regard to the triggered autonomic reactions. This study is described in chapter III.

Chapter IV and V deal with the role of learning processes in pain and related influences of stress. Processes of classical conditioning play an important role for symptom generation and manifestation. In both studies aversive eyeblink conditioning was used as a model for pain learning. In the study described in Chapter IV we compared classical eyeblink conditioning in healthy volunteers to patients suffering from fibromyalgia, a chronic pain disorder. Also, differences of the HPA axis, as part of the stress system, were taken in account. The study of Chapter V investigated effects of the very first stress reaction, particularly rapid non-genomic cortisol effects. Healthy volunteers received an intravenous cortisol administration immediately before the eyeblink conditioning. Rapid effects have only been demonstrated on a cellular level and on animal behavior so far.

In general, the studies presented in this work may give an impression of the broad variety of possible interactions between the pain and the stress system. Furthermore, they contribute to our knowledge about theses interactions. However, more research is needed to complete the picture.

Content

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Index of Publications

This doctoral thesis consists in general of four chapters (and one additional chapter that represents a general introduction and overview), which are published or submitted for publication as 'Original Research Articles' in international peer-reviewed journals. The author is first author of three articles (one of them with a shared first authorship; here, both authors contributed equally to the study) and co-author of one. All articles are presented here in the originally published form, except for changes in formatting (i.e. figure labeling, references).

Content	***has been published as/ submitted for publication as***
Chapter II	**Kuehl, L. K.**, Michaux, G. P., Richter, S., Schächinger, H., & Anton, F. (2010). Increased basal mechanical pain sensitivity but decreased perceptual wind-up in a human model of relative hypocortisolism. *Pain.* 149(3):539-46. (Impact Factor 2009: 5.37).
Chapter III	Streff*, A., **Kuehl*, L. K.**, Michaux, G., & Anton, F. (2010). Differential physiological effects during tonic painful hand immersion tests using hot and ice water. *European Journal of Pain*. 14(3): 266-72. * both authors contributed equally to the study (Impact Factor 2009: 3.61).
Chapter IV	Nees, F., Rüddel, H., Mussgay, L., **Kuehl, L. K.**, Römer, S., & Schächinger, H. (2010). Alteration of delay and trace eyeblink conditioning in fibromyalgia patients. *Psychosomatic Medicine.* 72(4):412-8. (Impact Factor 2009: 4.24).
Chapter V	**Kuehl, L. K.**, Lass-Hennemann, J., Richter, S., Oitzl, M. S., Blumenthal, T. D., & Schächinger, H. (submitted). Accelerated trace eyeblink conditioning after cortisol IV-infusion.

Index of Abbreviations

ACTH	adrenocorticotropic hormone
ADX	adrenalectomy
Ag/AgCl	Silver/Silver chloride
AM	arithmetic mean
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANCOVA	analysis of variance
ANOVA	analysis of variance
BL	baseline
BMI	body mass index
BP	blood pressure
BPM	beats per minute
BPT	beats per test
CES-D	Center for Epidemiologic Studies Depression- Scale
CFA	complete Freund's adjuvant
CNS	central nervous system
CR	conditioned response
CS	conditioned stimulus
cm	centimeters
CPT	cold pressure test
CRH	corticotrophin releasing hormone
dB(A)	Decibels (A-scale)
DNIC	diffuse noxious inhibition control
EBC	eyeblink conditioning
EDA	electrodermal activity
ELISA	enzyme-linked immunosorbent assay
EMG	electromyogram
FMS	fibromyalgia syndrome
GABA	γ-aminobutyric acid
GBB	Gießener Beschwerdebogen
GC	glucocorticoid
GR	glucocorticoid receptor
h	hours
HF	high frequency
HIT	hot water immersion test
HPA axis	hypothalamic-pituitary adrenal axis
HR	heart rate
Hz	Hertz
i.a.	inter alia
i.e.	that is
ISI	inter-stimulus interval

ITI	intertrial interval
IWP	inter-digital web pinching
IV	intravenous
kg	kilograms
kg/m^2	kilograms per squaremeters
L	liters
LC	locus coeruleus
LF	low frequency
LTD	long-term depression
LTP	long-term potentiation
MAD	mean absolute deviation
Md	median
Mg	magnesium
mg	milligrams
min	minutes
ml	milliliters
mmHg	Millimeters mercury
MPQ	McGill pain questionnaire
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
m/s	meter per second
µV	microvolts
NaCl	natriumchlorid
ng	nanograms
ng/ml	nanograms per milliliter
NGF	nerve growth factor
NMDA	*N*-methyl *D*-aspartate
NMDAR	*N*-methyl *D*-aspartate receptor
nmol/L	nanomol per liter
NRM	nucleus raphe magnus
NRS	numerical rating scale
NTS	nucleus tractus solitarius
PAG	periaquaeductal gray
pg	picograms
POMC	pro-opiomelanocortin
psi	pounds per square inch
PTSD	posttraumatic stress disorder
QST	quantitative sensory testing
RCT	randomized controlled trial
RR	respiration rate
RVM	rostral ventromedial medulla
s	seconds
SCID	Structured Clinical Interview for DSM-IV
SCL	Symptom Check List
SD	standard deviation

SE	standard error	S/R	stimulus-response
SEM	standard error of the mean	STAI	State-Trait-Anxiety Inventory
SES	Schmerzempfindungs-Skala	UR	unconditioned response
SIA	stress-induced analgesia	US	unconditioned stimulus
SIH	stress-induced hyperalgesia	VAS	visual analogue scale
SNS	sympathetic nervous system		

Chapter I:

General Rationale

The relationship between stress and pain is complex. Both, the stress and the pain system, have adaptive functions and try to protect the organism in case of harm and danger. They also have a regulatory function tending towards a homeostatic state. Therefore, it is not surprising that both systems are able to communicate with each other. The complex and various ways of interaction will be highlighted in this chapter. A famous example is stress-induced analgesia (SIA) when acute stress leads to an analgesic phenomenon. However, stress induced hyperalgesia (SIH) has been reported as well. In both phenomena the endogenous opioid system plays an important role. Additionally to the analgetic effects of endogenous opioids, pain diminishing effects of cortisol and the baroreflex system have been reported. Both effects are related to stress as well. On the other hand, pain can be regarded as a stressor itself. Especially in the case of chronic pain and the chronic suffering of the concerned patients, this relation becomes obvious. In chronic pain syndromes, such as fibromyalgia syndrome (FMS), pain persists without a well-delineated bodily reason. The pain constitutes no longer simply a symptom of an injury or disease, but it becomes a pain syndrome that needs treatment itself. Chronic pain is the result of multiple subtle physical and psychological factors. Inter alia, ongoing stress and dependent alterations of the HPA axis such as a relative hypocortisolism have been suggested to play an important role in the development of chronic pain. Here, the interaction of stress and pain effects may contribute to the maintenance of pain symptoms. For the development, and particularly the maintenance of symptoms also learning processes such as classical conditioning play an important role. These processes are modulated by effects of stress and cortisol as well and underline the complex relation of stress and pain.

The aim of the present work was to contribute to the wide field of research that tries to understand the complex interactions of stress and pain. The studies of this doctoral thesis are selected from different aspects of possible interactions including baroreflex related mechanisms, effects of hypocortisolism, and classical conditioning processes. Therefore, in the present chapter particular attention will be paid to these topics that may reflect the variety of interactions between stress and pain systems.

1.1 What is pain?

Pain is a common experience to all of us. Still, the difficulties that occur when we try to define "pain" are remarkable. The "International Association for the Study of Pain" (IASP) defines pain as "an unpleasant sensory and emotional experience of a body sensation associated with actual or potential tissue damage, or described in terms of such damage" (Merskey, 1986). This definition underlines the loose association between pain and injury and the emotional dimension of "unpleasantness" in addition to the sensory dimension of pain. Pain is always a subjective experience and depends on physiological and psychological factors such as the individual learning history, the cultural background, attention and anxiety (Melzack & Wall, 1996). Pain that is mainly caused by a perturbation of the body can be divided into nociceptive and neuropathic pain. The first is caused by an activation of nociceptors and the second by a disturbance of the nervous system. Nociceptors can be activated by thermical, mechanical and chemical stimulation. The caused tissue damage can result into a local inflammation via a cascade of reactions including synthesis and release of prostaglandin, bradykinin, histamine, cytokins and NGF (nerve growth factor). The developed inflammatory milieu leads directly and indirectly to an enhanced sensitivity of the nociceptors. Neuropeptides such as substance P released from nociceptors additionally enhance the nociceptor sensitivity by a positive feedback slope. In this way, an increased responsiveness to normally painful stimuli of the same modality, hyperalgesia, can develop (for a review see Treede, Meyer, Raja, & Campbell, 1992). Hyperalgesia has also been associated with spontaneous or ongoing pain in the absence of external stimulation and decreased pain thresholds. Enhanced sensitivity in the area of the injury is called primary hyperalgesia. Hyperalgesia can also occur in the surrounded undamaged area which is called secondary hyperalgesia. The two forms of hyperalgesia differ in their sensory characteristics and underlying mechanisms which also depend on the pain modality.

Nociceptors are nerve endings of fast myelinated Aδ- and slower C-fibres which transmit the pain information to the spinal cord. Here, a reflex arc is triggered that leads to flight behavior to avoid further injuries, which is mainly caused by the fast Aδ-fibres. These fibres are also responsible for the first perception of pain, a clear, exactly locatable and fast decading sensation. A second, longer-lasting pain, typically dull and difficult to detect, is caused by the C-fibres that transmit to dorsal horn nociceptive neurons. C-fibres are probably primarily responsible for inflammatory and modulatory processes. Processes initiated by C-fibres include the development of hyperalgesia and maintenance of central sensitization. For signal

transmission, the excitatory transmitter glutamate and the glutamate *N*-methyl *D*-aspartate (NMDA) and α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors play an important role. NMDA-receptors in dorsal horn neurons are usually inactivated by magnesium (Mg) $^{2+}$-blocked ion channels. Strong nociceptive stimulation is able to remove the Mg^{2+}-ion which leads to enhanced calcium influx and facilitated cell depolarization and excitability. Longer-lasting alterations of cell characteristics such as potentiated synaptic transmission of Aδ, C- fibres and dorsal horn neurons can follow. In these processes, also phosphorylation of AMPA-receptors is involved. In consequence of this long-lasting potentiation, even less intensive pain stimuli can lead to a strong activation of dorsal horn neurons (for a review see Dickenson, Chapman, & Green, 1997). These mechanisms underlie the phenomenon of central sensitization.

Via the tractus spinothalamicus, the nociceptive information is transmitted to the thalamus. Signals from the lateral thalamus are projected to the somatosensory cortex which is responsible for the sensory-discriminative dimension of pain including localization, intensity and modality of the stimulus. The medial thalamus projects to the limbic system, insula and frontal cortex which are responsible for the emotional-affective dimension. Further projections from the spinal cord to the brain stem via the tractus spinoreticularis pass to the thalamus, hypothalamus, limbic system and neocortex and contribute to the emotional-affective dimension of pain. Thus, the perception of pain can be described in an affective and sensory way. Affective experiences are feelings such as “intolerable” or “exhausting” and sensory experiences are perceptions such as “burning” or “cutting”. The quality of pain can be measured for example with the McGill pain questionnaire (MPQ; Melzack, 1975). The quantity or intensity of a pain stimulus can be assessed using a numeric rating scale or a visual analogue scale (quantitative sensory testing (QST); Granot, Sprecher, & Yarnitsky, 2003). Other measures for intensity are pain detection and tolerance thresholds (Reulen, Lansbergen, Verstraete, & Spaans, 2003). Additionally, as “less subjective” measures for pain conductance of single nociceptive cells, reactions of the motoric system (e.g. withdrawal reflexes) and of the autonomic system (e.g. sympathetic activation) are used.

Further important components of the pain system are the descending nociceptive pathways. They modulate the nociceptive information from the brain stem via periaquaeductal gray (PAG) and nucleus raphe magnus (NRM) to the spinal cord. The PAG receives pain related information from other brain areas, but also from afferent projections and provides the connection between brain and spinal cord. A stimulation of the PAG can lead to a complete

analgesia. Also, the locus coeruleus (LC) is involved in desceding transmission and projects to and from the brain, mainly to spinal interneurons. An important function of these pathways is the descending inhibition. They can provoke a tonic inhibition of spinal cord cells, and thus lead to increased pain thresholds and decreased responses. Together with segmental inhibitory interneurons, the tonic descending inhibition is an endogenous antinociceptive system that keeps pain within a limit. Besides the descending inhibitory pathways, there are also excitatory pathways that potentiate spinal nociceptive processing. Important mediators of the endogenous antinociceptive system are endogenous opioids such as endorphins, dynorphins, encephalins, and inhibitory transmitters such as γ-aminobutyric acid (GABA). The release of endogenous opioids and activation of their receptors inhibits neuronal nociceptive activity by reducing the release of excitatory transmitters and hyperpolarisation of postsynaptic neurons. Inhibitory functioning can be measured by investigating the phenomenon of diffuse noxious inhibition control (DNIC). DNIC relates to the inhibition of nociceptive dorsal horn activity causing decreased pain perception when an additional heterotopic stimulus is applied (Le Bars, Dickenson, & Besson, 1979a; Le Bars, Dickenson, & Besson, 1979b; Le Bars, Villanueva, Bouhassira, & Willer, 1992). DNIC has been proposed to serve as a contrast-sharpening filter process functioning as a barrier against the uncontrolled spread of pain and keeps pain regions regional and bearable. In general, nociception is regulated by a cooperation of excitatory and inhibitory systems.

Usually, pain has a warning function with survival value: it can occur before a serious injury happens (touching a hotplate), prevent further injuries (as a basis for avoidance learning), set limits on activity and enforce inactivity and rest, which is often essential to ensure recovery and survival. However, there are pains that serve no biologically useful purpose. Examples are chronic pain syndromes such as FMS where pain persists without a well-delineated bodily reason. The pain constitutes no longer simply a symptom of an injury or disease, but it becomes a pain syndrome that needs treatment itself. Chronic pain is the result of multiple subtle physical (e.g. central sensitization or disturbed mechanisms of pain inhibition) and psychological factors (e.g. attention or learning processes). Chronic stress and related neuroendocrinological alterations are supposed to be one of them (for a review see Heim, Ehlert, & Hellhammer, 2000).

1.2 What is stress?

Stress is a common phenomenon to all of us in the modern world, when we think e.g. of traffic jams, job deadlines or exams. From an evolutionary point of view it is quite an old concept that already helped our ancestors to survive in potentially life-threatening situations such as being attacked during hunting. Even though the nature of potential stressors has changed, the organism´s reaction remained more or less the same. If a stressor occurs or is anticipated, a cascade of biological mechanisms follows which redirect the physiology and behavior towards survival: the characteristic "fight or flight" reaction. It includes a fast mobilization of energy, and a down-regulation of those processes that are not necessary for the immediate survival of the organism (Sapolsky, 1998).

To deal with a stressful situation the organism uses two systems – the sympathetic nervous system (SNS) and the hypothalamus-pituitary-adrenal (HPA) axis – to prepare for the characteristic "fight or flight"-reaction. The SNS is part of the autonomic nervous system and very fast activated within seconds when a potential threat occurs. It helps to mediate vigilance, arousal, activation and mobilization. Activation of the SNS leads to release of the catecholamines epinephrine (adrenaline) and norepinephrine from the adrenal glands (norepinephrine is additionally produced in the LC). In turn, an increase in heart rate and blood pressure and a facilitation of respiration and energy release will be initiated. Besides from hormonal functioning, norepinephrine can also work as a neurotransmitter in the brain.

As a second system the HPA axis is activated when a stressor occurs or is anticipated. The hypothalamus secretes releasing hormones into the hypothalamic-pituitary circulatory system. Most important for the stress reaction is the corticotrophin releasing hormone (CRH) which triggers the pituitary within about 15 seconds to release the adrenocorticotropic hormone (ACTH). After ACTH is released into the blood, it triggers the adrenal gland to release glucocorticoid (GC) hormones within a few minutes. The most important GCs in humans are cortisol or corticosterones in rodents, respectively. GCs are steroid hormones which describes the chemical hormone structure that is derived from cholesterine. Steroids are lipophilic and therefore able to cross the cellular membrane without second messengers. GCs bind on glucocorticoid (GRs) or mineralocorticoid receptors (MRs) of the target tissues in the brain and periphery. By mechanisms of translation and transcription, GCs have impact on protein synthesis. Therefore, they are able to influence different processes that prepare the organism to resist the stressor (for a review see Sapolsky, Romero, & Munck, 2000). For that reason a fast release of energy (e.g. release of glucoses) and SNS activation (e.g., cardiovascular

activation by enhancing adrenergic and noradrenergic effects) are promoted, whereas interfering projects such as digestion or reproduction are stopped. GCs also affect the immune system, inhibit inflammatory processes and alter pain sensitivity. However, GC effects on pain immune system functions are ambigious and both, facilitating and suppressive effects, have been reported (Chrousos, 1995). While acute stress suppresses the perception of pain to ensure behaviour of "fight or flight", afterwards this perception is enhanced to avoid further injuries and allow processes of healing. Importantly, GCs may also influence emotion and cognition, especially by binding on GRs and MRs in limbic structures (Kirschbaum & Hellhammer, 1999; Pearson Murphy, 2000). GC effects on learning and memory have been extensively investigated (for reviews see Joels, Pu, Wiegert, Oitzl, & Krugers, 2006; Het, Ramlow, & Wolf, 2005).

The stress response is an adaptive pattern directed towards homeostasis and in a usual case, the organism returns to a homeostatic state by a negative feedback mechanism of the HPA axis. In modern societies though, a growing problem rises from the phenomenon of chronic stress such as mobbing at work, money worries or ongoing conflicts in relationships. The same reaction that helps us in an acute stressful situation can result into negative outcomes if processed over a long time. Stress-related disorders are getting more and more common. Several of these disorders such as chronic fatigue, FMS, chronic pelvic pain, and asthma have been associated with a chronic disturbance of the HPA axis resulting in hypocortisolism (Demitrack, Dale, Straus, Laue, Listwak, Kruesi, Chrousos, & Gold, 1991; Crofford, Pillemer, Kalogeras, Cash, Michelson, Kling, Sternberg, Gold, Chrousos, & Wilder, 1994; Kruger & Spiecker, 1994; Heim, Ehlert, Hanker, & Hellhammer, 1998). An over-activation of the hormonal stress–response system as a result of ongoing strain often leads to a down-regulated adrenocortical responsiveness characterized by relative primary adrenal hypocortisolism with increased feedback inhibition of the HPA axis (Heim et al., 2000; Tsigos & Chrousos, 2002). This paradox phenomenon of reduced cortisol levels has mainly been described in patients with posttraumatic stress disorder (PTSD; Yehuda, 1997), but also in healthy individuals under conditions of ongoing stress (Friedman, Mason, & Hanburg, 1963; Bourne, Rose, & Mason, 1968; 1967; Mason, 1968; Caplan, Cobb, & French, 1979). However, it remains unclear if the stress induced down-regulations of the HPA axis are a maladaptive or a protective mechanism. The damaging effects of GCs on target tissues, such as degeneration of hippocampal neurons (Sapolsky, 1993; Stein-Behrens, Mattson, Chang, Yeh, & Sapolsky, 1994), may be reduced at the expense of the characteristic symptoms of chronic hypocortisolism: stress sensitivity, fatigue and pain (Fries, Hesse, Hellhammer, &

Hellhammer, 2005). In the concerned pain syndromes, it remains still unclear if the pain is cause or consequence of the observed hypocortisolism. Several correlative and prospective studies speak in favour of experienced ongoing stress and depending alterations of the HPA-axis at first resulting in chronic pain (McBeth, Silman, Gupta, Chiu, Ray, Morriss, Dickens, King, & Macfarlane, 2007; Geiss, Rohleder, Kirschbaum, Steinbach, Bauer, & Anton, 2005). However, in general the methods of those studies do not allow any conclusions about a causal relation. Hypocortisolism has been hypothesized to be a relevant factor for mediating the effect of stress on pain chronicity based on the immunosuppressive effects of GCs (Chrousos, 2000; Chrousos & Gold, 1992). Thus, reduced GC levels might disinhibit the secretion of inflammatory mediators and thereby promote the sensitization of peripheral or central nociceptive neurons (Sommer, Häuser, Gerhold, Joraschky, Petzke, Tölle, Uçeyler, Winkelmann, & Thieme, 2008). However, further research is needed.

1.3 Pain as a stressor

The experience of pain can be regarded as a stressor. A stressor is any experience of positive or negative valence that disrupts homeostasis in a physiological or (social-) psychological way. Pain can work as physiological stressor among others such as hypoglycaemia, sleep deprivation or exposition to extreme heat or cold. Therefore, pain stimulation is used in several established laboratory stress tests. One of the most frequently used is the cold pressure test (CPT) that elicits a well-documented stress reaction with increase in heart rate and blood pressure (Wolf & Hardy, 1941; Lovallo, 1975). Additionally, painful hot water immersion tests, application of electro shocks and pressure pain stimulation are common to induce stress. Important moderators are psychological variables such as perceived controllability of the pain and feelings of fear which become especially important in chronic pain.

A tissue trauma, e.g. caused by pain eliciting tests, generates noxious signals and excitation of nociceptors as described above. For example, repetitive stimulation and inflammation sensitize both peripheral nerves and cells of spinal transmission. Thus, firing thresholds are decreased and peripheral and central mechanisms can exacerbate the noxious signalling. Cascades of further central transmission lead to excitation of the paraventricular nucleus of the hypothalamus which initiates the neuroendocrine stress reaction of the HPA axis. As already mentioned, the stress response also affects the immune system (Torpy & Chrousos, 1996). The immune system identifies and destructs foreign substances and communicates

injury related events and tissue pathology to the brain via the release of cytokines activating the vagus nerve and thus the solitary nucleus (Blalock, Smith, & Meyer, 1985). The immune system and the brain seem to communicate in a bidirectional way (Maier & Watkins, 1998). The brain controls the immune system via the SNS and HPA axis. Therefore, a stressor can trigger a constellation of physiological and behavioural changes that is experienced as sickness. Usually, these changes are adaptive and conserve energy and increase body temperature to suppress reproduction of infectious microbial organisms. However, if this pattern of sickness response is extended over some time by activation of the SNS and HPA axis, but without microbial invasion, it gets maladaptive. Via these interactions of immune and stress system, chronic pain can promote an extended and destructive stress response characterized by neuroendocrine dysregulation, fatigue, dysphoria, myalgia, and impaired mental and physical performance. Furthermore, this constellation of discomforts and functional limitations can foster negative thinking and create a vicious cycle of stress and disability (Chapman & Gavrin, 1999). Second psychological stressors activities, productive work, family life and supportive social interactions may potentiate this cycle.

1.4 Stress affecting pain

Acute effects of stress on pain perception are somewhat ambiguous. It is well admitted that stress can induce analgesia (stress induced analgesia (SIA); for a review see Ford & Finn, 2008) mainly via endogenous opioid release (Akil, Mayer, & Liebeskind, 1976; Lewis, Cannon, & Liebeskind, 1980) on the one hand. On the other hand, also stress induced hyperalgesia (SIH) has been reported, but less is known about underlying mechanisms. Furthermore, there is evidence that stressful events and continually experienced stress play a role in the pathogenesis of chronic pain.

Exposure to aversive stimuli or contexts can result in the experience of analgesia. Physical as well as psychological stressors have been shown to induce analgesia in humans (e.g. marathon running or mathematical operation tasks; Bandura, O´Leary, Taylor, Gauthier, & Gossard, 1988; Droste, Greenlee, Schreck, & Roskamm, 1991; Frid, Singer, Oei, & Rana, 1981; Janal, Colt, Clark, & Glusman, 1984; Scott & Gjisbers, 1981) and animals (e.g. inescapable electric footshock or forced swimming; Amit & Galina, 1986). Furthermore, also conditioned SIA has been reported after re-exposure to an environment, context or cue that was paired with an unconditioned aversive stimulus (Fanselow, Calcagnetti, & Helmstetter,

1989; Fanselow & Helmstetter, 1988; Finn, Beckett, Richardson, Kendall, Marsden, & Chapman, 2004; Finn, Jhaveri, Beckett, Madjd, Kendall, Marsden, & Chapman, 2006; Roche, O'Connor, Diskin, & Finn, 2007).

Animal models of conditioned and unconditioned SIA helped to understand the underlying neurobiological mechanisms. Endogenous opioids are suggested to play a key role in mediating endogenous analgesia by activating descending inhibitory pathways. Projections from the amygdala to the opioid sensitive neurons in the PAG and the rostral ventromedial medulla (RVM) are suggested to be critical for expression of conditioned SIA. Certain opioid receptors seem to play a role for conditioned and unconditioned SIA (Helmstetter, 1992; Helmstetter & Landeira-Fernandez, 1990; Helmstetter & Tershner, 1994). Furthermore, the endogenous cannabinoid system in the amygdala, PAG and RVM may mediate a non-opioid form of unconditioned as well as conditioned SIA and its extinction (Hohmann, Suplita, Bolton, Neely, Fegley, Mangieri, Krey, Walker, Holmes, Crystal, Duranti, Tontini, Mor, Tarzia, & Piomelli, 2005; Finn et al., 2004; Roche et al, 2007). Also the central rennin angiotensin system and monoamines may play a role in analgesia after immobilization stress (Haulica, Neamtu, Stratone, Petrescu, Branisteanu, Rosca, & Slatineanu, 1986). Mediating effects of corticosterone and CRH have been shown after forced swim SIA. These effects are probably related to local anti-inflammatory actions and/or promoting release of opioid peptides, such as ß-endorphin (Lariviere & Melzack, 2000; MacLennan, Drugan, Hyson, Maier, Madden, & Barchas, 1982). Local anti-inflammatory GC actions that affect arachidonic acid metabolism show inhibiting effects on pro-inflammatory mediators (Malcher-Lopes, Franco, & Tasker, 2008; Tasker, Di, & Malcher-Lopes, 2006) such as prostanoids that are involved in mechanically induced hyperalgesia (Forster, Anton, Reeh, Weber, & Handwerker, 1988; Growcott, Stone, Beise, Stammer, Tetzloff, & Demey, 2000). Pharmacological studies in humans correlate strongly with data from animal SIA studies (Flor, Birbaumer, Schulz, Grusser, & Mucha, 2002; Willer, Dehen, & Cambier, 1981; Willer & Ernst, 1986; Willer, Von Frenkell, Bonnet, & Le Fur, 1986), but findings still need to be completed.

Another pain-related system that is sensitive for stress is the cardiovascular system. A relation between elevated blood pressure and diminished pain sensitivity has been observed, probably mediated by baroreceptor related, noradrenergic and endogenous opioid mechanisms. In humans, most evidence has been reported for baroreflex related mechanisms (for a review see Bruehl & Chung, 2004).

However, stress exposure can also lead to enhanced pain perception. Hyperalgesia following stress (or SIH) has been reported in animals (Vidal & Jacobs, 1982), especially when stress is repeated for a long time (Satoh, Kuraishi, & Kawamura, 1992; da Silva Torres, Cucco, Bassani, Duarte, Silveira, Vasconcellos, Tabajara, Dantas, Fontella, Dalmaz, & Ferreira, 2003; Bradesi, Schwetz, Ennes, Lamy, Ohning, Fanselow, Pothoulakis, McRoberts, & Mayer, 2005; Khasar, Green, & Levine, 2005; Gameiro, Gameiro, Andrade Ada, Pereira, Arthuri, Marcondes, & Veiga, 2006). The endogenous opioid system seems to play a major role in this case as well. Thermal SIH induced by delayed swim stress could be prevented by opioid receptor antagonists (Suarez-Roca, Silva, Arcaya, Quintero, Maixner, & Pinerua-Shuhaibar, 2006). Exogenous opioid administration induces analgesia, but delayed and longer-lasting hyperalgesia appears dose-dependent after analgesia (Laulin, Larcher, Célèrier, Le Moal, & Simonnet, 1998; Laulin, Maurette, Corcuff, Rivat, Chauvin, & Simonnet, 2002; Célèrier, Laulin, Corcuff, Le Moal, & Simonnet, 2001). A single opioid administration can enhance hyperalgesia following inflammation or surgical incision until 1 week later indicating a facilitated development of long-term pain vulnerability (Richebe, Rivat, Laulin, Maurette, & Simonnet, 2005; Rivat, Laboureyras, Laulin, Le Roy, Richebé, & Simonnet, 2007; Rivat, Laulin, Corcuff, Celerier, Pain, & Simonnet, 2002). Acute restraint stress accompanied by GC release has been shown to enhance pain sensitization (i.e. mechanical allodynia) in an animal neuropathic pain model that could be prevented by a GC antagonist (Alexander, DeVries, Kigerl, Dahlman, & Popovich, 2009). The results suggest that the GC stress response can exacerbate neuropathic pain through enhanced central sensitization involving glutamatergic signaling. Moreover, drugs that inhibit GCs and/or NMDA receptor signaling could ameliorate pain syndromes caused by stress. But the underlying mechanisms of SIH and delimitation to SIA need further research.

Chronic stress has been hypothesized to take part in the pathophysiology of chronic pain syndromes. Various chronic pain syndromes are associated with relative hypocortisolism, a marker for HPA axis dysregulation related to ongoing stress (Crofford et al., 1994; Crofford, Engleberg, & Demitrack, 1996; Elwan, Mohamed Abdella, El Bayad, & Hamdy, 1991; Gaab, Baumann, Budnoik, Gmünder, Hottinger, & Ehlert, 2005; Galli, Gaab, Ettlin, Ruggia, Ehlert, & Palla, 2009; Griep, Boersma, & de Kloet, 1993; Heim et al., 1998; 2000; Lentjes, Griep, Boersma, Romijn, & de Kloet, 1997; Straub, Kittner, Heijnen, Schedlowski, Schmidt, & Jacobs, 2002; Strittmatter, Grauer, Fischer, Hamann, Hoffmann, Blaes, & Schimrigk, 1996; Wingenfield, Heim, Schmidt, Wagner, Meinlschmidt, & Hellhammer, 2008; Zoli, Lizzio, Ferlisi, Massafra, Mirone, Barini, Scuderi, Bartolozzi, & Magaro, 2002). Hypocortisolism has been

hypothesized to be a relevant factor for mediating the effect of stress on pain chronicity based on the immunosuppressive effects of GCs (Chrousos, 2000; Chrousos & Gold, 1992). Reduced glucocorticoid levels might disinhibit the secretion of inflammatory mediators and thereby promote the sensitization of peripheral or central nociceptive neurons (Sommer et al., 2008). Correlative and prospective studies speak in favour of experienced ongoing stress and depending alterations of the HPA-axis at first, resulting in chronic pain (Geiss et al., 2005; McBeth et al., 2007).

1.5 Examples of interactions between pain and stress systems

1.5.1 Stress related hypocortisolism and pain processing

Several chronic pain syndromes have been associated to ongoing stress exposure and a related dysfunctional reactivity of the HPA axis resulting in a relative hypocortisolism as described above. As an underlying mechanism it has been hypothesized that the disinhibiting effects of GCs on the secretion of proinflammatory mediators may result in an ongoing sensitization of nociceptive neurons and in enhanced pain sensitivity (Chrousos, 2000). Prospective studies support this hypothesis. Chronically stressed patients with stress-related HPA axis abnormalities and associated changes in pro-inflammatory cytokine levels are at a higher risk for a poor outcome of lumbar disc surgery (Geiss et al., 2005). Among a group of psychologically at risk subjects, stress-related dysfunction of the HPA axis constituted a risk factor for the development of chronic widespread musculoskeletal pain as seen in FMS for instance (McBeth et al., 2007). Several pain related changes have been reported in patients suffering from chronic pain. Cortical reorganizations in states of chronic pain have been demonstrated (Flor, 2000). Patients with pain diseases such as temporomandibular joint disease (Maixner, Fillingim, Sigurdsson, Kincaid, & Silva, 1998), complex regional pain syndrome (Price, Long & Huitt, 1992), post-herpetic neuralgia (Eide, Jorum, Stubhaug, Bremnez, & Breivik, 1994) and FMS (Meeus & Nijs, 2007; Staud, Vierck, Cannon, Mauderli, & Price, 2001) do not only report disease related enhanced pain, but show also exaggerated or abnormally triggered wind-up. (Perceptual) wind-up is induced by brief repetitive pain stimulation and used as a model for central sensitization. It is probably due to sensitized NMDA receptors on central neurons (Herrero, Laird, & Lopez-Garcia, 2000; Koltzenburg & Handwerker, 1994; Price et al., 1992). In contrast, results of pain threshold alterations in chronic pain are less explicit. Furthermore, FMS patients show enhanced psychophysiological

responses to stress (Thieme, Rose, Pinkpank, Spies, Turk, & Flor, 2006b), which may also contribute to the development and maintenance of this syndrome. However, if the observed hypocortisolism could causally contribute to the development of chronic pain still remains unclear, because the methods of the mentioned studies investigating this relation do not allow any conclusions about a causal relationship. To clarify if reduced cortisol levels could directly lead to alterations in pain perception we investigated the effects of a pharmacologically induced hypocortisolism on different pain paradigms as described in Chapter II. In a randomized placebo-controlled crossover trial, effects of metyrapone induced hypocortisolism on mechanical pain sensitivity, perceptual wind-up and hyperalgesia (temporal summation of pressure pain) were measured by individual quantitative ratings. Experimentally induced hypocortisolism significantly decreased pain detection thresholds and augmented hyperalgesic effects of temporal pain summation depending on the relative reduction in cortisol levels, whereas perceptual wind-up was decreased. The latter result is in line with findings from animal studies showing a reversal of NMDA receptor activation by GC receptor antagonists in neuropathic pain models. Also, acute restraint stress has been shown to enhance other forms of central sensitization (i.e. mechanical allodynia) in an animal neuropathic pain model (Alexander et al., 2009; Wang, Lim, Yang, Sung, & Mao, 2006). Of course, it cannot be excluded that our findings result from a rise in ACTH and other pro-opiomelanocortin (POMC)-derived peptides (e.g. β-endorphin) as well as in CRH. However, that a pharmacological blockade of cortisol synthesis by metyrapone was able to alterate pain perception, even on a behavioral level, supports the hypothesis of a causal involvement of chronic disturbances of the HPA axis, resulting in hypocortisolism, in the pathophysiology of pain chronicity. Chronic hypocortisolism, as a neuroendocrinological correlate of chronic stress, could be a modulating factor in the relation of ongoing stress and alterations in pain perception of chronic pain.

1.5.2 Stress induced hypoalgesia via baroreceptor related mechanisms

An important component of the pain regulatory process is the functional interaction between the cardiovascular and pain regulatory system. A relation between elevated resting blood pressure (BP) and diminished pain sensitivity has been observed in hyper- and normotensives (Bruehl, Carlson, & McCubbin, 1992; Bruehl, Chung, Ward, Johnson, & McCubbin, 2002; Fillingam & Maixner, 1996; Friedman, Murphy, Persons, & McCaughran, 1984; Maixner, Fillingim, Kincaid, Sigurdsson, & Harris, 1997; McCubbin & Bruehl, 1994; Myers,

Robinson, Riley, & Sheffield, 2001, Zamir & Segal, 1979; Zamir, Simantov, & Segal, 1980). Apart from the influence of actual BP levels even healthy people with a familiar risk for hypertension show diminished responsiveness to acute pain stimulation (al´Absi, Petersen, & Wittmers, 2000; France, Froese, & Stewart, 2002; Stewart & France, 1996). This relationship is proposed to reflect an adaptive homeostatic feedback loop that helps to restore arousal levels in the presence of pain (Bruehl, McCubbin, & Harden, 1999; Ghione, 1996) via baroreceptor related, noradrenergic and endogenous opioid mechanisms.

The initial response to nociceptive stimuli engages descending inhibitory mechanisms that allow the organism to escape from the cause of the injury without interference from the sensation of pain to ensure survival (Maixner, 1991; Millan, 2002). Afterwards, the pain regulatory system shifts to rather descending facilitation. Thus, the pain becomes more salient to avoid further injuries and allow healing. Beyond this initial healing, descending inhibitory pathways are activated to facilitate the resumption of normal activities that are required for survival (Millan, 2002). Elevated central descending pain inhibitory activity has been shown to partly mediate the effects of diminished pain perception related to BP (France et al., 2002). Interestingly, the brain regions of the cardiovascular system overlap substantially with those contributing to antinociception (Randich & Maixner, 1984). The nucleus tractus solitarius (NTS) serves as the interface between autonomic and sensory systems and plays an important role in the processing of visceral information, receiving afferent input from the vagus nerve (subserving the baroreflex) and spinal laminae (involved in nociceptive processing). Stimulation of the NTS induces antinociception (Aicher & Randich, 1990) probably by its direct and indirect efferent projections to PAG, nucleus raphe magnus (NRM) and RVM which are involved in modulation of pain pathways. Further efferent projections to the LC might also contribute substantially to BP-related antinociception given that direct LC stimulation leads to analgesia (Burnett & Gebhart, 1991; Jones, 1991; Miller & Proudfit, 1990). Pathways from the NTS to spinal cord nuclei modulating cardiovascular tone also interact with descending pain modulation pathways (Lewis, Baldrighi, & Akil, 1987; Millan, 2002). Via a baroreceptor feedback loop, descending pain inhibitory pathways may be able to self-regulate their activity via autonomic centers of the spinal cord that modulate cardiovascular function. Baroreceptor activation plays a significant role in a functional model that is proposed to explain the BP/ pain sensitivity relationship (Ghione, 1996; Zamir & Maixner, 1986): Pain increases sympathetic arousal via a somatosensory reflex which leads to increased BP. Resulting increased baroreceptor stimulation triggers inhibitory pain activity and helps thereby to return arousal levels to a state of homeostasis. Evidence for the important

role of baroreceptor related mechanisms comes from studies showing that electrical (Bossut & Maixner, 1996; Takeda, Tanimoto, Ojima, & Matsumoto, 1998) and pharmacological (Randich & Maixner, 1984) stimulation of baroreceptor (vagal) afferents leads to antinociception. Normotensives showed diminished pain sensitivity when BP increased spontaneously during a stressful task (al`Absi & Petersen, 2003) or when baroreceptors were experimentally stimulated (Angrilli, Mini, Mucha, & Rau, 1997; D´Antono, Ditto, Sita, & Miller, 2000; Droste, Kardos, Brody, Greenlee, Roskamm, & Rau, 1994; Dworkin, Elbert, Rau, Birbaumer, Pauli, Droste, & Brunia, 1994; Rau & Elbert, 2001). Other studies in normotensives could show that the sensitivity to brief electrical pain stimuli covaried with the cardiac cycle suggesting that the degree of phasic and not tonic baroreceptor stimulation is related to the cardiac cycle (Bruehl & Chung, 2004; Edwards, McIntyre, Carroll, Ring, France, & Martin, 2003; Edwards, Ring, McIntyre, & Carroll, 2001). This assumption is supported by a greater effect size of systolic BP on pain sensitivity (al´Absi & Petersen, 2000; Bruehl et al., 1992; 2002; McCubbin & Bruehl, 1994). At least in normotensives the relationship of BP and pain seems to reflect a homeostatic feedback system that is regulated i.a. by baroreceptor activity. Animal studies suggest an additional importance of endogenous opioid mechanisms, but human data is not that clear. Also, alpha-2 adrenergic mechanisms are discussed to play a role. There is much direct and indirect evidence from animal studies, but evidence from human data is missing.

The complex interaction between SNS and pain, as described above, is reflected in the usage of the cold pressure test (CPT) which is commonly used in research on stress, pain, and cardiovascular activity (al'Absi, Petersen, & Wittmers, 2002; Gluck, Geliebter, Hung, & Yahav, 2004; Hines & Brown, 1936; Mitchell, MacDonald, & Brodie, 2004). The well-known interactions between the three systems suggest potential confoundations. Cold water immersion is often used for experimental characterization of endogenous pain modulation, especially as a trigger stimulus for diffuse noxious inhibition control (DNIC). However, the validity of cold water immersion as a trigger stimulus for DNIC may be confounded by interactions of the cardiovascular and pain regulatory systems. As mentioned above, experimentally induced as well as constitutional hypertension is associated with reduced pain sensitivity caused by baroreflex related mechanisms (Bruehl & Chung, 2004). Thus, observed cold water related decreases in subjective pain may not only be attributed to DNIC, but also to baroreflex related pain inhibition induced by thermoregulatory vasoconstriction. Besides cold water, hot water immersions are used interchangeably, although little is known about specific physiological characteristics. To validate the relative usefulness of the two paradigms for

investigation of DNIC effects, we contrasted both tests with regard to their psychophysical and physiological characteristics in a study described in chapter III. 35 participants accomplished a cold and hot water immersion test in a randomized order. Cardiovascular, respiratory and electrodermal activity, subjective pain intensity as well as pain detection and tolerance thresholds were measured. Even though, time course and subjective pain intensity were comparable in both tests, a significantly higher increase of blood pressure was observed during cold water immersion. Thus, the hot water immersion seems to be less confounded with thermoregulatory baroreflex activity and therefore a more appropriate model to produce experimental tonic pain with less autonomic arousal. Future research using pain stimulating paradigms should pay more attention to confounding effects such as cardiovascular influences or stress interactions to get more validate and generalizable results.

1.5.3 Stress related cortisol effects on aversive conditioning as a model of pain learning

Learning processes such as classical or operant conditioning are believed to play an important role in the development and maintenance of chronic pain (Flor, 2000). Operant mechanisms were first proposed by Fordyce (Fordyce, Fowler, Lehmann, DeLateur, Sand, & Treischman, 1973): (social) positive reinforcement for pain behaviors such as moaning and limping, negative reinforcement such as inactivity and intake of medication and lack of reinforcement of well-behaviors such as working or sports may contribute to the development from acute to chronic pain. Over time, they may mainly be maintained by reinforcement contingencies. The presence of a spouse that habitually reinforces pain can even influence pain related cortical responses (Flor, 2000). Flor and colleagues (2002) could show that patients with chronic back pain were more easily influenced by operant conditioning factors than healthy controls. Therefore, operant treatments such as extinction of pain behaviors, withdrawal of positive reinforcement (e.g. medication) or changes in the environment of the patient (Turk & Flor, 1984) have been recommended. Besides cognitive behavioral treatments, operant behavioral treatments are very effective in treating FMS patients (Thieme, Flor, & Turk, 2006a).

Processes of classical conditioning may contribute to chronic pain as well. Gentry and Bental (1977) suggested that classical conditioning of pain and tension may occur in an acute pain state due to some form of physical damage leading to a pain-tension circle. Pain can be regarded as an antecedent and reaction to muscular hypertension. Avoidance of movement may be used to reduce pain, leading to increased immobility. That may increase the tension

and pain still more. With time, more and more situations may elicit pain and anxiety. For example, stress is among others one of the most commonly reported triggers for headache (Martin, Milech, & Nathan, 1993). The pain-tension cycle may be further intensified if depression and medication follow. Additionally, conditioned fear of movement resulting in motivating avoidance of activity may not only lead to immobilization, but also result in muscular atrophy and increasing disability (Caldwell & Chase, 1977). Data suggests that muscle tension levels are easily conditioned and sensitized when painful stimuli are applied and support conditioning accounts of muscle tension increases in chronic pain patients (Diesch & Flor, 2007; Flor, Birbaumer, Schugens, & Lutzenberger, 1992; Schneider, Palomba, & Flor, 2004). Enhanced muscular responding during aversive conditioning using electric stimuli has been observed in patients with chronic back pain. Muscular and central processes seemed to be dissociated which may contribute to pain chronicity (Schneider et al., 2004).

In a study described in Chapter V we investigated learning processes in FMS patients using a non-painful aversive conditioning paradigm of eyeblink conditioning (EBC). As described above, FMS has often been associated with chronic alterations of the HPA axis resulting in a relative hypocortisolism. Our data of morning cortisol levels from FMS patients support these observations. FMS patients showed facilitated trace EBC and impaired delay EBC compared to healthy controls. A slower extinction of trace conditioning was observed, but no difference for delay conditioning. EBC correlated significantly with lower cortisol levels in trace conditioning exclusively. Our data supports on the one hand the hypothesis of enhanced conditioning in chronic pain patients that may contribute to the chronicity of pain. On the other hand, the facilitation of EBC was related to the alterations of the HPA axis, the relative hypocortisolism. The reduced cortisol levels seemed to play a role in the conditioning processes of trace EBC. They were associated with facilitated learning and slower extinction. Aversive conditioning can be regarded as model for pain learning, and thus, our data suggests that the altered function of the HPA axis may contribute to a facilitated learning of symptoms and a slower extinction of learned symptoms.

An important role of GCs in trace EBC has been reported before. Pharmacologically induced and endogenous hypercortisolism impaired trace, but not delay conditioning (Grillon, Smith, Haynos, & Nieman, 2004; Vythilingam, Lawley, Collin, Bonne, Agarwal, Hadd, Charney, & Grillon, 2006). Facilitated trace EBC was observed in healthy volunteers with pharmacologically suppressed cortisol production whereas delay EBC was not affected (Nees,

Richter, Lass-Hennemann, Blumenthal, & Schächinger, 2008). Trace conditioning is mainly mediated by the cerebellum and the hippocampus (Berger & Thompson, 1978; Clark & Squire, 1998; Moyer, Deyo, & Disterhoft, 1990; Woodruff-Pak & Papka, 1996), whereas for delay conditioning only the cerebellum is needed (Lavond, Kim, & Thompson, 1993). The hippocampus shows a great amount of glucocorticoid and mineralocorticoid receptors (de Kloet, Reul, & Sutanto, 1990) and is thus sensitive for cortisol. Most studies that investigated effects of stress and GCs on learning and memory refer to hippocampus-based declarative memory. Stress has been shown to influence the quantity and quality of learning and memory. Depending on intensity and timing of the stressor or the administration of exogenous GCs, the effects show a remarkable diversity. Stress and GCs can on the one hand change the amount of *what* is learned, on the other hand the quality of learning (*how* we learn). Under stress, rigid habit memory gets favored over more flexible cognitive memory. This shift between differential behavioral strategies on environmental demands may facilitate adaptive responses (Schwabe, Wolf, & Oitzl, 2010). The timing of the stressor or GC administration leads to different effects depending on the affected stage of the memory process. The memory process consists of encoding/acquisition, consolidation, and retrieval stage. Thus, learning can be regarded as a first step in the memory process. In general, stress or GC administration before acquisition have rather ambiguous effects on memory (Buchanan & Lovallo, 2001; Kirschbaum, Wolf, May, Wippich, & Hellhammer, 1996; Newcomer, Craft, Hershey, Askins, & Bardgett, 1994), whereas enhancing effects were found when applied before consolidation (Cahill, Gorski, & Le, 2003; Roozendaal, 2000; Roozendaal, 2002) and impairing effects before retrieval (Buss, Wolf, Witt, & Hellhammer, 2004; De Quervain, Roozendaal, & McGaugh, 1998; de Quervain, Roozendaal, Nitsch, McGaugh, & Hock, 2000; Kuhlmann, Kirschbaum, & Wolf, 2005; Kuhlmann, Piel, & Wolf, 2005). However, also the time of the day when cortisol is administered plays an important role (Het et al., 2005), and can reverse the effects. Another important aspect is the intensity of the stressor or amount of GC administration. The relation of stress intensity and memory corresponds to an inverse U-shaped curve (Baldi & Bucherelli, 2005), whereas medium stress intensities or amounts of GCs, respectively, show the greatest increase in memory. Additionally, the effects depend on the quality of the learned material. Emotional contents are in general better learned and remembered (Buchanan & Lovallo, 2001; Cahill et al., 2003; Payne, Jackson, Ryan, Hoscheidt, Jacobs, & Nadel, 2006). Stress affects physiological processes underlying learning and memory such as NMDA-receptor dependent changes during stress in the CA1 of the hippocampus that are assumed to alter the inducibility of long-term potentiation (LTP) and

long-term depression (LTD) (Kim, Foy, & Thompson, 1996). GCs also seem to be involved in the plasticity of the hippocampus (McEwen, 1999). They can modulate the excitability of hippocampal neurons and the strength of LTP. Together with amino acids, they have impact on the neurogenesis in the dentate gyrus and on the stress induced atrophy in the CA3. The diverse effects of stress on memory have been explained by a model of Joels et al. (2006). According to that model, stress leads to facilitated learning and memory if stress is experienced in the context and around the time of the concerned event, and if the hormones and transmitters released in response to stress exert their actions on the same circuits as those activated by the situation. The model suggests that GCs, CRH and norepinephrines on the one hand facilitate the current cognitive processes, on the other hand they impede interfering memory processes such as acquisition of new or retrieval of old contents by a gene-mediated pathway. Thus, the impairing effects of stress on memory are probably due to an adaptive function which ensures that ongoing memory processes are not disturbed.

The mechanisms that are suggested in this model to underly the influence of stress and GCs on learning and memory are based on genomic actions of GCs. However, besides the genomic actions of GCs, several studies have shown rapid non-genomic GC action. These rapid GC effects occur within seconds or up to a few minutes, but may also last longer, depending on the tissue. Most genomic effects of cortisol are expected within hours and hardly before an interval of 10 to 20 minutes has passed. Even faster genomic effects have onset latencies of at least 20 min in lymphocytes (McEwen, Krey, & Luine, 1978) and 30 min in neurons (Dayanithi & Antoni, 1989). Modulating effects of GC release on gene expression require several steps until the gene product is expressed and available to take effect on the relevant system. The first step, GC dependent transcription, does not result in detectable mRNA until 7.5 minutes after addition of the steroid ‘in vitro’ (Groner, Hynes, Rahmsdorf, & Ponta, 1983). However, rapid non-genomic GC effects have been demonstrated on a cellular level, and even on behavior in animals (Borski, 2000; Joels, Krugers, Lucassen, & Karst, 2009; Stellato, 2004). Reduced reproductive behavior in male amphibians (Moore & Miller, 1984; Rose, Moore, & Orchinik, 1993) as well as increased locomotion in novelty situations (Sandi, Venero, & Guaza, 1996), impaired memory retrieval (Khaksari, Rashidy-Pour, & Vafaei, 2007) and increased aggressive behavior in rats (Mikics, Kruk, & Haller, 2004) have been observed. In a study described in chapter VI, we wanted to investigate if rapid cortisol effects may also influence classical conditioning processes in humans. Thus, we administered intravenously a mild dosage of cortisol or placebo, respectively, immediately before the conditioning protocol was started. As a conditioning paradigm we chose trace EBC because

its sensitivity for GC influence has been clearly demonstrated (Grillon et al., 2004; Nees et al., 2008; Nees, Rüddel, Mussgay, Kuehl, Römer, & Schächinger, 2010; Vythilingam et al., 2006) and its underlying mechanisms are well understood (Christian & Thompson, 2003). Furthermore, the duration of the EBC protocol did not last longer than ten minutes, so that potentially interfering effects from genomic GC action could be excluded. By using EBC it is possible to have a detailed look at the time course of the learning process, because single trials can be regarded. Our results show a facilitation of EBC after cortisol infusion during the first trials, but no differences in the general learning performance. Thus, cortisol seemed to accelerate the learning, probably via a fast non-genomic mechanism. A faster learning of an adaptive response such as the protective eyeblink might be very helpful in stressful and potentially threatening situations. Therefore, this reaction could reflect an adaptive strategy of the early stress reaction. This may also be the case in situations when pain related learning is required. As already mentioned, the EBC protocol can be regarded as a model for pain learning. We chose this paradigm instead of a pain paradigm for ethical reasons. We investigated fast cortisol effects on human classical conditioning processes for the first time, and thus did not know about the potential effects. However, pain related classical conditioning processes should be investigated in future research, because fast GC effects may also play an important role in pain related learning. An example for pain related learning with a proposed adaptive function is conditioned SIA. SIA has been suggested to have under normal circumstances an evolutionary advantage, permitting the organism to respond quickly in the face of danger despite the injury. It has been proposed that the function of conditioned SIA is the advantage to learn adaptive responses to pain even more quickly and in a more coordinated fashion to signals of impending stress by the mechanisms of classical conditioning (Finn et al., 2004). In such cases, but also for other pain protecting responses, an even faster learning of adaptive responses would be of great importance. Stress related fast cortisol effects may additionally contribute to pain related learning by classical conditioning mechanisms. In general, the findings of our study add that the stress hormone cortisol may, besides well-known effects on quantity and quality of learning, also affect the speed of learning.

1.6 Summary, limits and concluding remarks

This work deals with the complex interaction of stress and pain. On the one hand stress affects pain by different mechanisms such as endogenous opioids, GCs, or immune system and baroreflex related mechanisms. Especially, chronic stress has been associated with chronic pain syndromes. An often observed relative hypocortisolism, probably as a result of a down-regulated HPA axis after ongoing overactivation, seems to be involved. On the other hand, pain can act as a stressor which becomes obvious in patients suffering from chronic pain. Here, the caused stress may contribute to the maintenance of pain. For the development and maintenance of chronic pain, also learning processes such as classical conditioning play an important role. In turn, learning can be affected by stress. The pain system also interacts with the cardiovascular system through baroreflex related mechanisms (besides effects of the adrenergic and opioid system) which are sensitive to stress via the reactive sympathetic activation. The studies described in this work reflect different aspects of pain and stress interactions and give an impression of their broad variety. Furthermore, the results contribute to the knowledge about mechanisms and implications of these interactions.

We were able to show that pharmacologically reduced cortisol levels caused alterations in pain sensitivity when compared to a placebo condition. This result speaks in favor of a causal involvement of hypocortisolism in chronic pain. In another study we could show that lower cortisol levels in patients suffering from FMS were related to facilitation of conditioned responses in a trace EBC paradigm. Thus, classical conditioning processes that contribute to symptom generation and maintenance may be fostered by stress related hypocortisolism. A faster learning in trace EBC was found in a study in which we investigated the effects of rapid cortisol actions. These rapid cortisol effects are probably part of an early stress reaction and differ from the well-known delayed cortisol effects that are due genomic mechanisms. A faster learning of an adaptive response such as the protective eyeblink seems also feasible for pain related learning. In a last study, we compared two commonly used pain tests regarding the elicited physiological and psychophysical reactions. We could show that hand immersion in cold water led to greater sympathetic activation than hot water, even though pain ratings were comparable. Thus, interferences from elevated blood pressure and baroreceptor activation leading to decreased pain sensitivity cannot be excluded. This aspect is especially important when both tests are interchangeably used to investigate pain inhibitory effects.

Of course, these studies cannot represent the whole pattern of interactions between pain and stress systems. Furthermore, the studies show some limitations. In the first study, we

used the cortisol blocker metyrapone for pharmacological manipulations to investigate effects of reduced cortisol levels on pain perception. Due to the negative feedback mechanism of the HPA axis the reduced cortisol levels lead to enhanced levels of CRH and ACTH. Thus, we cannot exclude that our results are caused by changes of levels of these hormones. Additionally, we could block cortisol synthesis only for a short time. But hypocortisolism found in chronic pain patients has been lasting for longer times, so that effects and underlying mechanisms may differ. This may explain why we found decreased pain ratings in a paradigm of perceptual wind-up, whereas chronic pain patients usually show opposite ratings. Another limitation rises from the selection of participants. We only investigated young healthy males, so that conclusions about potential effects of gender and age cannot be drawn. This was also the case in the study in which we investigated rapid cortisol effects on trace EBC. Here, we used metyrapone to avoid interferences from endogenous cortisol pulsatility. Thus, we measured effects of a depleted system which may differ from the effects in a natural situation. Again, we cannot exclude interactions from the increased ACTH levels or further endocrinological changes. Because of the time limitation, avoiding interference with genomic effects of cortisol, data of extinction and subjective ratings of the used stimuli could not be assessed. Thus, no conclusions can be drawn about other forms of learning and memory and we can only speculate about other influences, such as the use of painful stimuli. In the study investigating EBC in FMS patients, we found an association between reduced cortisol levels and facilitation of conditioning. Of course, we do not know about the causality. Even though, the healthy control group in this study was matched for age and gender, the group was not controlled for socioecological factors. We also did not use any other patient groups as a control. In the study comparing cold and hot water tests regarding physiological measures, pain tolerance thresholds and pain ratings we suggested implications especially for pain inhibition, but an empirical verification still needs to be done. In all studies we measured behavioral, physiological and endocrinological variables. However, we can only assume the underlying processes on a cellular level. In general, the experimental setting delimitates generalization of the results. However, our studies provide useful contributions to the knowledge of stress and pain interactions and sets new approach for future research.

Interactions between pain and stress system play an important role, especially in the modern world where stress is a common phenomenon. Chronic stress seems to be involved in several disorders following the disturbed homeostasis of the organism. Both, the stress and the pain system have adaptive functions and try to protect the organism in case of harm and danger. But in some cases these processes become maladaptive such as in chronic pain. The usually

functional interactions between the systems may facilitate and foster such developments. Therefore, it is of great importance to know about interactions and underlying mechanism for prevention, therapeutical interventions and rehabilitation which has great impact for society and the health system.

Chapter II:

Increased basal mechanical pain sensitivity but decreased perceptual wind-up in a human model of relative hypocortisolism

(Kuehl et al., 2010)

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2.0 Abstract

Clinical data have accumulated showing that relative hypocortisolism, which may be regarded as a neuroendocrinological correlate of chronic stress, may be a characteristic of some functional pain syndromes. However, it has not been clarified yet whether deregulations of the hypothalamus–pituitary–adrenal (HPA) axis may directly alter pain perception and thus be causally involved in the pathophysiology of these disorders. To test this hypothesis, we performed a randomized placebo-controlled crossover trial in N = 20 healthy drug-free volunteers (median age 24 yrs) and analyzed the effects of metyrapone induced hypocortisolism on quantitatively assessed basal mechanical pain sensitivity (1.5–13 m/s impact stimuli), perceptual wind-up (9 m/s impact stimuli at 1 Hz) and temporal summation of pain elicited by inter-digital web pinching (IWP; 10 N pressure stimuli for 2 min). Experimentally induced hypocortisolism significantly decreased pain detection thresholds and augmented temporal summation of IWP induced pain ($p < .05$). The latter effect was dependent on the relative reduction in cortisol levels, and seemed to rely on a potentiated sensitization and not merely on the observed changes in basal pain sensitivity. Perceptual wind-up by contrast was reduced when cortisol synthesis was blocked ($p < .05$). This result is reminiscent of findings from animal studies showing a reversal of NMDA receptor activation by glucocorticoid receptor antagonists in neuropathic pain models. Our results speak in favor of a potential causal role of HPA axis alterations in pain chronicity.

***Keywords*:** Hypocortisolism, Metyrapone, Pain, Quantitative sensory testing, Randomized controlled trial

2.1 Introduction

Despite our growing understanding of the physiological mechanisms involved in nociceptive and neurogenic pain, we still know very little about the factors involved in pain chronicity. In case of functional pain syndromes - where pain persists without a welldelineated bodily cause - psychological factors such as chronic stress have been postulated to play an important role (Diatchenko, Nackley, Slade, Fillingim, & Maixner, 2006). But how the physical effects of these factors are mediated and how they may enhance pain intensity and duration is still not fully understood. Modern stress research focuses mainly on neuroendocrine perturbations such as dysfunctional alterations of the hypothalamic–pituitary–adrenal (HPA) axis. An over-activation of the hormonal stress–response system as a result of ongoing strain often leads to a down-regulated adrenocortical responsiveness characterized by relative primary adrenal hypocortisolism with increased feedback inhibition of the HPA axis (Heim, Ehlert, & Hellhammer, 2000; Tsigos & Chrousos, 2002). Hypocortisolism has been observed in healthy people undergoing prolonged psychological stressors as well as in patients with post-traumatic stress disorder (Liberzon, Abelson, Flagel, Raz, & Young, 1999; Rohleder & Karl, 2006; Yehuda, 2002). Furthermore, many chronic pain syndromes are associated with ongoing stress and hypocortisolism. Based on the immunosuppressive effects of glucocorticoids, hypocortisolism has been hypothesized to be a relevant factor for mediating the effect of stress on pain chronicity (Chrousos, 2000; Chrousos & Gold, 1992). Reduced glucocorticoid levels might disinhibit the secretion of inflammatory mediators and thereby promote the sensitization of peripheral or central nociceptive neurons (Sommer, Häuser, Gerhold, Joraschky, Petzke, Tölle, Uçeyler, Winkelmann, & Thieme, 2008).

In this context, various chronic pain syndromes have been investigated for relative hypocortisolism as a marker for HPA axis deregulation. Accumulated evidence has been reported for an associationbetween fibromyalgia and HPA axis dysfunction (Crofford, Engleberg, & Demitrack, 1996; Crofford, Pillemer, Kalogeras, Cash, Michelson, Kling, Sternberg, Gold, Chrousos, & Wilder, 1994; Griep, Boersma, & de Kloet, 1993; Griep, Boersma, Lentjes, Prins, van der Korst, & de Kloet, 1998; Wingenfield, Heim, Schmidt, Wagner, Meinlschmidt, & Hellhammer, 2008). Interestingly, this association has also been observed in rheumatoid arthritis (Straub, Kittner, Heijnen, Schedlowski, Schmidt, & Jacobs, 2002; Zoli Lizzio, Ferlisi, Massafra, Mirone, Barini, Scuderi, Bartolozzi, & Magaro, 2002), migraine and tension-type headache (Elwan, Mohamed Abdella, El Bayad, & Hamdy, 1991), trigeminal neuralgia (Strittmatter, Grauer, Fischer, Hamann, Hoffmann, Blaes, & Schimrigk,

1996) chronic facial pain (Galli, Gaab, Ettlin, Ruggia, Ehlert, & Palla, 2009) and whiplash-associated disorder (Gaab, Baumann, Budnoik, Gmünder, Hottinger, & Ehlert, 2005) as well as in chronic pelvic and certain types of low back pain (Geiss, Varadi, Steinbach, Bauer, & Anton, 1997; Griep et al., 1998; Heim, Ehlert, Hanker, & Hellhammer, 1998; Lentjes, Griep, Boersma, Romijn, & de Kloet, 1997). More to the point, prospective studies showed that stress-related HPA axis abnormalities and associated changes in pro-inflammatory cytokine levels may predict surgical outcomes in lumbar disc patients (Geiss, Rohleder, Kirschbaum, Steinbach, Bauer, & Anton, 2005) and constitute a risk factor for the development of chronic widespread musculoskeletal pain as seen in fibromyalgia for instance (McBeth, Silman, Gupta, Chiu, Ray, Morriss, Dickens, King, & Macfarlane, 2007; Uçeyler, Valenza, Stock, Schedel, Sprotte, & Sommer, 2006).

The correlative methodology of the aforementioned studies, however, precludes causal conclusions whether HPA axis downregulations are the cause or the consequence of chronic pain, which can by itself be regarded as a stressor. As a first experimental approach to assess the potential impact of decreased cortisol levels on acute pain, we conducted a study in healthy humans based on a pharmacological manipulation of cortisol levels and quantitative sensory testing (QST) of several mechanical pain models. We induced acute hypocortisolism by administering metyrapone, which blocks the regeneration of cortisol from its inactive 11-keta-derivates via the inhibition of the rate-limiting enzyme 11-b-hydroxylase (Sampath-Kumar, Yu, Khalil, & Yang, 1997). The blocked cortisol synthesis results in the removal of negative feedback from the pituitary and hypothalamus, leading to reduced circulating cortisol, increased adrenocorticotropic (ACTH) and corticotropin-releasing hormone (CRH) (Fiad, Kirby, Cunningham, & McKenna, 1994; Hagendorf, Koper, de Jong, Brinkmann, Lamberts, & Feelders, 2005; Otte, Lenoci, Metzler, Yehuda, Marmar, & Nylan, 2007; Rotllant, Ons, Carrasco, & Armario, 2002). Our hypothesis was that this would result in an enhanced pain perception indicated by higher pain intensity ratings.

2.2 Methods

The current study investigated the influence of metyrapone-induced hypocortisolism on psychophysically assessed pain sensitivityin a randomized placebo-controlled trial in humans. The study was double blind and based on a repeated measures crossover designcomprising one metyrapone and one placebo condition in counterbalanced order. Changes in basal pain

and hyperalgesic sensitivity were assessed through QST using different mechanical pain models. Besides the assessment of mechanical pain perception thresholds and stimulus–response (S/R) functions for noxious mechanical stimuli, we measured perceptual wind-up of pain sensation to controlled ballistic mechanical impacts (Kohllöffel, Koltzenburg, & Handwerker, 1991) and temporal summation of pain evoked by tonic inter-digital web pinching (IWP) (Forster, Anton, Reeh, Weber, & Handwerker, 1988). The various models were chosen to differentiate effects related to basal nociception and hyperalgesia, and to obtain indications about the site of action (i.e. central vs. peripheral sensitization). It has been hypothesized that wind-up relies mainly on short-term central potentiating mechanisms (Herrero, Laird, & Lopez-Garcia, 2000), whereas IWP-induced sensitization is predominantly attributable to peripheral processes (Magerl, Geldner, & Handwerker, 1990). For reasons of interpretability, we chose the same sensory modality (here: mechanical) for all pain types.

2.2.1 Subjects

The study included 20 healthy drug-free volunteers with a median age of 24 (range 20–31) yrs. Participation was restricted to male subjects in order to avoid interferences with menstrual cycle-related changes in adrenocortical regulation. All the participants were right handed and free from dermatological disorders or skin lesions. Candidates were medically checked (via anamnestic interview and auscultatory blood pressure assessment) and excluded from participation if they were suffering from any acute or chronic medical (including psychiatric) disease, known lactose intolerance or drug allergies. Further exclusion criteria were: body mass index > 27, illicit drug use and smoking. All subjects participated in the two experimental placebo and metyrapone sessions, and were randomly assigned to treatment order. No analgesics or alcohols were taken <72 h before the beginning of each session. Subjects were not allowed to consume caffeinated drinks beforeand during the experiment. The participants did not leave the laboratory until the end of the session, and filled the pauses with calm activities such as reading. The Ethics Committee of the State Medical Chamber of Rhineland-Palatinate (Ref. No. 837.412.07 [5947]) endorsed the experimental procedures, which are in accordance with the ethical guidelines of IASP and the Declaration of Helsinki. All candidates gave written informed consent.

2.2.2 Treatment

For the induction of hypocortisolism, we chose an oral metyrapone (Metopiron®; Novartis Pharma AG) administration protocol that has been validated for effective down-regulation of plasma cortisol levels (Broadley, Korszun, Abdelaal, Moskvina, Jones, Nash, Ray, Deanfield, & Frenneaux, 2005; Young, Lopez, Murphy-Weinberg, Watson, & Akil, 1997) and consisted in a cumulative dosing regimen with two intakes of 750 mg separated by 3½ h. The placebo was composed of coated lactose powder. All drugs had the same galenic form (i.e. gelatin capsule). Drugs were administered with 200 ml of water, and the participants were asked to drink 100 ml of waterevery half an hour. The first dose was taken with a standard snack at 9.00 a.m., the second one post-prandial (i.e. 30 min after a standard meal) at 12.00 a.m. At the end of each metyrapone session, an oral substitution dose of 10 mg synthetic cortisol (Hydrocortisone ®, Hoechst AG) was administered to bring cortisol levels back to normal values (4.30 p.m.). The treatment was continuously monitored by a physician and well tolerated by all subjects.

2.2.3 Algesimetry

QST of pain sensitivity was carried out on the non-dominant (i.e. left) hand. A verbally anchored numerical rating scale (NRS) ranging from 0 (no pain) to 100 (strongest pain imaginable) was used for all algesimetric tests.

2.2.3.1 Assessment of basal pain sensitivity

Basal pain sensitivity was assessed using the method of constant stimuli and derived from S/R functions to phasic impact stimuli. Stimulation was applied on the middle digital phalanges via lightweight (mass 0.5 g) plastic projectiles, which were vertically accelerated through a custom-built pneumatically driven stimulator as described by Kohllöffel et al. (1991). Each test procedure comprised nine stimulus intensities ranging from 1.5 (innocuous) to 13 m/s (noxious) in a geometric series with a ratio of 2. All stimulusintensities were presented ten times in pseudo-randomized order with an inter-stimulus interval (ISI) of 5 s. All stimuli had a duration of 0.5 s. During the ISI, participants evaluated each stimulus on the NRS.

Mechanical pain threshold was defined as the intensity level identified as painful (>0) in 50% of its presentations.

2.2.3.2 Statistical Analysis

The impact stimulation was used likewise for the assessment of perceptual wind-up. A single test block consisted of five stimulus series. During one series 10 stimuli with an identical impact velocity of 9 m/s were applied at a repetition rate of 1 Hz. Stimulus location was alternated between the middle phalanges of the index, middle and ring fingers for each stimulus series to avoid sensitization. Subjective magnitude of each stimulus in a series was numerically estimated.

2.2.3.3 Induction and monitoring of hyperalgesia

Mechanical hyperalgesia was induced by controlled tonic noxious squeeze stimuli (force 10 N; duration 2 min) applied to the interdigital webs between the index, middle and ring fingers (Sieweke, Birklein, Riedl, Neundoerfer, & Handwerker, 1997). Pressure was applied through a feedback-controlled pneumatically driven forceps (modified version of the device described by Forster et al. (1988)) with round-shaped plastic tips (diameter 6 mm). A given test trial consisted of five tonic stimuli that were repeated with an ISI of 5 min. Subjective pain intensity was numerically rated at 15-s intervals.

2.2.4 Endocrinological measures

HPA axis reactivity was monitored via cortisol measurements in saliva and plasma. Saliva samples were collected using Salivette® tubes (Sarstedt AG & Co.) with synthetic swabs. Sampling was performed by the subjects themselves and started at 7.00 a.m. immediately after controlled awakening (i.e. upon receiving a wake-up call by the experimenter). Participants were requested to stay in bed until 7.15 a.m., when the second sample was collected. Two further samples were taken at 7.30 and 7.45 a.m. Subjects were asked to abstain from eating or drinking until the first standard snack at 9.00 a.m. From 8.00 a.m. on, saliva samples were taken on an hourly basis in the laboratory until 4.00 p.m. Tubes were stored at -20 °C until analysis. After thawing for biochemical analysis, the fraction of free

cortisol in saliva was determined using a time-resolved immunoassay with fluorometric detection, as described in detail elsewhere (Dressendörfer, Kirschbaum, Rohde, Stahl, & Strasburger, 1992). This assay had a sensitivity (95% confidence interval [CI]) of 0.15 ng/ml and satisfactory precision with inter- and intra-assay coefficients of variation (CV) < 9% at cortisol concentrations of 2 ng/ml.

Blood samples were taken at 1.30 p.m. using the S-Monovette® (EDTA K_2-gel preparation; Sarstedt AG & Co.). Tubes were immediately placed on ice and centrifuged at 4000 rpm at 6 °C for 10 min. Plasma was separated and stored at -80 °C until assayed by using a commercial enzyme-linked immunosorbent assay (ELISA) for cortisol (RE52061; IBL International GmbH) and ACTH (Ref. 7023; BIOMERICA Inc.). The analytical sensitivity (95% CI) was 0.5 pg/ml and 2.5 ng/ml for the ACTH and cortisol ELISA, respectively (as indicated by the manufacturer). Precision was satisfactory according to the manufacturer with inter- and intra-assay CV < 8% for both assays at mean concentrations of 35.5 pg/ml for ACTH and 50 ng/ml for cortisol. All endocrinological analyses were run at the in-house biochemical laboratory at the University of Trier.

2.2.5 Experimental protocol

The two experimental sessions were separated by at least 6 days. Fig. 1 illustrates the medication, QST and saliva/blood sampling schedules. QST of basal pain sensitivity and perceptual windup was realized before (pre-medication baseline) and 90 min after (post-medication assessment) the medication protocol. In addition, the post-medication assessment included QST for hyperalgesia induced by IWP. While salivary cortisol was repetitively sampled over the course of the experiment, blood sampling took place at 60 min post-drug, i.e. 30 min before the start of the QST procedures. All sessions took place in the same mechanically ventilated laboratory room and were conducted by the same investigator.

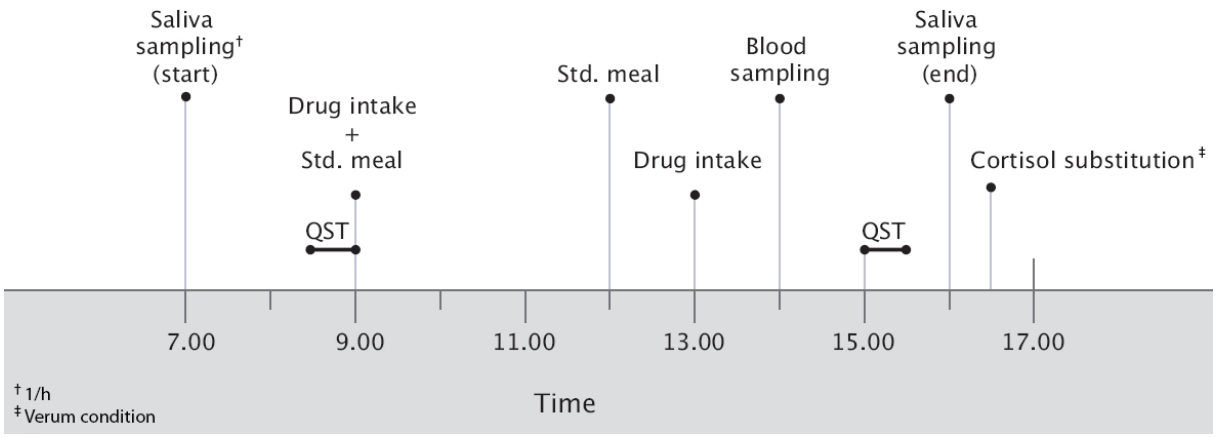

Figure 1. Experimental protocol for both sessions. QST = quantitative sensory testing

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2.2.6 Data evaluation and statistical analysis

Statistical analyses were computed using the SPSS (SPSS Inc.) and G*Power (Faul, Erdfelder, Lang, & Buchner, 2007) software, whereas data illustrations were generated with SigmaPlot® (SPSS Inc.). Data were analyzed by univariate analysis of variance (ANOVA) and covariance (ANCOVA) for repeated measures or paired *t*-test. Two-tailed *t*-tests were computed in case descriptive data were in contradiction with our directional hypotheses (unless otherwise stated indicated *p*-values are one-tailed). Greenhouse-Geisser adjustments were made to correct for nonsphericity in AN(C)OVAs when necessary. Significance level was fixed at $p = .05$. Supra-threshold and S/R data were relativized to the individual minimal rating over all sessions before all analyses. To reach linearization and normalization, data of the S/R functions were additionally log-transformed. A constant of 0.1 was added to the corresponding raw data to ensure that all values were greater than zero (Bartlett transformation). In order to quantify absolute changes in hyperalgesic sensitivity (i.e. unrelated to pain threshold alterations), we computed (a) the ratios of the average of the three last ratings to the initial rating and (b) the slopes (Dy/Dx) of the linear regression curve fitting pain ratings (relative to the initial rating in a corresponding test trial) to stimulus duration. For the analysis of perceptual wind-up, we calculated the ratios of the average of the five last ratings to the first rating in a series. The rationale for the additional slope computations was based on the fact that they might be a more sensitive indicator for the temporal summation of IWP-induced pain, which generally takes longer to reach its apex than wind-up (Forster et al.,

1988). All data are represented as arithmetic mean values ± standard error of the mean (*AM* ± *SEM*), unless otherwise stated. One participant was excluded from all analyses, because he did not show any decreases in cortisol levels after metyrapone intake. Thus, the final statistical sample was $N = 19$.

2.3 Results

2.3.1 Endocrinological data

As expected, metapyrone treatment induced a pronounced hypocortisolism, with an average reduction of cortisol levels in saliva by approx. 60% (verum relative to placebo). Fig. 2A shows the diurnal time course of salivary cortisol for the placebo and the metyrapone condition. ANOVA confirmed the observed time ($F_{12,18} = 48.35$, $p < .001$) and treatment ($F_{1,18} = 25.84$, $p < .001$) as well as the treatment x time interaction ($F_{1,12} = 3.68$, $p < .001$) effects. While pretreatment salivary cortisol levels (≤ 9.00 a.m.) did not differ between sessions ($F_{1,18} = .54$, $p = .50$; test power 1- $\beta = .99$), post-treatment cortisol levels (≥ 2.00 p.m.) were significantly decreased under metyrapone compared to placebo ($F_{1,18} = 16.97$, $p = .001$). Statistical analysis of the plasma values (1.30 p.m.) further corroborated the above-mentioned findings by revealing a significant post-treatment difference between the metyrapone (27.6 ± 5.8 nmol/L) and placebo condition (87.2 ± 8.1 nmol/L; $t_{18} = 6.85$, $p < .001$; see Fig. 2B). The significantly increased ACTH levels ($t_{18} = 7.45$, $p < .001$) indicate that the down-regulated cortisol secretion is attributable to the negative feedback inhibition of the HPA axis (Fig. 2B).

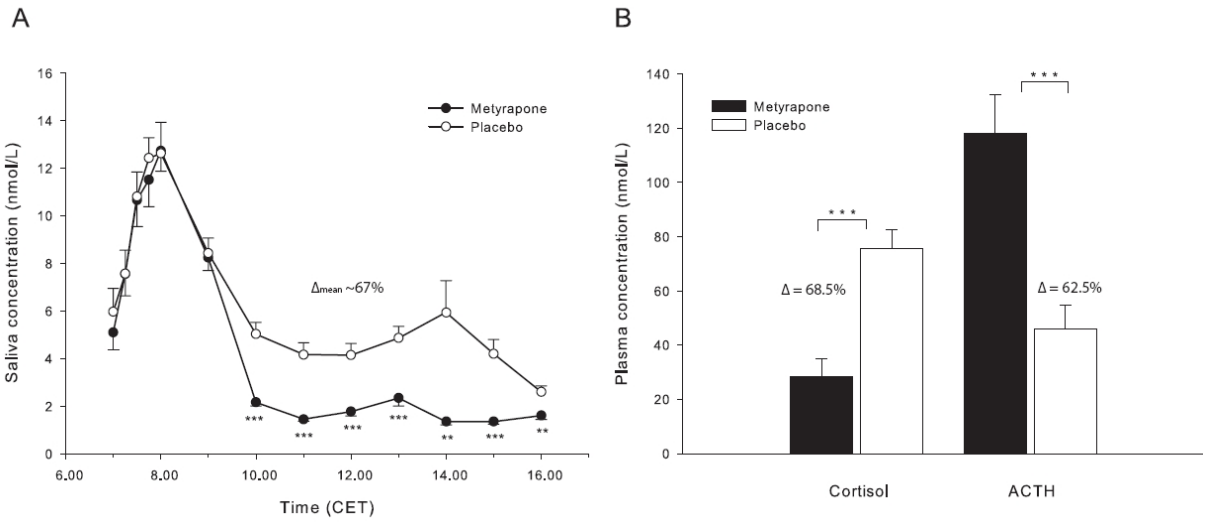

Figure 2. (A) Time course of cortisol levels in saliva for the metyrapone and placebo session. (B) Plasma levels of cortisol and ACTH 90 min after oral medication. $N = 19$; *AM* ± *SEM*; $^{**}p < .01$, $^{***}p < .001$.

2.3.2 Algesimetric data

2.3.2.1 Basal pain sensitivity

As shown in Fig. 3, metyrapone treatment significantly reduced mechanical pain thresholds by approx. 0.2 m/s compared to baseline ($t_{18} = 1.90$, $p = .03$) and placebo ($t_{18} = 2.13$, $p = .02$) with a medium effect size of 0.7 (Cohen's *d*). This increase in sensitivity corresponded in average to a 29% threshold reduction relative to baseline ($t_{18} = 2.11$, $p = .02$; Fig. 3). There were no visible changes in pain thresholds under placebo treatment. Consistent with the threshold data, S/R functions revealed significantly higher post-drug pain ratings in the metyrapone session compared to the placebo session (Bonferroni-corrected paired *t*-tests: $t_{18} = 2.17$, $p = .02$; $t_{18} = 2.52$, $p = .01$; $t_{18} = 1.79$, $p = .04$; $t_{18} = 2.09$, $p = .03$; data not illustrated), except for very low (≤ 2.6 m/s) and very high stimulus intensities (≥ 10.6 m/s). Baseline S/R functions did not differ, indicating a high reliability of the algesimetric assessments.

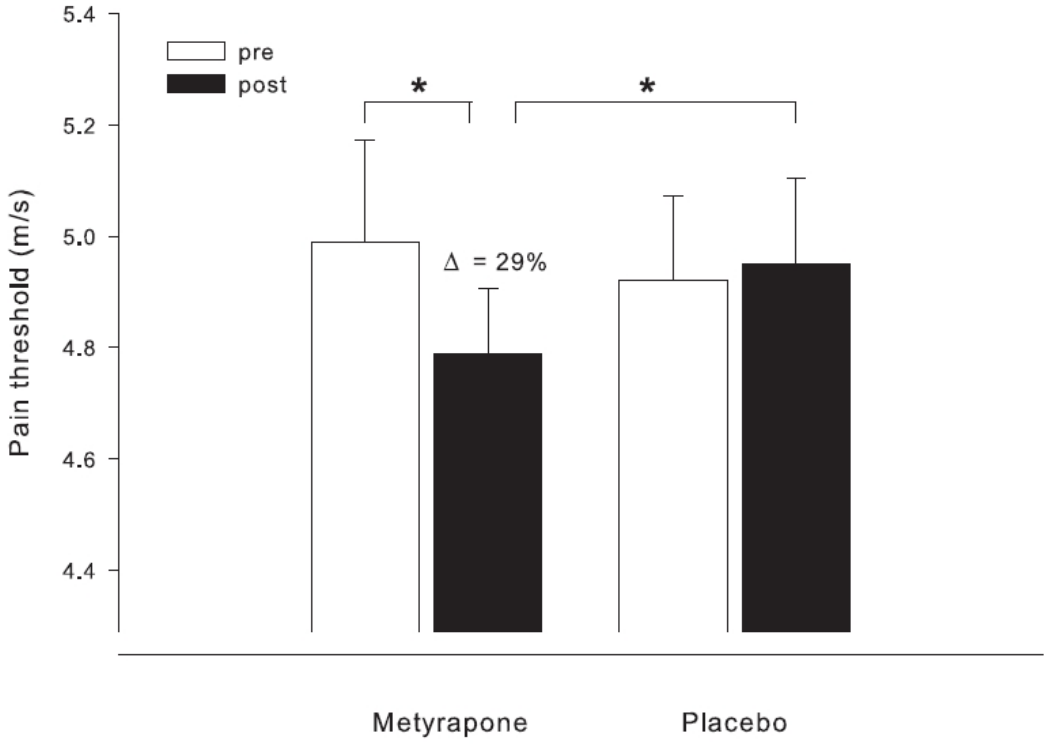

Figure 3. Mechanical pain detection thresholds before (pre) and 90 min after (post) oral drug administration (n.b. ordinate origin corresponds to lowest noxious stimulus intensity; $N = 19$). $AM \pm SEM$; $*p < .05$.

2.3.2.2 Perceptual wind-up

In contrast to the enhanced basal pain sensitivity, we observed a prominent reduction of short-term sensitization (i.e. perceptual wind-up ratio) by a factor of approx. 0.3 after metyrapone intake in proportion to placebo ($t_{18} = 1.94$, $p = .03$). In the placebo condition, post-drug ratings tended even to be slightly higher than the baseline levels (n.s.). The metyrapone effect (comparison to predrug values: $F_{1,4} = 4.24$, $p < .05$; effect size: partial $\eta^2 = .19$) appeared only with a certain latency (treatment x repetition interaction: $F_{1,4} = 3.20$, $p = .02$; Fig. 4). This observation speaks in favor of a genuine effect on wind-up not confounded by changes in basal nociceptive processing.

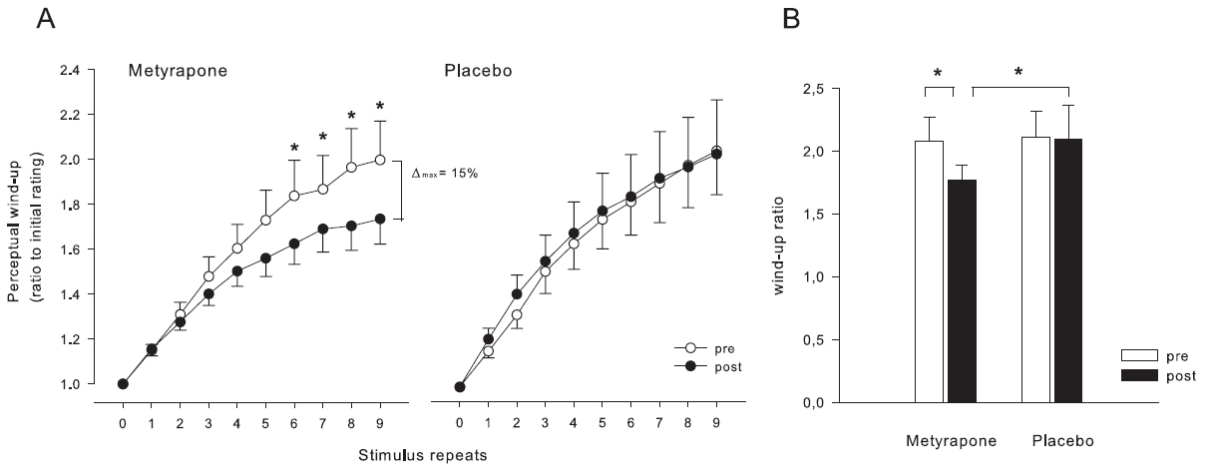

Figure 4. Perceptual wind-up (i.e. ratios of NRS-ratings to the first rating in a stimulus series) before (pre) and 90 min after (post) oral drug administration ($N = 19$). $AM \pm SEM$; *$p < .05$.

2.3.2.3 Hyperalgesia

Metyrapone effects on IWP-induced pain were linearly dependent on the amount of cortisol reduction ($r = .50$, $p = .03$) and statistical significance could only be achieved through ANCOVA when afternoon salivary cortisol levels (sampling point 3.00 a.m.) were considered as a covariate (non-correlative analyses showed only a tendency toward significant differences with $p \leq .08$). Contrary to the relative reduction of perceptual wind-up, we found an enhancement of temporal summation of IWP-induced pain by metyrapone, which grew noticeably stronger with longer stimulus duration (see Fig. 5; treatment x time interaction: $F_{7,112} = 3.94$, $p = 0.001$; partial $\eta^2 = .20$). An ANCOVA on the relativized hyperalgesic sensitivity gradients also revealed a significant pain-enhancing metyrapone effect with regard to percent slopes (estimated marginal means: 100% and 85% for metyrapone and placebo, respectively; $F_{1,16} = 3.97$, $p = .03$; partial $\eta^2 = .20$) as well as to ratios (estimated marginal means: 1.3 and 1.2 for metyrapone and placebo, respectively; $F_{1,16} = 2.90$, $p = .05$; partial $\eta^2 = .15$).

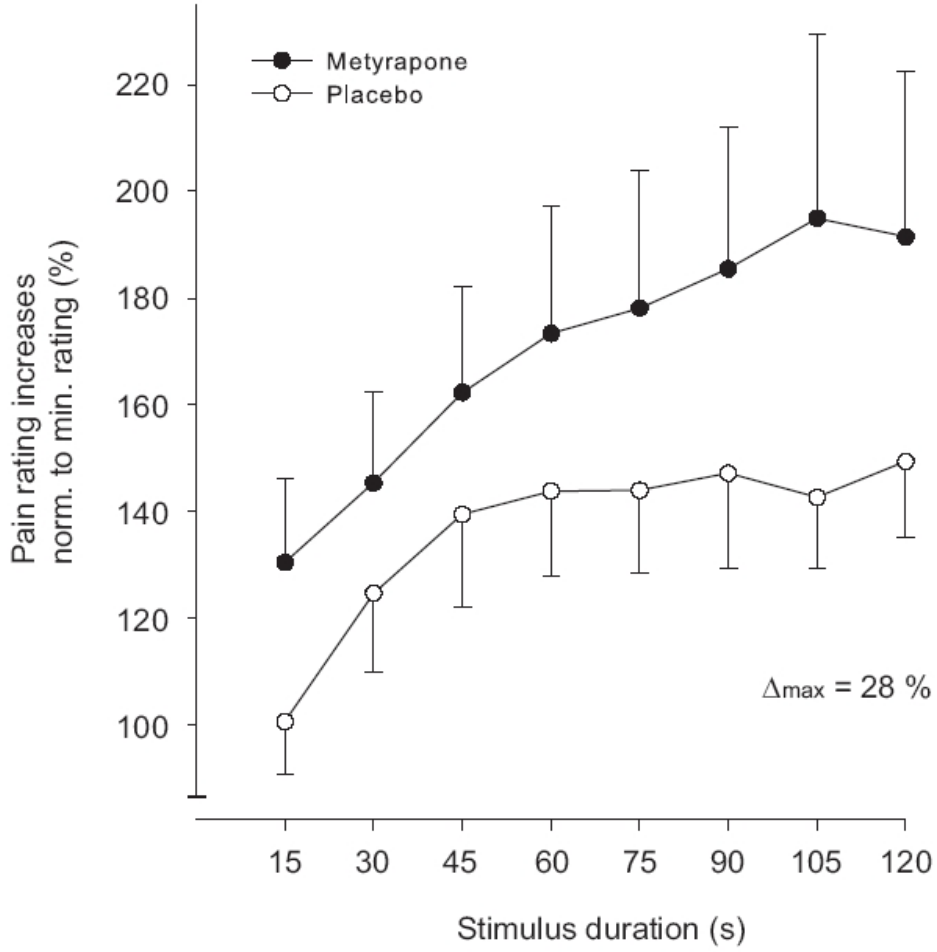

Figure 5. IWP-induced temporal summation of subjective pain intensity (normalized NRS-ratings; $N = 19$) over stimulus duration. Estimated marginal means (ANCOVA with salivary cortisol level sampled at 3.00 a.m. as a covariate) ± *SEM*; *$p < .05$.

2.4 Discussion

The purpose of the present study using a randomized controlled trial (RCT) was to investigate the causal relationship between acute HPA axis disturbances and (mechanical) pain perception in humans. Compared to conditions of normal HPA axis regulation, we predicted an intensified pain experience (i.e. increased basal pain sensitivity and potentiated hyperalgesia) as a result of metyrapone-induced hypocortisolism. With the model at hand, we were able to induce a pronounced, but not complete cortisol blockade, which was accompanied by heightened ACTH levels. This closely mimicked the (patho-)physiological condition of relative primary adrenal insufficiency (Oelkers, 2000). Consequently, the observed changes in pain processing were not exclusively attributable to the reduced cortisol levels but could also be associated with the concomitant rise in ACTH and other pro-

opiomelanocortin (POMC)-derived peptides (incl. b-endorphin) as well as in CRH (Papadimitriou & Priftis, 2009).

2.4.1 Basal pain sensitivity

As expected from clinical findings (see Section 1), our S/R and pain threshold data confirmed an increased sensitivity to noxious stimuli under conditions of acute hypocortisolism. This might not necessarily be to the result of a specific anti-nociceptive effect. It could also reflect a more general sensory modulation by cortisol levels, since increased and decreased detection thresholds have been documented for other exteroceptive (viz. auditory, olfactory and gustatory) sensory systems in patients suffering from hyperand hypocortisolism, respectively (Henkin, 2001). Congruently, experimental studies investigating the effects of oral hydrocortisone administration or acute laboratory stress in humans report a reduced sensitivity regarding detection and discrimination of acoustic and gustative stimuli (Beckwith, Lerud, Antes, & Reynolds, 1983; Fehm-Wolfsdorf, Scheible, Zenz, Born, & Fehm, 1989; Fehm-Wolfsdorf, Soherr, Arndt, Kern, Fehm, & Nagel, 1993).

Observations from recent animal studies employing for instance the glucocorticoid receptor (GR) antagonist mifepristone (RU486) would suggest a diminished suppression of glutamate and an inhibited facilitation of c-aminobutyric acid (GABA) release or even a negative GR-mediated regulation of spinal glutamate transporters as putative underlying mechanisms for the hypocortisolemia-induced threshold lowering (Di, Malcher-Lopes, Marcheselli, Bazan, & Tasker, 2005; Wang, Lim, Yang, Sung, & Mao, 2006). In line with this, exposure to stress has been shown to promote a glucocorticoidmediated extracellular accumulation of glutamate in the hippocampus and other areas of the central nervous system (Moghaddam, Bolinao, Stein-Behrens, & Sapolsky, 1994).

Although data on anti-nociceptive ACTH effects are somewhat ambiguous - possibly due to the differences in the dosages used -, physiological doses have been shown to produce a reductive effect on nocifensive reflex behavior (Yarushkina, 2008). CRH on the other hand seems to lack direct threshold-modulating efficacy as may be inferred from a RCT by Lautenbacher and colleagues using a noninflammatory pain model (Lautenbacher, Roscher, Kohl, Vedder, & Krieg, 1999). Intriguingly, the observed CRH-related increases in b-endorphin and cortisol produced no pain modulating effect either in the latter study. Taken

together with our results, this fact leads us to the assumption that basal circulating cortisol levels would rather play a housekeeping role in normal pain threshold regulation, whereas de novo liberation by CRH would not necessarily suppress the processing of strong suprathreshold pain stimuli (Sapolsky, Romero, & Munck, 2000).

2.4.2 Perceptual wind-up

Somewhat unexpectedly and contrary to clinical studies in fibromyalgia patients (many of which display a relative hypocortisolism; see Section 1) reporting enhanced perceptual wind-up (Klauenberg, Maier, Assion, Hoffmann, Krumova, Magerl, Scherens, Treede, & Juckel, 2008; Staud, Vierck, Cannon, Mauderli, & Price, 2001), we found anti- but not pro-nociceptive effects for the human perceptual model of central wind-up sensitization under conditions of acute hypocortisolism. More to the point, animal data point out that chronic stress might enhance the sensitivity of Nmethyl-D-aspartate receptors (NMDARs) (Kole, Swan, & Fuchs, 2002), which underlie the wind-up phenomenon (Herrero et al., 2000). Notwithstanding, our data are reminiscent of the involvement of neural GRs in the early development of neuropathic pain behaviors, and especially the effects of RU486 in spinal nerve injury resulting in a diminished expression of NMDARs and reversal of nociceptive reflex behavior (Wang, Lim, Zeng, Sung, Yang, & Mao, 2005). In line with this, acute restraint stress has been shown to enhance other forms of central sensitization (i.e. mechanical allodynia) in an animal neuropathic pain model. Again, pre-treatment with mifepristone prevented this effect (Alexander, DeVries, Kigerl, Dahlman, & Popovich, 2009). While under acute hypocortisolism a permissive pro-nociceptive glucocorticoid action may prevail, it is possible that for chronic hypocortisolism the intricate negative relationship between NMDARs in dorsal root ganglions and b-endorphin might in the long term lead to central hyper-sensitization (Zhang, Min, Seol, Lee, Han, Kim, & Han, 2009). Prolonged over-activation of the HPA might engender NMDAR-mediated μ-opioid tolerance (Adam, Bonnet, & Le Bars, 2006; Adam, Dufour, & Le Bars, 2008), which could assumedly result in the disinhibition of NMDAR-induced sensitization processes. Interestingly, Rivat et al. (Rivat, Laboureyras, Laulin, Le Roy, Richebé, & Simonnet, 2007) could demonstrate that acute innocuous environmental stress can produce pro-nociceptive effects in animals with previous pain or opioid experience. Regarding CRH, whole-cell patch clamp studies have shown its ability to depress NMDAR-induced currents in neurons, which would in principle correspond

to a wind-up modulation into the same direction as found in our study (Sheng, Zhang, Sun, Gao, Ma, Lu, & Ni, 2008). Although ACTH-like peptides have been shown to modulate NMDA-induced behavioral changes (Spruijt, Josephy, Van Rijzingen, & Maaswinkel, 1994), specific data for pain behavior are elusive. At this point, it should be kept in mind that although wind-up is a manifestation of functional central plasticity, it is not equivalent to the classical concept of central sensitization or secondary hyperalgesia (Herrero et al., 2000). This issue will be considered in the subsequent studies.

2.4.3 Hyperalgesia

With regard to IWP-induced temporal summation of pain we found an increased subjective pain experience under the metapyrone condition. Analysis of sensitivity level-corrected changes in pain perception revealed that this effect was not solely attributable to a linear leftward shift of the pain threshold but to an additional supra-threshold summation of pain. This conclusion that is also supported by the divergence of the time curves between the metyrapone and placebo condition (cf. Fig. 5). The fact that this genuine hyperalgesic effect was only identifiable when the relative cortisol levels were considered as a covariate corroborated its dependence on cortisol concentrations. All in all this clearly indicates that reduced cortisol levels may aggravate hyperalgesic states via amplifying sensitization caused by local tissue irritation (Meeus & Nijs, 2007).

Since prostanoids are involved in this form of hyperalgesia (Forster et al., 1988; Growcott, Stone, Beise, Stammer, Tetzloff, & Demey, 2000), the hypocortisolemia-induced effect may be explained by a disinhibition of pro-inflammatory mediators normally kept under control by diverse local anti-inflammatory glucocorticoid actions that affect arachidonic acid metabolism (Malcher-Lopes, Franco, & Tasker, 2008; Tasker, Di, & Malcher-Lopes, 2006). Application of glucocorticoids has consistently been shown to dampen the development of mechanical hyperalgesia in various animal injury model (Takeda, Sawamura, Sekiyama, Tamai, & Hanaoka, 2004; Xie, Liu, Xuan, Luo, Zhao, Zhou, & Xu, 2006). But from studies on lowered corticosterone levels induced by adrenalectomy (ADX) conflicting results are obtained. Taylor and coworkers (Taylor, Akana, Peterson, Dallman, & Basbaum, 1998) failed to find an effect on nociceptive processing in the formalin test in rats. On the other hand, Zhang et al. (Zhang, Lao, Qiao, Malsnee, & Ruda, 2004) could demonstrate—in agreement with our human data—demonstrate that ADX was able to amplify hyperalgesia in rats with peripheral

hind paw inflammation induced by complete Freund's adjuvant (CFA). This discrepancy may be explained by the different time courses and mechanisms of the algogenic substance and glucocorticoid actions. Formalin produces for instance a short-term and CFA a long-term inflammatory pain state (Honoré, Menning, Rogers, Nichols, Basbaum, Besson, & Mantyh, 1999). Glucocorticoids manifest short-term pro-inflammatory and longterm anti-inflammatory actions, but they are specifically mediated by mineralo- and glucocorticoid receptors, respectively (Sapolsky et al., 2000).

CRH and ACTH have been shown to exert anti-hyperalgesic action, a property that seems to be specifically confined to tonic inflammation-induced hyperalgesia either induced by chemical irritants or observed in post-operative pain (Lariviere & Melzack, 2000; Schäfer, Mousa, & Stein, 1997; Zhou, Li, Li, Ruan, & Zhao, 1999). Nevertheless, our data do not allow us to refute the possibility that the mostly HPA independent effects of CRH or those of ACTH and pituitary opioids counteracted the pro-inflammatory effects of acute hypocortisolism to a certain degree (Vit, Clauw, Moallem, Boudah, Ohara, & Jasmin, 2006). If relative hypocortisolism and the concomitant endorphin hyper-secretion persist, hyperalgesia symptoms are expected even to aggravate due to both insufficient antiinflammatory glucocorticoid action and chronic opioid-induced activation of pro-inflammatory neuro-immune responses (DeLeo, Tanga, & Tawfik, 2004).

2.4.4 Conclusion

The current study demonstrated a causal relationship between HPA function and acute pain processing in humans, corroborating foregoing data from animal and clinical studies. Although our results require replication with other pain models (e.g. thermal models including the assessment of central sensitization and secondary hyperalgesia), they support the potential role of relative hypocortisolism in the pathophysiology of chronic pain syndromes in the sense of a risk factor for pain exacerbation. To clarify the impact of the duration of relative hypocortisolism on pain sensitivity, prospective or longitudinal (pre-)clinical studies are clearly needed. Future studies may ascertain the scope of altered HPA axis reactivity on changes in pain perception and explore potential clinical implications for prophylaxis, diagnosis and therapy of chronic pain syndromes.

2.i REFERENCES – Chapter II

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2.ii AUTHOR NOTES

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Chapter III:

Differential physiological effects during tonic painful hand immersion tests using hot and ice water

(Streff, Kuehl, Michaux, & Fernand Anton, 2010)

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3.0 Abstract

The cold pressor test (CPT) is an empirically validated test commonly used in research on stress, pain and cardiovascular reactivity. Surprisingly, the equivalent test with water heated to noxious temperatures (hot water immersion test, HIT) has not been thoroughly investigated. The aim of the present study was to characterize the physiological effects and psychophysics of both tests and to analyze whether the autonomic responses are mainly induced by baroreflexes or a consequence of the pain experience itself. The study consisted of a single session including one CPT (4 ± 0.2 °C) and one HIT (47 ± 0.5 °C; cut-off point 5 min) trial performed on 30 healthy drug free volunteers aged 19–57 (median 24) yrs. The sequence of both trials was alternated and participants were randomly assigned to sequence order and parallelized with respect to gender. Physiological parameters (cardiovascular, respiratory and electrodermal activity) and subjective pain intensity were continuously monitored. In addition, pain detection and tolerance thresholds as well as pain unpleasantness were assessed. Both tests were comparable with regard to the time course and intensity of subjective pain. However, a significantly higher increase of blood pressure could be observed during the CPT when compared to the HIT. The HIT appears less confounded with thermoregulatory baroreflex activity and therefore seems to be a more appropriate model for tonic pain.

Keywords: Baroreflex hypoalgesia, Cold pressor test, Endogenous pain modulation, Human pain models, Psychophysiology, Psychophysics

3.1 Introduction

The cold pressor test (CPT; Hines & Brown, 1936) was originally conceived as a clinical cardiovascular challenge test to identify blood pressure (BP) and heart rate (HR) reactivity after hand immersion into ice water. It also proved to be a reliable experimental model for tonic pain or pain tolerance assessment (Mitchell, MacDonald, & Brodie, 2004). It has been hypothesized that the relationship between cardiovascular excitability and pain induction is primarily due to the extensive rise in BP caused by the thermoregulatory vasoconstriction of blood vessels in deep tissue (Wolf & Hardy, 1941).

Hand immersion in painful cold or hot water has also been used for experimental characterization of endogenous pain modulation, especially as a trigger stimulus for diffuse noxious inhibitory controls (DNIC). The DNIC phenomenon relates to the inhibition of nociceptive dorsal horn activity and pain sensations induced byadditional heterotopic noxious stimulation, and has been postulated to serve as a contrast-sharpening filter process (Le Bars, Dickenson, & Besson, 1979a; Le Bars, Dickenson, & Besson, 1979b; Le Bars, Villanueva, Bouhassira, & Willer, 1992). Animal studies have shown that it is mediated via an extra-segmental inhibitory process involving the medullary subnucleus reticularis dorsalis (Villanueva, Bouhassira, & Le Bars, 1996) and a reticular involvement may also be assumed in humans (cf. Le Bars et al., 1992).

The validity of cold-water immersion as a heterotopic noxious counter-stimulus for DNIC induction may however be hampered by confounding interactions of cardiovascular and pain regulatory systems. Experimentally induced, as well as constitutional hypertension is associated with reduced pain sensitivity, a phenomenon commonly referred to as baroreflex hypoalgesia (for overview see Bruehl & Chung, 2004; Randich & Maixner, 1984; Ring, Edwards, & Kavussanu, 2008). Observed cold-pressor related reductions in pain ratings may thus not selectively be attributable to DNIC, baroreflex mechanisms induced by thermoregulatory vasoconstriction may be involved as well. Painful hot and cold water stimulations are comparable with regard to their inhibitory effects on subjective pain experience (Granot, Weissman-Fogel, Crispel, Pud, Granovsky, Sprecher, & Yarnitsky, 2008). The two stimulation paradigms are thus interchangeably used, although little is known about possible physiological specificities and underlying mechanisms.

In the present study we contrasted the hot and ice water immersion tests with regard to their psychophysical and physiological (cardiovascular, respiratory and electrodermal activity

[EDA]) characteristics. Our main goal was to validate the relative usefulness of the two paradigms for studies investigating DNIC effects.

3.2 Methods

3.2.1 Subjects

N = 35 healthy (18 female and 17 male; two left-handed) volunteers aged between 19 and 57 years (median [Md] age 24 yr.) participated in the study. The subjects were recruited at the University of Luxembourg and received monetary compensation for their participation. All participants gave informed written consent, were drug free (no drug or alcohol intake >24 h before the study, except oral contraceptives) and did not suffer from any medical, neurological, psychiatric or psychological disorder nor did they manifest any substance (incl. nicotine) abuse.

The study consisted of a single session (duration: 75 min) including one hot water immersion trial (HIT) and one cold pressor trial (CPT). The sequence of both trials was alternated (AB–BA scheme) and participants were randomly assigned to sequence order and parallelized with respect to gender. The experimental protocol was in accordance with the ethical guidelines of IASP (Charlton, 1995) and met the criteria for an exemption from local ethical committee approval.

3.2.2 Algesimetry

Tonic thermal pain was induced by immersing the right hand up to the wrist in a 12 L tank with circulating hot (47-48 °C) or cold (3-4 °C) water. A cut-off point of 5 min was predefined, which guaranteed a time interval sufficient for reliable psychophysiological recordings of cardiovascular parameters (Sollers JJ, personal communication, 03/09/2008). The temperature of the hot water bath was held constant with a commercially available submergible heater and a digital controller, whereas an external chiller was used for the cold water bath (Aqua Medic GmbH, Germany). External aquarium pumps ensured water circulation in both water containers.

Subjective pain intensity was numerically rated on a verbally anchored scale (0 corresponding to no pain and 100 to the maximal imaginable pain) every 15 s during both pain tests. Pain

unpleasantness was quantified using a 10-cm visual analogue scale (VAS; verbal anchors: not at all unpleasant and extremely unpleasant) immediately after each test. Apprehension (nervous tension) associated with the pain test was determined using a 5-point Likert scale (1 = minimal tension; 5 = maximal tension). Furthermore, qualitative (i.e. affective and sensory) aspects of the pain experience during cold/hot water immersion were assessed with an adjective scale (Schmerzempfindungs-Skala, SES [pain sensation scale]; Geissner, 1996).

In addition, detection thresholds for cold and warm sensation (method of limits) as well as cold and heat pain (staircase-method) were evaluated, employing a 30 x 30 mm contact thermode attached to the volar surface of the left forearm (TSA-II NeuroSensory Analyzer; Medoc Advanced Medical Systems Ltd., Israel).

3.2.3 Psychophysiological recording

BP was continuously monitored on the wrist of the left arm with a noninvasive BP amplifier (NIBP100A; BIOPAC Systems, Inc., USA). Cardiac activity was assessed with a pre-cordial lead II electrocardiograph (ECG100C; BIOPAC Systems, Inc., USA; with 0.5-Hz high pass and 35 Hz low pass filtering) employing disposable pre-gelled Ag–AgCl electrodes. Subjects were grounded through a surface electrode attached to the chest. Respiration rate (RR) was obtained (with 0.05-Hz high pass and 1-Hz low pass filtering) using strain gauge belts positioned on the thorax and the abdomen (TSD201; BIOPAC Systems, Inc., USA). EDA was recorded with two 6-mm diameter domed Ag–AgCl electrodes (SS3LA; BIOPAC Systems, Inc., USA) and processed through a constant voltage (0.5 V) coupler (GSR100C; BIOPAC Systems, Inc., USA; with 5 lS/V signal gain and 1-Hz low pass filtering). Transducers were filled with isotonic electrode paste (formulated with 0.5% saline in a neutral base) and fixed on the mid-phalanx of the third and the fourth finger of the left hand. The skin temperature of both hands was measured on the palms by using a digital infrared thermometer (Sanowell Scaneo; Hofmann GmbH, Germany). The laboratory room was mechanically ventilated with ambient temperature maintained at 23.5 ± 0.5 °C. The AcqKnowledge software package (BIOPAC Systems, Inc., USA) was used for the collection and analysis (online and offline transformations) of the psychophysiological data.

3.2.4 Psychometrics

To test whether inter-individual differences in behavioral inhibition or activation systems might influence reactivity in the CPT and HIT, subjects were asked to fill out the BIS/BAS-scales (Carver & White, 1994).

3.2.5 Procedure

Each session began with the installation of the subject in upright position onto the experimental chair (approximately 90° inclination) and electrode/transducer placement. This was followed by a 5-min adaptation period and the measurement of detection thresholds for thermal sensation and pain (see experimental protocol in Fig. 6).

Subsequently, the registration of physiological parameters was started with the recording of a 5-min resting baseline (BL1), succeeded by the first water test (CPT or HIT, depending on the individual sequence). The subjects were instructed to immerse their right hand up to the wrist in the corresponding water tank and to verbally indicate the time point of the first pain sensation (i.e. pain threshold). Further, they were instructed to rate their pain sensation every 15 s on a numerical rating scale (NRS). The subjects were asked to leave their hand in the water container until the pain tolerance level was reached. The alternate water immersion test (CPT or HIT, respectively) followed after a 10-min rest period serving for BL assessment (BL2). For adaptation of skin temperature, the test hand was immersed in a container with tepid water (32 °C) during the first 3 min of this pause. Skin temperature on both hands was measured before and after each BL and test recording. Only the last two minutes of the corresponding BLs (BL1 and 2, respectively) were used for standardization of physiological data.

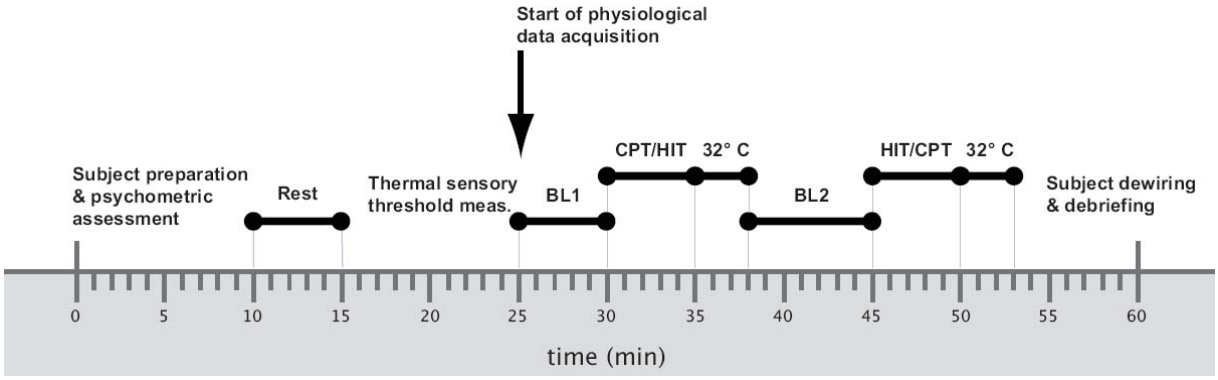

Figure 6. Experimental protocol: cold pressor test (CPT), and hot water immersion test (HIT).

3.2.6 Data reduction and analysis

Due to technical problems during psychophysiological recording, the data of three subjects were incomplete and thus not included in analysis. Furthermore, two participants felt no pain sensations during one or both water tests and had to be excluded as well, leaving a statistical population of N = 30.

The mean systolic BP and HR were calculated separately for both test periods and relativized to mean BL (1-min recording 2 min before the beginning of CPT and HIT, respectively) values. The mean RR was computed for thoracic and abdominal respiration separately (re-sampling rate = 50 Hz). The standard deviation of nonspecific EDA amplitudes for the first test minute was calculated offline and served as tonic EDA parameter (cf. Besthorn, Schellberg, Pfleger, & Gasser, 1989). The 1-min recording preceding test onset served as BL for RR and EDA. Overall pain experience during the immersion tests was computed as the geometrical grand mean of all subject's ratings different from zero.

All statistical analyses were conducted using the Statistical Package for Social Sciences (SPSS Inc., USA). Graphs were created with SigmaPlot (Systat Software Inc., USA) and Temporis (Bartas Technologies LLC, USA). Effect size computations were carried out with the G*Power program (Faul, Erdfelder, Lang, & Buchner, 2007). Parametric (*t*-tests for paired samples; Pearson product-moment correlation coefficient [*r*]), non-parametric tests and correlation coefficients (Wilcoxon's signed rank test; Spearman's rho [r_s]) were computed as appropriate (e.g. non-parametric tests in the case of skewed data distributions). For normally distributed data, the arithmetic mean and standard error of the mean (*AM* ± *SEM*) were used as measures of central tendency and variability, whereas asymmetrically distributed data are represented as median plus mean absolute deviation (*MAD*) or range. As in the analysis of psychometric data we tested for the null hypothesis (that there is no difference between both tests), a more conservative two-tailed significance level of $\alpha = .20$ was chosen. For the analysis of psychophysiological data, a one-tailed *p*-value of less than .05 was considered significant.

3.3 Results

3.3.1 Psychophysical and psychometric data

Pain thresholds (i.e. latency to detection of first pain) correlated moderately between both tests (r_s = .33, p < .05) and were significantly higher for the CPT than for the HIT ($z_{29,1}$ = 2.9, p = .003, effect size [d] = .52), although the absolute time difference of 3 s (CPT: *Md* = 13 s, range = 5-30 s; HIT: *Md* = 10 s, range = 1-28 s) may be considered negligible (see Fig. 7 and Table 1). Pain tolerance levels (CPT: *Md* = 300 s, range = 63-300 s; HIT: *Md* = 150 s, range = 35-300 s) were also higher during cold-water immersion ($z_{29,1}$ = -1.91, p = .06) and highly correlated between both tests (r_s = .48, p < .01). As expected, both immersion tests were comparable with regard to the time course of subjective pain experience (see Fig. 7a, and c) and pain increase (see Fig. 7; 63 compared to 67 NRS-units for CPT and HIT, respectively; $t_{29,1}$ = -1.22, p = .22 and r_s = .41, p < .05). However, when analyzing relative summation of pain as percent difference between the first and last pain rating, a significant difference could be shown between both tests (Δ% = 30-56% for CPT and HIT, $z_{29,1}$ = -2.57, p = .01; cf. (Fig. 7e). No sequence effects were found with respect to subjective pain intensity (sequence CPT-HIT: *AM* ± *SEM* = 62 ± 4.7 and 67 ± 5.5 for CPT and HIT, respectively; $t_{29,1}$ = -.72, p = .49; sequence HIT-CPT: *AM* ± *SEM* = 63 ± 6.1 and 66 ± 5.5 for HIT and CPT, respectively; $t_{29,1}$ = -.50, p = .63). Nonetheless, pain thresholds were negatively correlated with the percent increases in pain for both tests (r_s = -.40, p < .05 for CPT and r_s = -.54, p < .01 for HIT). Interestingly, pain thresholds did not correlate with the pain tolerance levels, but with overall subjective pain intensity (see Fig. 7d), although this relationship became significant for the CPT only (r_s = .63, p < .01).

Both tests were perceived as highly unpleasant and were evaluated similarly with regard to the affective and sensory dimensions of the pain experience (cf. Fig. 7b). Unpleasantness correlated with overall subjective pain intensity in both tests (r_s = .43, p < .05 for the CPT and r_s = .55, p < .01 for the HIT) as well as with pain tolerance (r_s = -.40, p < .05), which again was only true for the CPT. On the other hand, significant correlations between unpleasantness (r_s = .38, p < .05), subjective pain intensity (r_s = .43, p < .05), pain tolerance level (r_s = -.40, p < .05) and the affective SES scale could only be observed during hot water immersion, but not for the CPT. These observations may constitute a first indication of a more discernable pain sensation induced by the HIT. There were no consistent relations between the quantitative sensory parameters and inter-individual differences in behavioral inhibition or activation (i.e. on the BIS/BAS scales) with the exception of a positive correlation between unpleasantness

and behavior inhibition during CPT (total BIS score; r = .48, p < .01). Thus a more intense pain experience may be associated with a stronger avoidance behavior, which is further supported by the fact that the total BIS score showed a negative correlation with pain tolerance (r = -.40, p < .05).

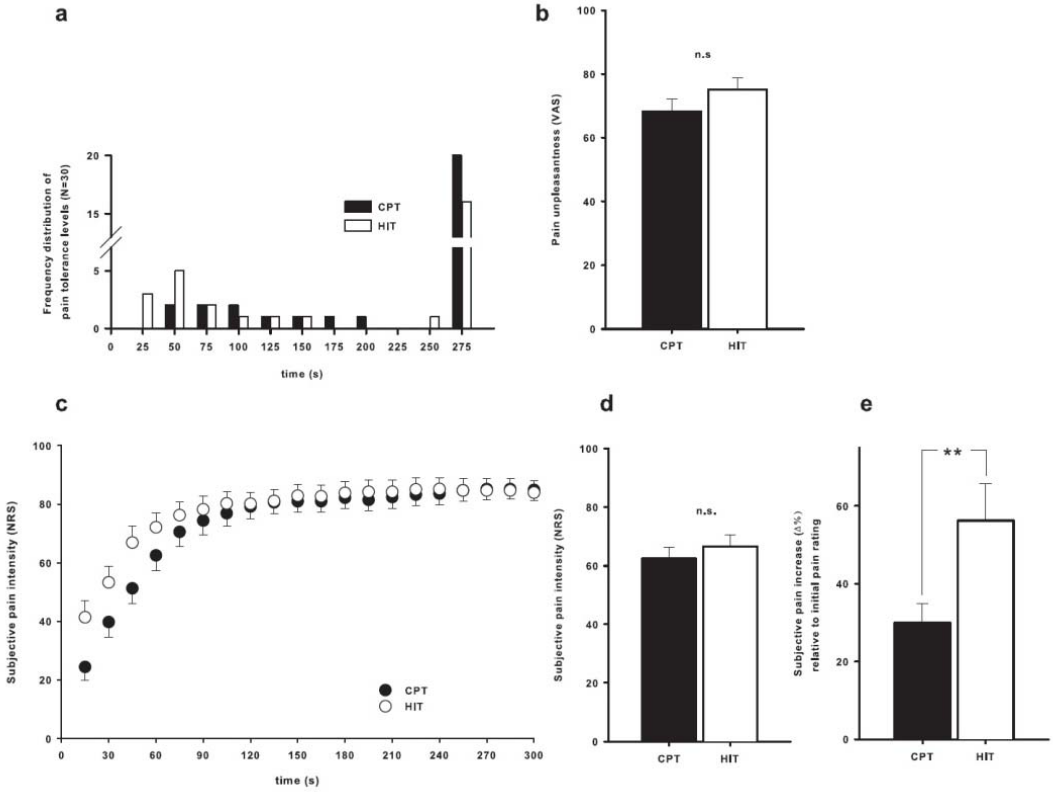

Figure 7. Psychophysical data. (a) Frequency distribution of pain tolerance levels (N = 30) for both immersion tests. (b) Overall pain unpleasantness. (c) Time course of subjective pain intensity. (d) Overall subjective pain intensity (individual geometric means aggregated over test duration). (e) Temporal summation of subjective pain intensity (percent increase relative to initial pain rating). All data expressed as *AM* ± *SEM*. **p < .01.

Table 1. Psychophysical data.

	CPT		HIT				
	Measures of central tendency + dispersion	Range	Measures of central tendency + dispersion	Range	Correlation CPT/HIT	Test value (df = 29)	p-value (2-tailed)
Pain threshold (s)	13 ± 6[a]	5–30	10 ± 6	1–28	r_s = .33*	–2.93[b]	.003**
Pain tolerance level (s)	300 ± 93	63–300	150 ± 124	35–300	r_s = .48**	–1.91	.06
Overall subjective pain intensity (aggregated over time)	63 ± 4[c]	30–93	67 ± 4	22–96	r = .41*	–1.22[d]	.22
Subjective pain increase (%Δ) relative to initial rating	30 ± 5	1–88	56 ± 10	0–250	r = .34	–2.57	.01**
Unpleasantness (VAS)	68 ± 4	18–100	75 ± 4	28–100	r = –.18	–1.19	.24
Affectivity (SES)	39[e]	34–59	40	33–62	r = .34	–.32	.75

* p < .05.
** p < .01.
*** p < .001.
[a] Md ± *MAD* (*mean absolute deviation*).
[b] z-value.
[c] *AM* ± *SEM*.
[d] t-value.
[e] T-value (mean = 50, SD = 10).

3.3.2 Psychophysiological data

Significantly different overall (aggregated over test time) BP levels were observed during both tests (absolute values of 159-152 mmHg for CPT and HIT, respectively; $t_{29,1} = 2.81$, $p = .009$). More to the point, the CPT produced a stronger rise in BP ($\Delta\% = 16\%$) than the HIT ($\Delta\% = 8\%$; $t_{29,1} = 0.85$, $p = .0002$), calculated as percent differences relative to BL (see Fig. 8 and Table 2).

Both tests also differed with respect to HR variability (ratio between low and high frequency components [LF/HF ratio] of the HR variability spectra relative to BL: 2.5 for CPT and 1.5 for HIT; $t_{29,1} = 2.49$, $p = .019$) and with respect to the first test minute of EDA (or skin conductance level: 0.14–0.22 mS for CPT and HIT, $t_{29,1} = -1.81$, $p = .003$).

HR on the other hand was highly correlated ($r = .80$, $p < .01$) during both tests and consequently did not differ significantly (80-81 BPM for CPT and HIT, $t_{29,1} = -.97$, $p = .17$). HRs recorded during BL were however significantly different from the ones recorded during test periods (76 to 80 BPM for BL and CPT, $t_{29,1} = -2.31$, p = .01; and 76–81 BPM for BL and HIT, $t_{29,1} = -4.92$, $p = .00002$). A significantly different HR between BL and test time was a result that could only be replicated for the HIT (76–81 BPM for BL and HIT, $t_{29,1} = 2.10$, $p = .04$) when the initial 15-s phase was taken into consideration. The subjective pain intensity and the increase of the HR during this initial phase correlated ($r_s = .46$, $p < .05$).

The calculated percent difference in BP correlated with the EDA ($r = .43$, $p < .05$) and with the mean HR ($r = .44$, $p < .05$). This was again the case only for the HIT.

As to RR, no difference was found in thoracic (197-191 beats per test [BPT] for CPT and HIT, respectively; $t_{29,1} = .77$, $p = .22$; $r = .47$, $p < .05$) nor in abdominal respiration (184-188 BPT for CPT and HIT, respectively; $t_{29,1} = -.57$, $p = .29$, $r = .45$; $p < .05$) over the entire test duration. Additionally, no differences relative to BL (thoracic RR: 188-189 BPT for BL and CPT, $t_{29,1} = 0.04$, $p = .9$; 191-183 BPT for BL and HIT, $t_{29,1} = -0.88$, $p = .4$; abdominal RR: 183-177 BPT for BL and CPT, $t_{29,1} = -0.94$, $p = .4$; 184-179 BPT for BL and HIT, $t_{29,1} = -0.52$, $p = .6$) could be observed. A high correlation between thoracic and abdominal RR was only identified for the CPT ($r = .59$, $p < .01$). During the CPT, but not during the HIT, the respiration parameters correlated with the mean HR (thoracic RR x HR: $r = .58$, $p < .01$; abdominal RR x HR: $r = .38$, $p < .05$).

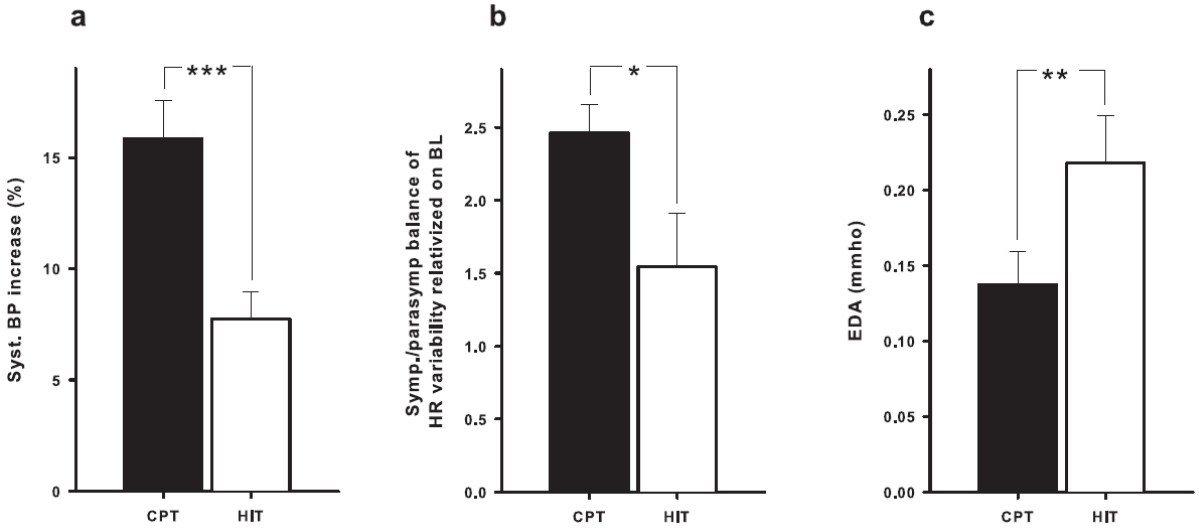

Figure 8. Psychophysiological data. (a) Percent blood pressure increase relative to baseline (BL). (b) Sympathetic/parasympathetic balance rel. to BL. (c) Spontaneous electrodermal fluctuations rel. to BL. All data expressed as *AM* ± *SEM*. ***$p < .001$, **$p < .01$, *$p < .05$.

Table 2. Psychophysiological data

	CPT		HIT		Correlation CPT/HIT	*t*-value (*df* = 29)	*p*-value (2-tailed)	Effect size (*d*)
	Mean ± *SEM*	Range	Mean ± *SEM*	Range				
Syst. blood pressure (mmHg)	159 ± 4	118–211	152 ± 4	113–196	$r = .74^{***}$	2.81	$.009^{**}$	.46
Increase of syst. blood pressure (%Δ)	16 ± 2	4–48	8 ± 1	7–21	$r = .11$	4.00	$.0004^{***}$	.72
Heart rate variability (symp./parasymp.balance relative to BL)	2.5 ± 0.2	0.2–4	1.5 ± 0.4	−6-4	$r = .25$	2.49	$.019^{*}$	.52
Heart rate (BPM)	80 ± 2	65–104	81 ± 2	64–114	$r = .80^{***}$	−.97	.17	–
Thoracic respiration rate (BPT)	197 ± 8	131–342	191 ± 7	119–284	$r = .47^{*}$	0.77	.22	–
Abdom. respiration rate (BPT)	184 ± 7	109–298	188 ± 8	101–333	$r = .45^{*}$	−0.57	.29	–
EDA (mS)	0.14 ± 0.02	0.004–0.45	0.22 ± 0.03	0.003–0.6	$r = .62^{***}$	−1.81	$.003^{**}$	.53

* $p < .05$.
** $p < .01$.
*** $p < .001$.

3.4 Discussion

The major goal of the present more methodologically oriented study was to investigate the internal validity of noxious water immersion as a tonic pain stimulus for DNIC induction. Internal validity refers to the validity of causal inferences (cf. Campbell & Stanley, 1963) and here to the degree to which a test non-spuriously induces the target phenomenon it purports to elicit (i.e. that its effects on the dependent variables under study are not confounded with another moderating factor and thus may only be attributed to the hypothesized underlying phenomenon). Since the cardiovascular regulations induced by local cooling of the extremities may themselves engender a reduced pain sensitivity in the sense of a baroreflex

hypoalgesia (Duschek, Mück, & Reyes del Paso, 2007), using noxious cold as a DNIC trigger could result in reactive testing by producing extra-segmental pain reduction through the thermal and not the nociceptive qualities of the stimulus. Accordingly, it has already been postulated that pain processing and modulation may be highly intermingled with cardiovascular changes induced during the CPT (see Peckerman, Saab, McCabe, Skyler, Winers, Llabre, & Schneiderman, 1991 for overview). More to the point, local cooling of the finger has been shown to cause a prominent pain-associated vasoconstriction for several minutes (Kreh, Anton, Gilly, & Handwerker, 1984).

By contrasting cold to hot water immersion and analyzing the concurrent physiological arousal, especially cardiovascular reactivity, we wanted to investigate whether the HIT would be a less sympathetically confounded tonic pain model. We did not explicitly test for the capacity of both tests to induce pain inhibition, since both tests are analogous in this regard as Granot et al. (2008) documented.

We observed that both immersion tests were quite comparable with respect to temporal summation, unpleasantness and subjective intensity of pain. With the stimulation temperatures chosen in this study, on the order of those commonly used in DNIC investigations (cf. Granot et al., 2008; Lautenbacher, Kunz, & Burkhardt, 2008), the HIT produced, however, a slightly higher subjective pain experience and was tolerated for a shorter period of time.

Both tests produced pronounced EDA fluctuations and tachycardia during the beginning of the immersion, an increase that returned to baseline levels within the second minute of the test. Spontaneous fluctuations of EDA were higher during the HIT, but contrary to Dowling (1983), who found a positive correlation between skin conductance level and pain tolerance, we could not identify any relationship between respiratory, electrodermal and algesimetric parameters. Correlations between mean thoracic and abdominal RR and HR were only found for the CPT (Steptoe, Melville, & Ross, 1984; see also Weise, Laude, Girard, Zitoun, Siché, & Elghozi, 1993), which could be the result of a potential respiratory sinus arrhythmia. This finding further supports a relatively higher baroreflex activity during coldwater immersion. The results of the HR variability parameter substantiate this conclusion as well, since we observed a higher sympathetic activity during the CPT than during the HIT.

With regard to HR, we found enhanced values compared to BL in both tests, which is largely documented for the CPT and congruent with data from Kondo et al. (Kondo, Shibasaki, Aoki,

Koga, Inoue & Crandall, 2001), who observed an overall increased HR during lower leg immersion even in innocuous 42 °C water. Interestingly, the forehead CPT has even been shown to cause HR decreases (Peckerman et al., 1991), which could be explained by a reduced sympathetic innervation of the forehead.

Both immersion tests lead to increases in BP, which is also in line with data from former investigations (see Lovallo, 1975, and Lovallo, Wilson, Pincomb, Edwards, Tompkins, & Brackett, 1985, for review on CPT and Tousignant-Laflamme et al., 2005, for HIT). The less pronounced cardiovascular effects during the HIT compared to the CPT are compatible with the observed inverse relationship between water temperature range (0–28 °C) and size of HR rise (Kregel, Seals, & Callister, 1992). Despite the observed increases in both tests and a more pronounced pain experience during the HIT, the cardiovascular changes were more prominent during the CPT with a higher increase of BP and a lower LF/HF ratio (i.e. sympathetic–parasympathetic balance).

The postulation that physiological changes induced by hot water are due to a genuine nocifensive rather than a thermoregulatory reaction was further corroborated by the positive correlation between pain tolerance and BP increase in the HIT trial, but the lack of such a correlation during the CPT. A positive, albeit gender-specific relationship between HR and pain experience was also found by Tousignant-Laflamme and colleagues (Tousignant-Laflamme, Rainville, & Marchand, 2005) in an investigation using only the HIT. The absence of a correlative relationship between pain ratings during CPT and HR on the other hand, were in line with findings by other investigators (Peckerman et al., 1991). Interestingly, Dowling (1983) found a negative correlation between HR level and pain tolerance level during the resting and anticipation period before a CPT. This correlation became insignificant 40 s after the immersion, i.e. when pain had started to develop. This divergence between indicators of pain perception and cardiovascular reactivity observed in the two immersion tests is likely to be related to a lower sympathetic or thermoregulatory involvement during the HIT (Appenzeller, 2000).

Trying to differentiate between DNIC and baroreflex hypoalgesia using pharmacological manipulations has proven to be complicated. Although it has been demonstrated that opiates may reduce increases in subjective pain and BP induced by CPT, the causality and moderation of this effect remains elusive, due to the additional vasodilatory effectiveness of these substances (Posner, Telekes, Crowley, Phillipson, & Peck, 1985; see also Edwards Ness, & Fillingim, 2004). The analgesic ibuprofen has, on the other hand, failed to reduce

pain during CPT despite of its vasodilatory effects. The fact that pain was even increased in this study could speculatively be attributed to an inhibition of baroreflex hypoalgesia (Peckerman et al., 1991).

In summary, our data indicate that the HIT is less confounded with thermoregulatory baroreflex activity and therefore a more appropriate model to produce experimental tonic pain with less autonomic arousal. Nonetheless, the HIT might also provoke significant increases in BP, so that the induction of baroreflex hypoalgesia may not be excluded for this model. Due to the complex interactions between baroreflex, opioid and descending pain modulation mechanisms (see France, 1999 for review and discussion), it is difficult to experimentally differentiate between baroreflex and multi-segmental DNIC-induced hypoalgesia in humans. Although pain models employing water immersion as well as the ischemic tourniquet pain test (Smith, Egbert, Markowitz, Mosteller, & Beecher, 1966) are able to induce hypoalgesia, they are massively confounded with cardiovascular regulations that may be majorly responsible for this effect (cf. Pertovaara, Kemppainen, Vuolteenaho, & Leppäluoto, 1984). Thus, the pain modulation provoked by these two models should strictly speaking be described as an unspecified form of descending inhibition rather than a perceptual correlate of DNIC. Further research with other tonic pain models, using psychophysics combined to psychophysiology, is needed to characterize tonic pain models that are less likely to induce interfering vegetative reactions, and therefore more appropriate for induction of distinct forms of descending pain control.

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3.ii AUTHOR NOTES

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Chapter IV:

Alteration of delay and trace eyeblink conditioning in fibromyalgia patients

(Nees, Rüddel, Mussgay, Kuehl, Römer & Schächinger, 2010)

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4.0 Abstract

Objective: Classical conditioning processes are important for the generation and persistence of symptoms in psychosomatic disorders, such as the fibromyalgia syndrome (FMS). Pharmacologically induced hyper- and hypocortisolism were shown to affect trace, but not delay classical eyeblink conditioning. Since previous studies revealed a relative hypocortisolism in FMS patients, we hypothesized that FMS patients also show altered eyeblink conditioning.

Methods: FMS patients (n = 30) and healthy control subjects (n = 20) matched for gender and age were randomly assigned to a delay or trace eyeblink conditioning protocol, where conditioned eyeblink response probability was assessed by electromyogram (EMG). Morning cortisol levels, ratings of depression, anxiety as well as psychosomatic complaints as well as general symptomatology and psychological distress were assessed.

Results: As compared to healthy controls FMS patients showed lower morning cortisol levels, corroborating previously described disturbances in neuroendocrine regulation of the hypothalamus-pituitary-adrenal (HPA) axis in these patients. Trace eyeblink conditioning was facilitated in FMS patients whereas delay eyeblink conditioning was reduced, and cortisol measures correlate significantly only with trace eyeblink conditioning.

Conclusion: We conclude that FMS patients characterized by decreased cortisol levels differ in classical trace eyeblink conditioning from healthy controls, suggesting that endocrine mechanisms affecting hippocampus-mediated forms of associative learning may play a role in the generation of symptoms in these patients.

***Keywords*:** Eyeblink conditioning, Fibromyalgia, Cortisol

ACTH = adrenocorticotropic hormone; CES-D = Center for Epidemiologic Studies Depression- Scale; CNS = central nervous system; CR = conditioned response; CRH = corticotropin releasing hormone; CS = conditioned stimulus; FMS = fibromyalgia syndrome; EMG = electromyogram; GBB = Gießener Beschwerdebogen; HPA axis = hypothalamus-pituitary-adrenal axis; ITI = intertrial interval; psi = pounds per square inch; SCID = Structured Clinical Interview for DSM-IV; SCL = Symptom Check List; STAI = State-Trait-Anxiety Inventory; UR = unconditioned response; US = unconditioned stimulus

4.1 Introduction

Fibromyalgia syndrome (FMS) is a common clinical syndrome characterized by chronic widespread pain and tenderness (Wolfe, Smythe, Yunus, Bennett, Bombardier, Goldenberg, Tugwell, Campbell, Abeles, & Clark, 1990). Elevated levels of depression, anxiety and psychosocial stress are frequently reported in FMS patients (Wolfe, Ross, Anderson, Russell, & Hebert, 1995; Wolfe, 1989). While the precise pathophysiological mechanisms are still poorly understood, recent studies suggested a neurobiological basis for FMS (altered central nervous system (CNS) pain processing) (Gracely, Petzke, Wolf, & Clauw, 2002; Cook, Lange, Ciccone, Liu, Steffener, & Natelson, 2004), and the hypothalamic-pituitary-adrenal (HPA) axis has been implicated as essential. Although inconsistent findings are reported, in FMS a chronic hypoactivity of the HPA axis including low 24h urinary free (Crofford, Pillemer, Kalogeras, Cash, Michelson, Kling, Sternberg, Gold, Chrousos, & Wilder, 1994; Griep, Boersma, Lentjes, Prins, van der Korst, & de Kloet, 1998; Lentjes, Griep, Boersma, Romijn, & de Kloet, 1997) and basal blood cortisol levels (Griep et al., 1998; Lentjes et al., 1997) could be observed repeatedly. This hypoactivation has been shown to be associated with HPA axis perturbation in terms of a sensitized pituitary with adrenal insufficiency. Several studies showed an exaggeration of adrenocorticotropic hormone (ACTH) during the CRH test, while the insuline tolerance test was accompanied by unchanged cortisol levels (Crofford et al., 1994; Griep, Boersma, & de Kloet, 1993; Griep et al., 1998; Riedel, Layka, & Neeck, 1998). This relatively mild hypocortisolism might develop after prolonged periods of

stress that are first characterized by a hyperactivity of the HPA axis including an excessive release of glucocorticoids (Hellhammer & Wade, 1993).

Basal learning processes such as classical conditioning are involved in physiological and neurochemical processing as well as subjective and behavioral expression of pain and thus are relevant in the generation of pain symptoms and their persistance (Flor, 2000; Linton, Melin, & Gotestam, 1984). Classical eyeblink conditioning has been studied intensively in animals (e.g. Christian & Thompson, 2003) and humans (Clark & Squire, 1998; Fortier, Disterhoft, Capozzi, Kilduff, Cronin-Golomb, & McGlinchey, 2003). It can be seen as a translational tool for clinical populations. There are two frequently used kinds of eyeblink conditioning paradigms: delay and trace eyeblink conditioning. The unconditioned stimulus (US), e.g. a weak air puff to the cornea, induces an eyeblink response that serves as unconditioned response (UR). In delay eyeblink conditioning the conditioned stimulus (CS), e.g. a tone of short duration (e.g. 400 ms) overlaps the US, with both stimuli terminating together. After repeated tone-air puff pairings, the CS is able to elicit an eyeblink without the application of the US. Delay eyeblink conditioning represents an example of learning without the necessity of voluntarily directing attention to stimuli. Here, the cerebellum is the essential neural system (Lavond, Kim, & Thompson, 1993). In trace eyeblink conditioning, the tone (CS) and air puff (US) are separated by an empty interval (e.g. 600 ms) and an awareness of CS-US contingency is essential (Clark & Squire, 1998). Contingency learning permits prediction of the appearance of one stimulus based on the presence of another, and evidence suggests that conscious awareness of a contingency is dependant on conditioned associations (Allan, 1993; Price & Yates, 1995). On the neural level, trace eyeblink conditioning requires both the cerebellum and the hippocampus (Berger & Thompson, 1978; Clark & Squire, 1998; Moyer, Deyo, & Disterhoft, 1990; Woodruff-Pak & Papka, 1996). Stress hormones, in particular glucocorticoids, have been shown to modulate classical conditioning (Grillon, Smith, Haynos, & Nieman, 2004) and thus may affect the generation and persistence of pain symptoms by influencing learning and memory processes (Het, Ramlow, & Wolf, 2005). Animal studies have shown the involvement of stress-sensitive neurons from the hippocampal CA1 and CA3 regions in trace conditioning (McEchron & Disterhoft, 1997; Weiss, Kronforst-Collins, & Disterhoft, 1996), and human studies as well demonstrated the critical role of glucocorticoids in eyeblink conditioning. An impairment of eyeblink conditioning during pharmacologically induced mild hypercortisolism and in persons with endogenous hypercortisolism was observed for trace but not delay conditioning processes (Grillon et al., 2004; Vythilingam, Lawley, Collin, Bonne, Agarwal, Hadd, Charney, & Grillon, 2006), findings supported by the

high concentration of glucocorticoid receptors in the hippocampus. A facilitation of hippocampus-based conditioning could be observed after pharmacologically induced endogenous mild hypocortisolism (Nees, Richter, Lass-Hennemann, Blumenthal, & Schächinger, 2008). These results may be of theoretical and clinical significance for pain syndromes such as fibromyalgia in which a relatively mild hypocortisolism is postulated. However, so far classical eyeblink conditioning has not been investigated in fibromyalgia patients.

The purpose of the present study was to examine delay and trace eyeblink conditioning in fibromyalgia patients and healthy matched control persons. The existence of a relatively mild hypocortisolism was assessed by morning cortisol profiles. We hypothesized a facilitation of trace eyeblink conditioning in fibromyalgia patients showing a relatively mild hypocortisolism compared to healthy controls, while delay eyeblink conditioning was assumed to be unaffected.

4.2 Method

4.2.1 Participants

The present study, which was approved by the local ethics committee, involved 30 fibromyalgia patients (11 male and 19 female) with a mean age of 40.73 years (range from 30 to 54 years) and 20 healthy matched controls (9 male and 11 female) with a mean age of 40.95 years (range from 31 to 55 years). Data were collected from June 2007 to December 2008. Control subjects were recruited from an unselected general population using flyers and ads in the local media. The patient population comprised consecutive FMS patients, recruited from the Hospital for Psychosomatic Medicine Bad Kreuznach, Germany and diagnosed according to the criteria of the American College of Reumathology (Wolfe et al., 1990). Mean duration of pain was 14.33 years (SD = 8.3), mean number of tender points was 14.6 and the patients reported pain in an average of 7 regions of their bodies. FMS patients were excluded from participation if they were taking centrally acting pain medication (e.g. morphine derivates), were suffering from mental disorder, neurologic complications, another severe disease such as a tumor, liver, or renal disease, or if they reported a duration of pain of less than 6 months or drug abuse. Mental disorders were diagnosed using the Structured Clinical Interview for DSM-IV (SCID-I/-II; First, Spitzer, Gibbon, & Williams, 1996; Wittchen & Fydrich, 1997). SCID I and II show high validity and reliability in American and German

studies (First et al., 1996; Wittchen & Fydrich, 1997; Strakowski, Keck, McElroy, Lonczak, & West, 1995). Ratings of depression, anxiety, as well as psychosomatic complaints and global symptomatology and psychological distress were acquired using validated standard questionnaires. For the assessment of depressive symptoms we used the German version of the Center for Epidemiologic Studies Depression-Scale (CES-D; Hautzinger & Bailer, 2005). This measure is a reliable and valid indicator of depressed mood in both clinical and research populations. Its 20 items are relatively free of content related to pain and functional limitations associated with rheumatologic disorders. The German version of the State-Trait-Anxiety Inventory (Laux, Glanzmann, Schaffner, & Spielberger, 1981) was used to measure current feelings and a stable disposition characterized by tension and apprehension across time and setting. Both, the state and trait version are reliable and valid, and are the most commonly used measures of anxiety in psychological and behavioral medicine research. The short version of the Gießener Beschwerdebogen (GBB-24; Brähler, Schumacher, & Scheer, 2004) was used to assess psychosomatic complaints. The 24 unspecific symptoms are grouped in the following 4 subscales: fatigue, stomach trouble, rheumatic pain, heart trouble. For the assessment of somatization, obsessive-compulsive symptomatology, and interpersonal sensitivity we used the SCL-90-R (Franke, 2002) that was designed to characterize global symptomatology and psychological distress.

Control subjects were healthy and carefully matched for gender and age. Exclusion criteria for healthy controls were the same as for FMS patients. Furthermore, none of the control subjects reported the presence of any pain at the time of participation in the study. The study adhered to the guidelines of the Declaration of Helsinki, the local institutional review board approved the study (Landesärztekammer Rheinland-Pfalz), and informed consent was obtained from all subjects prior to participation.

4.2.2 Salivary Cortisol Sampling

Saliva samples were collected on two consecutive days directly before the test day: at awakening, + 15 min, + 30 min, + 45 min, + 60 min (awakening cortisol profile). Furthermore, we obtained one saliva sample for each subject immediately before the assessment of delay and trace eyeblink conditioning.

Saliva samples were stored at -20°C and analyzed for cortisol with a time-resolved fluorescence immunoassay (Dressendörfer, Kirschbaum, Rohde, Stahl, & Strasburger, 1992).

Intra- and interassay variabilities were below 6 % and 12 %, respectively. The data of 4 FMS patients had to be excluded because of technical problems during laboratory data analysis.

4.2.3 Design

Participants entered the research room at 4 p.m. Saliva samples were taken and a Coulbourn bioamplifier EMG system was attached for measuring muscle activity of the orbicularis oculi. All participants were randomly assigned to complete a delay or trace eyeblink conditioning protocol and blinded to group assignment. They were asked to fixate their gaze on the wall, to move as little as possible, and to blink naturally. Furthermore, they were informed that an air puff would be delivered to one eye and that they would hear tones.

In both delay and trace eyeblink conditioning protocols, the CS was a 75 dB(A), 400 ms, 1000 Hz pure tone presented binaurally via headphones. The US was a 10 psi, 50 ms air puff to the left cornea delivered through a tube attached to the headphones.

Both protocols consisted of three periods: an initial air puff familiarization period including six air puffs alone without CS, an acquisition period including three blocks of 20 trials, with each block consisting of 18 CS-US trials and two CS alone trials, and an extinction period including 10 trials with CS alone. In trace conditioning, there was a 600 ms free interval between CS offset and US onset. The intertrial interval (ITI) varied between 10 and 14 s, with a mean interval of 12 s.

4.2.4 Psychophysiological Recordings

We assessed the eyeblink response as peak EMG activity of the left musculus orbicularis oculi. Two electrodes were placed below the left eye with an interelectrode distance of 1.5 cm, and a third (reference) electrode was taped to the forehead. EMG was recorded with a Coulbourne bioamplifier and DasyLab software at a sampling rate of 1000 Hz (50 Hz notch filter; band-pass filter 30 to 500 Hz). Data were rectified and integrated with a 10 ms time constant. In a visual analysis we categorized the trials with respect to artifacts (i.e. voluntary or spontaneous eyeblinks at or near the startle stimulus onset, trials with excessive background noise, multiple peaks). For data analysis we used only data of participants with at least 75 % of trials without artifacts.

4.2.5 Data Analysis

In both delay and trace eyeblink conditioning, the unconditioned response (UR) was represented as eyeblink response between a stable baseline (50 ms before US onset) and the maximum amplitude in the time interval of 20 – 100 ms after US onset. No participant had to be excluded because of not responding to the air puff.

Eyeblinks with amplitudes of at least 15 µV in the time window of 500 ms before CS onset were defined as spontaneous eyeblinks. In both eyeblink conditioning protocols, those trials with spontaneous eyeblinks were rejected.

Eyeblinks with amplitudes of at least 15 µV in the first 100 ms after CS onset were classified as alpha responses. Alpha responses are unconditioned (orienting) responses to the tone (Gormezano, 1966). For both eyeblink conditioning protocols, we observed few alpha responses during acquisition and extinction period. Their probability did not differ significantly between FMS patients (acquisition: delay: mean = 2.97 %, trace: mean = 3.61 %; extinction: delay: mean = 2.12 %, trace = 2.43 %), and healthy control persons (acquisition: delay: mean = 3.48 %, trace: mean = 3.22 %; extinction: delay: mean = 2.33 %, trace: mean = 2.68 %). Thus, conditioned responses were not influenced by alpha responses.

In delay eyeblink conditioning, the conditioned response (CR) is represented as an eyeblink with an amplitude of at least 15 µV in the time interval of 100 - 300 ms after CS onset.

In trace eyeblink conditioning, eyeblinks with amplitudes of at least 15 µV in the time interval of 600 - 1000 ms post-CS (in a period of 400 ms that precede the US) were categorized as conditioned responses (“adaptive”, true CRs; Spence & Ross, 1959). Further, eyeblinks that occurred during the empty interval of 100 - 600 ms after the CS were considered as “nonadaptive” responses, because of their poor timing relative to the CS/US, i.e. closure of the eyelid occurs too early, and the eyelid is no longer closed upon delivery of the air puff (Grillon et al., 2004; Vythilingam, 2006). The probability of nonadaptive CRs was low and did not differ significantly between FMS patients (mean = 6.23 %) and control persons (mean = 5.58 %).

All CR probabilities were calculated based on CS-US acquisition trials, only. CS alone trials, that were used to implement a partial reinforcement schedule, were not included in the calculation of CR probabilities.

4.2.6 Statistical Analysis

Cortisol data were analyzed with a group (patients vs. controls) X cortisol awakening profile (1-5) repeated measures analyses of variance (ANOVA). The magnitudes of unconditioned eyeblink responses during the air puff familiarisation period were averaged over the six trials and the data during acquisition and extinction periods were averaged within blocks. Acquisition data were analyzed with a group (patients vs. controls) X block (1-3) repeated ANOVA for both delay and trace eyeblink conditioning. Extinction data were analyzed with a group (patients vs. controls) X trial (1-10) one-way ANOVA. In order to investigate the impact of cortisol, ratings of depression, anxiety and psychosomatic complaints as well as global symptomatology and psychological distress on CR probability of delay and trace eyeblink conditioning, we used Pearson correlation analyses.

For all statistical analyses, α was .05 (two-tailed) and we applied the Greenhouse-Geisser-adjustment in the case of violation of the assumption of homogeneity of variances, and adjusted degrees of freedom are reported. In the case of significant main effects or interactions, paired t-tests with Bonferroni-adjustment were performed. We used Statistical Package of the Social Sciences, Version 14.0.1 for Windows.

4.3 Results

4.3.1 Symptom Ratings

In comparison to control persons, FMS patients reported significantly increased total scores of depression ($t(48) = 5.83$, $p < .001$), anxiety ($t(48) = 6.12$, $p < .001$), as well as psychosomatic complaints ($t(48) = 4.89$, $p < .001$) and global symptomatology and psychological distress ($t(48) = 4.67$, $p < .001$), but below the border to clinical characteristic (see Table 3).

Table 3. Symptom rating of anxiety symptoms, depression, psychosomatic complaints and general symptomatology and psychological distress of FMS patients and healthy controls.

	Controls (N = 20) M (SD)	FMS patients (N = 30) M (SD)	Sign. (between both groups) p
ADS	3.6 (2.1)	12 (4.3)	<.001
STAI			
State	13.1 (3.3)	29.4 (5.1)	<.001
Trait	12.3 (4.2)	27.6 (6.7)	<.001
SCL-90			
Total score	0.5 (0.3)	1.2 (0.7)	<.001
Somatization	0.4 (0.2)	1.2 (1)	<.001
Compulsivity	0.1 (0.2)	0.3 (0.5)	.004
Uncertainty in social contact	0.4 (0.3)	1.7 (1.1)	<.001
GBB			
Total score	20.8 (8.4)	37.6 (8.7)	<.001
Fatigue	6.3 (3)	13.1 (7.1)	<.001
Stomach trouble	4.5 (2.4)	6.8 (4.8)	.026
Rheumatic pains	6.1 (2.7)	13.5 (6.9)	<.001
Heart trouble	4 (2.6)	7.5 (6.3)	.022

ADS, Allgemeine Depressionsskala; STAI, State-Trait-Anxiety-Inventory; SCL-90, Symptom-Check-List; GBB, Gießener Beschwerdebogen

4.3.2 Salivary Cortisol Data

Figure 9 illustrates the awakening cortisol profile of FMS patients and healthy controls obtained two days before the test day of eyeblink conditioning assessment. A significant effect of cortisol awakening profile ($F(3,111) = 71.058$; $p < .001$) and group ($F(1,44) = 4.558$; $p = .038$), and a significant cortisol awakening profile X group interaction ($F(3,111) = 25.328$; $p < .001$) were found.

Furthermore, we found significantly decreased cortisol values, obtained immediately before the assessment of delay and trace eyeblink conditioning, in FMS patients [mean = 3.12] compared to healthy controls [mean = 4.98] ($t(48) = 2.132$; $p < .05$).

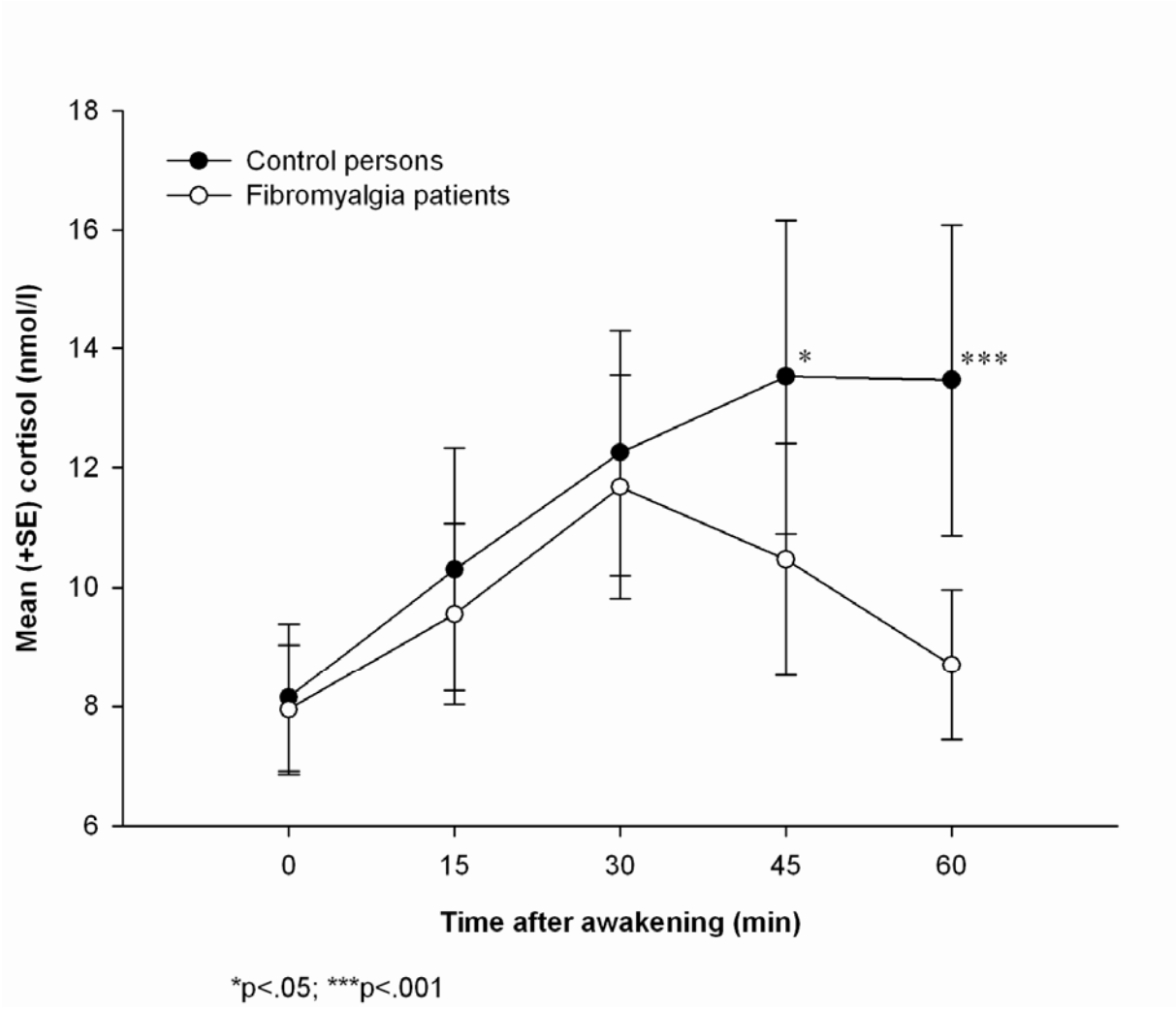

Figure 9. Awakening cortisol profiles (averaged over data of two consecutive days) of control and patient group.

4.3.3 Eyeblink Conditioning

Baseline eyeblinks

In delay as well as trace eyeblink conditioning, the eyeblink magnitude to the air puff during familiarization did not differ significantly between FMS patients and control persons (delay: mean = 117.7 μV; SD = 20.9; trace: mean = 134.3 μV; SD = 32.8). Probabilities of spontaneous eyeblinks, assessed during the 500 ms time window prior to the CS-US pairs, were not significantly different between the patient and control group.

Conditioned responses

Conditioning is normally slower using the trace paradigm, compared to the delay paradigm. To check for this difference under normal conditions, we compared both eyeblink conditioning protocols in the control group. As previously demonstrated, delay conditioning was more effective than trace conditioning ($F(1,18) = 33.384$; $p < .001$).

4.3.3.1 Acquisition

Delay conditioning

A group x block ANOVA revealed a significant main effect of group ($F(1,21) = 12.002$; $p = .002$). Furthermore, a significant block effect was seen ($F(1,30) = 169.924$; $p < .001$), with CR probability increasing from Block 1 to Block 2 ($p < .001$) to Block 3 ($p = .001$). The interaction between group x block was also significant ($F(1,30) = 12.504$; $p < .001$).

Thus, we found an impaired acquisition probability of delay-conditioned responses as well as slower increase in block by block CR probability during acquisition in patients compared to controls (see Figure 10).

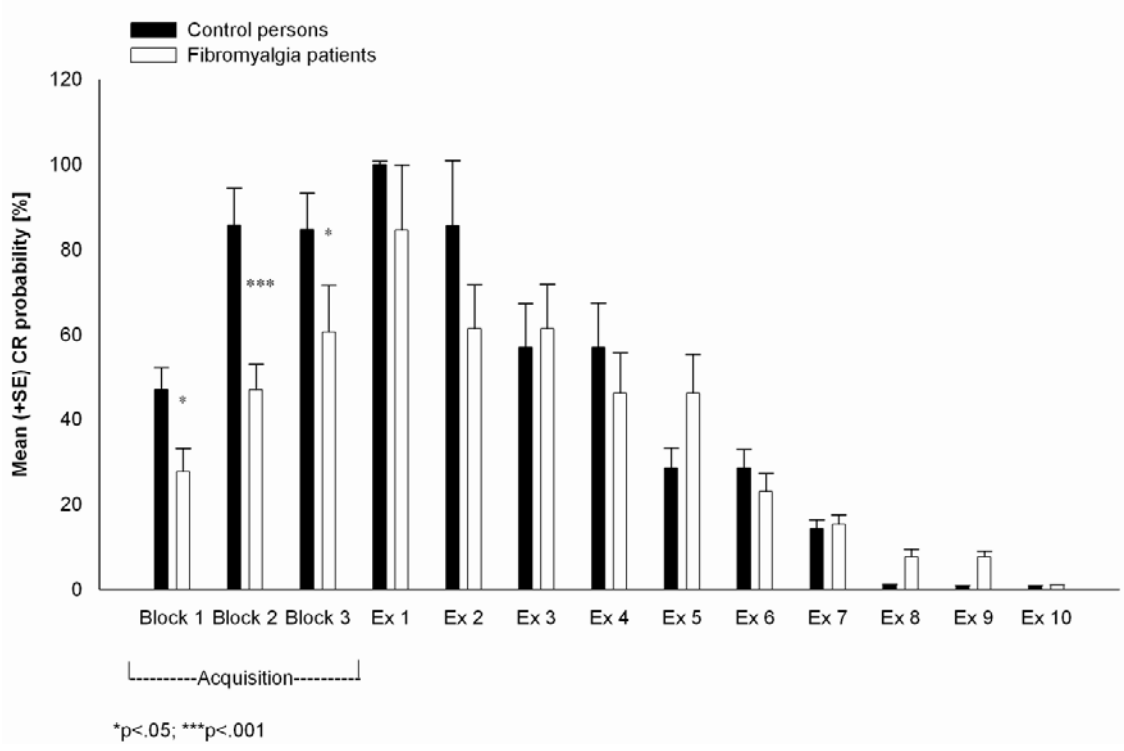

Figure 10. All three acquisition blocks and the extinction block of delay eyeblink conditioning in control and patient group.

Trace conditioning

We found a significant effect of group for adaptive CRs ($F(1,21) = 6.697$; $p = .017$) as well as a significant block effect ($F(2,38) = 7.351$; $p = .003$), with CR probability increasing from Block 1 to Block 2 ($p = 0.001$).

Thus, FMS patients showed a higher acquisition probability of trace-conditioned responses, with a comparable block by block increase of conditioned response probability to healthy controls (see Figure 11).

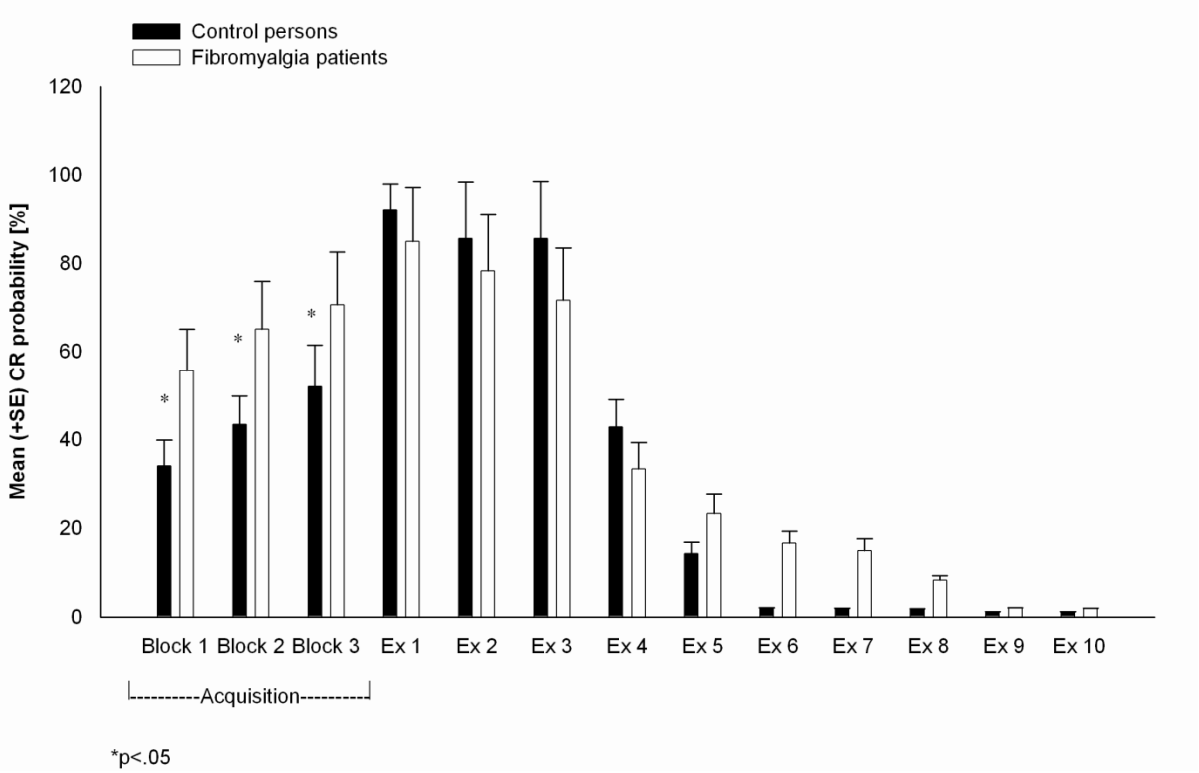

Figure 11. All three acquisition blocks and the extinction block of trace eyeblink conditioning in control and patient group.

4.3.3.2 Extinction

Delay conditioning

While there was no significant effect of group nor a significant difference in the time function between the two groups (no significant group x trial interaction effect), we found a significant effect of trial ($F(4,77) = 12.064$; $p < .001$).

Thus, both patients and controls showed similar delay-conditioned extinction indicated by a trial by trial decrease of CR probability.

Trace conditioning

While we found a significant trial effect ($F(5,77) = 18.046$; $p < .001$) as well as a significant group x trial interaction ($F(5,77) = 2.432$; $p = .048$), there was no significant group effect.

Thus, while both patients and controls showed extinction of trace-conditioned responses, patients and controls differed in the time course of extinction indicated by a slower decrease in CR probability during the last extinction trials in patients compared to controls.

4.3.4 Correlation analyses

We found no significant correlations between the CR probability in delay or trace eyeblink conditioning and the total scores of depression, anxiety, psychosomatic complaints or global symptomatology and psychological distress. With respect to the subscales, FMS patients showed bilateral relations between the CR probability in delay eyeblink conditioning and the GBB related subscale of rheumatic pain (r = -.604; $p = .029$) as well as between the CR probability in trace eyeblink conditioning and the SCL-90-R related subscale of uncertainty in social contact (r = .660; $p = .014$).

In respect to salivary cortisol levels and eyeblink conditioning, we found no correlation of mean morning cortisol level with CR probability during acquisition in delay eyeblink conditioning, but with acquisition-related CR probability in trace eyeblink conditioning (r = -.642; $p = .018$). Thus, low levels of morning cortisol were associated with an increase in trace eyeblink conditioning.

4.4 Discussion

Our data corroborate previously described disturbances in neuroendocrine regulation of the HPA axis in fibromyalgia patients. The main new finding of the present study is that FMS patients show facilitated trace eyeblink conditioning as well as impaired delay eyeblink conditioning. While cortisol measures in this patient group did not significantly correlate with

delay eyeblink conditioning, they are significantly correlated with trace eyeblink conditioning, with lower cortisol levels related to increased trace eyeblink conditioning. Furthermore, while extinction of delay-conditioned responses was not different between the patients and controls, patients showed a slower decrease in CR probability during the last trace-conditioned extinction trials in patients compared to controls.

It is well established that both pharmacologically induced and endogenous mild hypercortisolism impair trace, but not delay eyeblink conditioning (Grillon et al., 2004; Vythilingam, 2006). Further, in a recent study, a facilitation of trace eyeblink conditioning after a pharmacological suppression of endogenous cortisol production could be shown while delay eyeblink conditioning remained unaffected (Nees et al., 2008). However, the present results showed an alteration not only of trace eyeblink conditioning, but also of delay eyeblink conditioning in FMS patients characterized by lower cortisol levels compared to healthy control subjects – a finding that failed to confirm our hypothesis as FMS patients and controls were expected to be similar in acquiring the CR.

Previous neuroendocrine studies have found increased ACTH but normal cortisol responses after CRH stimulation test (Crofford et al., 1994; Ferraccioli, Cavalieri, Salaffi, Fontana, Scita, Nolli, & Maestri, 1990; McCain & Tilbe, 1989; Griep et al., 1998), suggesting an HPA axis perturbation in terms of a combination of sensitized pituitary with adrenal insufficiency (Griep et al., 1998; Griep et al., 1993; Riedel et al., 1998). While the cerebellum mediates acquisition of delay eyeblink conditioning (Lavond et al., 1993), the cerebellum and hippocampus are involved in the acquisition of trace eyeblink conditioning in both animals (Berger & Thompson, 1978; Moyer et al., 1990) and humans (Clark & Squire, 1998; Fortier et al., 2003). As the present findings of an impairment of delay eyeblink conditioning in FMS patients was not associated with cortisol levels, the facilitation in hippocampus-mediated trace eyeblink conditioning suggests that hippocampal function is supported by circulating or locally relatively decreased cortisol levels. Furthermore, the difference in delay conditioning between FMS patients and healthy controls seems to be not based on the cortisol levels, but might be mediated by other factors differing for people with FMS compared to healthy controls.

Since pain is characterized by both sensory and affective aberrations, its chronification can lead to changes in psychological state and affect. Anxiety, depression and anhedonia as the most prominent affective states in patients with chronic pain can interfere with the patient`s quality of life (Jensen, Hoffman, & Cardenas, 2005; Leo, 2005; Rhudy & Meagher, 2000).

Also, stressful life-events at the beginning of or during pain states were mostly reported in chronic pain patients (Aghabeigi, Feinmann, & Harris, 1992). Thus, the stress of being in pain for a long time (as it occurs in FMS patients) as well as the anxiety- and depression-related affective state might affect cortisol status and conditioning, as well, resulting in the current finding of altered delay and trace eyeblink conditioning in FMS patients compared to healthy controls.

Predictability, a process of contingency or associative learning, is fundamental to classical conditioning. Classical conditioning is an adaptive associative process that enables organisms to learn to anticipate events, aversive or otherwise and classical conditioning processes are assumed to play a role in pain symptom generation and persistance (Flor, 2000; Linton et al., 1984). Chronic pain is suggested to capture attention (e.g. Grisart & Plaghik, 1999) and thus may be detrimental to other parallel processing. The hypervigilance model of pain perception (Rollmann & Lautenbacher, 1993) assumes a heightened sensitivity to experimentally induced pain as well as to non-painful stimuli (generalized hypervigilance, McDermid, Rollman, & McGain, 1996). The state of hypervigilance can be viewed as a state of pain-specific anxiety with higher bodily awareness in which attention is directed towards the sources of a potential or actual threat (Grisart, Van der Linden, & Masquelier, 2002). As awareness is important for trace, but not delay eyeblink conditioning, one would suggest an increase in conditioned responses only during trace eyeblink conditioning in FMS patients compared to healthy controls. Thus, the present finding of enhancement of trace eyeblink conditioning, but decrease in delay eyeblink conditioning may indicate a facilitation of cognitive awareness based processing towards an aversive event while more automatically based associations might be slowed down.

The study has several limitations. First, we did not collect blood samples, and thus cannot provide plasma data. Recent studies have shown relative hypocortisolism in basal blood cortisol levels (Griep et al., 1998; Lentjes et al., 1997) and 24h urine free cortisol levels (Crofford et al., 1994; Griep et al., 1998; Lentjes et al., 1997) only. Thus, comparisons with these studies are not possible. Second, while control subjects were recruited from an unselected general population, the FMS population comprised consecutive patients. Thus, one could argue that this limits the validation of the comparison between patients and controls, even more so we did not match for differences in the socio-cultural level. To make samples comparable though, patients and controls were matched for gender and age. In addition, any comorbidity of depression or anxiety, often reported in recent studies, failed in the present

FMS sample. This might limit the generalizability to other FMS samples and make comparisons with other studies difficult.

The current results extend findings from eyeblink conditioning research conducted under conditions of variations in glucocorticoids and may have theoretical and clinical significance not only for FMS patients but also for other symptom groups characterized by a relative mild hypocortisolism helping to explain the high prevalence of psychosomatic symptoms in these disorders.

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4.ii AUTHOR NOTES

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Chapter V:

Accelerated trace eyeblink conditioning after cortisol IV-infusion

(Kuehl et al., submitted)

Co-Authors: Johanna Lass-Hennemann, Steffen Richter, Terry D. Blumenthal, Melly S. Oitzl, & Hartmut Schächinger.

5.0 Abstract

Impairing effects of cortisol on learning performance have been shown in human trace eyeblink conditioning. As the effect is observed from 30 min to hours after administration, a genomic action of cortisol is assumed. Here we report rapid cortisol effects that were observed during the first 10 min after cortisol administration in humans. Young healthy males (n=24) received the cortisol synthesis inhibitor metyrapone (1.5 g per os) to avoid interference of the endogenous pulsatile secretion of cortisol. Next, 2 mg cortisol or placebo was infused intravenously, immediately before the trace conditioning task. The probability of the conditioned eyeblink responses was assessed electromyographically during the trace eyeblink conditioning task (unconditioned stimulus: corneal air puff, 10 psi, 50 ms; conditioned stimulus: binaural pure tone, 75 dB, 1000 Hz, 400 ms; empty interval between CS and US: 550ms). Cortisol resulted in a faster increase of conditioning (p= .02), reaching a comparable level to placebo later on. This result extends the well-known effects of stress on the quality and amount of learning by showing that cortisol also affects the speed of learning. We propose that cortisol accelerates trace eyeblink conditioning via a fast, non-genomic mechanism. This fast action of cortisol is part of the adaptive strategy during the early stress response.

***Keywords*:** Cortisol; Stress; Trace conditioning; Eyeblink; Learning

5.1 Introduction

Stress has differential effects on learning: it can improve or impair learning performance depending on the content of learning (Schwabe, Oitzl, Philippsen, Richter, Bohringer, Wippich, & Schächinger, 2007) as well as the type, timing, and severity of the stressor (Het, Ramlow, & Wolf, 2005; Joels, Pu, Wiegert, Oitzl, & Krugers, 2006). Stress leads to a variety of autonomic nervous system and neuroendocrinological changes, including activation of the hypothalamus pituitary adrenal (HPA) axis. This activation results in an increased release of glucocorticoids (GCs), such as cortisol in humans and corticosterone in rodents. These hormones influence multiple target tissues, both peripherally and in the central nervous system (CNS). The distribution and density of cortisol receptors in the CNS suggest that cortisol plays an important role in mediating stress effects at the CNS-level.

The effects of GCs on learning and memory have usually been shown for longer time courses, such as 60 min after cortisol administration. Therefore, these effects may be explained by "genomic" actions of GCs on gene expression. However, besides the genomic actions of GCs, several studies have shown rapid non-genomic GC action. These rapid GC effects occur within seconds or up to a few minutes, but may also last longer, depending on the tissue. Modulating effects of GC release on gene expression require several steps until the gene product is expressed and available to take effect on the relevant system. The first step, GC dependent transcription, does not result in detectable mRNA until 7.5 minutes after addition of the steroid 'in vitro' (Groner, Hynes, Rahmsdorf, & Ponta, 1983). Even fast genomic effects have onset latencies of at least 20 min in lymphocytes (McEwen, Krey, & Luine, 1978) and 30 min in neurons (Dayanithi & Antoni, 1989). Thus, most genomic effects of cortisol are expected within hours and hardly before an interval of 10 to 20 minutes has passed. On the other hand, rapid non-genomic GC actions have been found in the feedback control of pulses during the daily cortisol profile (Lightman, Wiles, Atkinson, Henley, Russell, Leendertz, McKenna, Spiga, Wood, & Conway-Campbell, 2008), and after exogenous cortisol administration as well (Borski, 2000; Joels, Krugers, Lucassen, & Karst, 2009; Stellato, 2004). The effects of rapid GC action have been demonstrated on a cellular level, and even on behavior. Animal studies show reduced reproductive behavior in male amphibians (Moore & Miller, 1984; Rose, Moore, & Orchinik, 1993) as well as increased locomotion in novelty situations (Sandi, Venero, & Guaza, 1996), impaired memory retrieval (Khaksari, Rashidy-Pour, & Vafaei, 2007) and increased aggressive behavior in rats (Mikics,

Kruk, & Haller, 2004). In the latter study, the aggressive effects started at less than 7 min after GC injection.

However, whether rapid GC action can affect learning processes in humans has not, to our knowledge, been reported to date. A form of simple associative learning is realized in the classical eyeblink conditioning paradigm, which has been studied extensively in animals (Christian & Thompson, 2003) and in humans (Clark & Squire, 1998; Fortier, Disterhoft, Capozzi, Kilduff, Cronin-Golomb, & McGlinchey, 2003). It constitutes a cognitive cross-species test with a neurobiological basis that is fairly well understood. There are two frequently used forms of eyeblink-conditioning paradigms: delay and trace eyeblink conditioning. In delay eyeblink conditioning, the conditioned stimulus (CS, for example, a tone of 400 ms) overlaps and co-terminates with the unconditioned stimulus (US, for example, a 50 ms air puff delivered to the cornea) that induces an unconditioned eyeblink response (UR). After repeated tone-air puff pairing the CS alone is able to elicit an eyeblink before the airpuff occurs. This form of implicit learning is mediated by the cerebellum (Lavond, Kim, & Thompson, 1993). In trace eyeblink conditioning, an empty interval separates the tone and the onset of the air puff. Trace conditioning is an explicit memory task requiring awareness of the CS-US relationship (Clark & Squire, 1998), and depends on both the cerebellum and the hippocampus (Berger & Thompson, 1978; Clark & Squire, 1998; Moyer, Deyo, & Disterhoft, 1990; Woodruff-Pak & Papka, 1996). The influence of endogenous cortisol levels on trace eyeblink conditioning has been shown in clinical and preclinical contexts (Grillon, Smith, Haynos, & Nieman, 2004; Moyer et al., 1990; Nees, Richter, Lass-Hennemann, Blumenthal, & Schächinger, 2008; Nees, Rüddel, Mussgay, Kuehl, Römer, & Schächinger, 2010; Vythilingam, Lawley, Collin, Bonne, Agarwal, Hadd, Charney, & Grillon, 2006). Because the hippocampus may play a more important role in trace than in delay eyeblink conditioning and because of the high density of glucocorticoid and mineralocorticoid receptors in the hippocampus (de Kloet, Reul, & Sutanto, 1990), it is likely that cortisol effects would be most evident in trace conditioning. Rapid GC effects “in vivo” cannot be studied with oral cortisol administration because of delayed effectiveness (gastric transit, absorption). Thus, intravenous (IV) cortisol infusions were necessary in the present study. It may be assumed that in humans a dosage of 2 mg cortisol corresponds to the cortisol response of a mild to moderate stress reaction (Goldman, Gnerlich, & Hussain, 2007; Kok, Westenberg, Thijssen, & van Ree, 1995). Thus, the current study investigated physiologically-relevant cortisol levels, and dosages that are much lower than those used in many pharmacological investigations.

Cortisol is released with ultradian pulsatility. Recent work has suggested that endogenous pulses of cortisol may influence CNS sensitivity to stress (Lightman et al., 2008). To avoid the influence of these endogenous cortisol pulses on exogenous cortisol effects, endogenous cortisol levels of all participants were suppressed by the cortisol synthesis inhibitor metyrapone before the testing session. Metyrapone blocks the regeneration of cortisol from its inactive 11-keta-derivates via inhibition of the rate-limiting enzyme 11-β-hydroxylase (Sampath-Kumar, Yu, Khalil, & Yang, 1997). Via this suppression, we furthermore expected the cortisol depleted CNS system to be more sensitive to the cortisol injection.

We conducted a placebo controlled single-blind study in 24 healthy men who received (after endogenous cortisol suppression by metyrapone) an intravenous administration of cortisol (2 mg) or placebo, immediately before the trace eyeblink conditioning protocol was started. A brief conditioning protocol that consisted of three acquisition blocks resulted in a testing time of less than 10 min, so that longer-lasting genomic GC effects could be excluded. We expected that cortisol administration would lead to alterations in the learning performance of trace eyeblink conditioning.

5.2 Materials and methods

5.2.1 Participants

The study included 24 healthy male volunteers with a mean age of 25.4 (range 20-31) years. All participants gave their written informed consent. Participation was restricted to male subjects in order to avoid interference by menstrual cycle-related changes in adrenocortical regulation. Subjects were excluded from participation if they reported any acute or chronic medical or psychiatric disease in the last two years, especially hearing impairments and allergies against pharmaceutical products. Further exclusion criteria were familial risk for cardiovascular or convulsion diseases, body-mass index < 18 or >28, smoking or taking any pharmaceuticals, illicit substance abuse in the last year, or participation in a pharmaceutical study within the last two months. All experimental procedures were endorsed by the local ethics committee.

5.2.2 Drug manipulation

All participants received an oral dose of 750 mg metyrapone (Metopiron®; Novartis Pharma AG) together with a snack 15 min after arriving. A second 750 mg dose of metyrapone was administered 3.5 hrs later together with a sandwich lunch. This dosing schedule was well tolerated by all subjects and has been shown to significantly reduce plasma cortisol levels when measured 1-2 hrs after the last administration (Broadley, Korszun, Abdelaal, Moskvina, Jones, Nash, Ray, Deanfield, & Frenneaux, 2005; Nees et al., 2008; Roemer, Nees, Richter, Blumenthal, & Schächinger, 2009; Young, Lopez, Murphy-Weinberg, Watson, & Akil, 1997). Immediately before the eyeblink conditioning protocol was started, 2 mg cortisol (Rotexmedia, Trittau, Germany) or a placebo (isotonic NaCl), was intravenously administered over 2 min. At the end of the experimental session an oral substitution dose of 10 mg synthetic cortisol (Hydrocortisone®, Hoechst AG) was administered to bring cortisol levels back to normal values.

5.2.3 Eyeblink conditioning protocol

The eyeblink conditioning protocol consisted of an initial air puff familiarization period (6 air puffs alone without CS) and an acquisition period (3 blocks of 10 trials, with each block consisting of 9 CS-US trials and 1 CS alone trial). The CS was a 75 dB, 400 ms, 1000 Hz pure tone presented binaurally via headphones. The US was a 10 psi, 50 ms air puff to the left cornea delivered through a tube attached to the headphones at a distance of 2 cm from the eye. The US was presented 950 ms after CS onset (550 ms free interval between CS offset and US onset). The intertrial interval during the familiarization and acquisition period varied between 13 and 17 s, with a mean interval of 15 s.

5.2.4 Psychophysiological recording and response scoring

We assessed the eyeblink response as the peak EMG activity of the left musculus orbicularis oculi, using two electrodes (Ag/AgCl) placed below the left eye with an interelectrode distance of 1.5 cm. A third (reference) electrode was placed on the forehead. EMG was recorded with DasyLab software at a sampling rate of 1000 Hz (50 Hz notch filter; bandpass filter 30 to 500 Hz). Data were rectified and integrated with a time constant of 10 ms. In a visual analysis, we categorized the trials with respect to artefacts (i.e., voluntary or

spontaneous eyeblinks at or near the startle stimulus onset, trials with excessive background noise, multiple peaks). If a participant had more than 27.5% of trials contaminated by artefact, they would have been excluded, but no participants had to be excluded for this reason.

5.2.5 Saliva and plasma sample collection and determination

A total of six saliva samples was collected 45, 30, 15 min, and immediately before (PRE) the cortisol/placebo infusion as well as immediately (POST) and 10 min after the eyeblink conditioning. Samples were stored at -20° C until analysis. After thawing for biochemical analysis, the fraction of free cortisol in saliva was determined using a time-resolved immunoassay with fluorometric detection, as described in detail elsewhere (Dressendörfer, Kirschbaum, Rohde, Stahl, & Strasburger, 1992). This assay has a sensitivity (95% confidence interval [CI]) of 0.15 ng/ml and satisfactory precision, with inter- and intra-assay coefficients of variation (CV) < 9% at cortisol concentrations of 2 ng/ml.

A total of three blood samples was taken 195, 105, and 15 min before conditioning (or 90, 180, and 270 min after the first metyrapone administration, respectively) using the S-Monovette® (EDTA K_2-gel preparation; Sarstedt AG & Co.). Samples were immediately placed on ice and centrifuged at 4000 rpm at 6° C for 10 min. Plasma was separated and stored at –80° C until assayed by using a commercial enzyme-linked immunosorbent assay (ELISA) for cortisol (ref.-nr. RE52061; IBL International GmbH) and ACTH (ref.-nr. 7023; BIOMERICA, Inc.). The analytical sensitivity (95% confidence interval) was 2.5 ng/ml and 0.46 pg/ml for the cortisol and ACTH assay, respectively. Analytical precision was high, with coefficients of inter- and intra-assay variation < 8% for both tests.

5.2.6 Procedure

Participants entered the laboratory at 7:45 AM or 9:15 AM and were randomly assigned to receive either cortisol or placebo before the beginning of the conditioning protocol at noon. All participants received the first metyrapone dose 15 min after arrival and the second dose after a further 3.5 hrs (11:30 AM or 1 PM). A sandwich snack was given together with metyrapone both times. The participants were not allowed to leave the laboratory until the end of the experiment, nor were they allowed to consume caffeinated drinks. Blood samples were taken 90, 180, and 270 min after the first metyrapone administration by intravenous access.

After the last blood intake (30 min before the eyeblink conditioning protocol started) an infusor (Infusomat FM, B. Braun, Melsungen, Germany) was applied. Isotonic NaCl solution was administered constantly to avoid clotting. Saliva samples were taken 45, 30, and 15 min before starting the conditioning protocol. Electromyogram (EMG) electrodes were attached for measuring muscle activity of the orbicularis oculi. Participants were asked to fixate their gaze on the wall and to move as little as possible. They were informed that an airpuff would be delivered to one eye and that tones would be delivered over headphones during the experiment. Immediately after the participants provided a further saliva sample and while they were reading the experimental instructions, 2 mg cortisol or placebo (isotonic NaCl) was administered intravenously over 2 min. None of the participants was aware of the administration. After 1 min for distribution of the cortisol in the body, the trace eyeblink conditioning protocol was started (about 1.5 hrs after the second dose of metyrapone). Immediately and 10 min after the conditioning further saliva samples were taken. Awareness of the CS-US contingency was assessed by an open interview and showed that all participants had realized the contingency. At the end of the experimental session all participants received an oral substitution dose of cortisol (10 mg; Hydrocortison, Jenapharm, Germany). The experimental protocol is shown in figure 12.

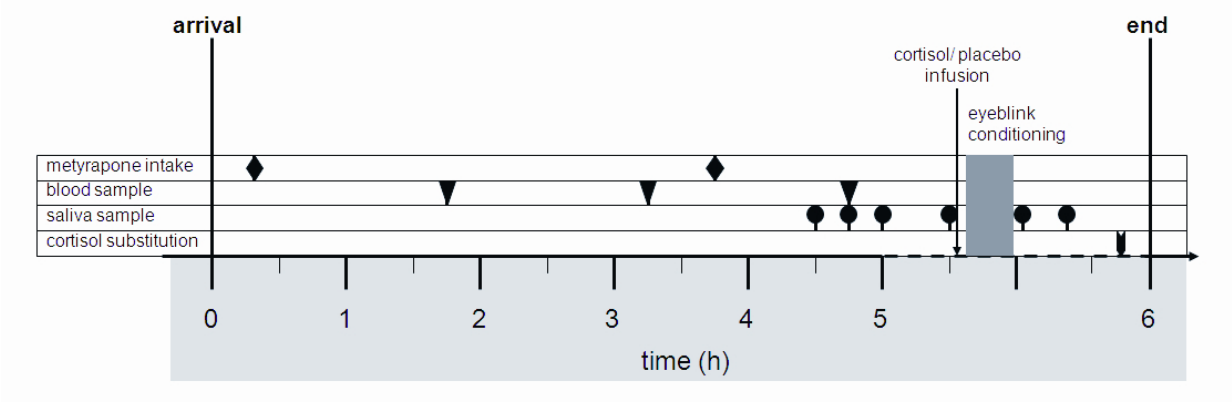

Figure 12. Experimental protocol.

5.2.7 Data scoring and reduction

We assessed EMG eyeblink responses as peak activity of the left musculus orbicularis oculi. Eyeblink responses between a stable baseline (50 ms before US onset) and maximum amplitude 20-100ms after US onset represented the unconditioned response (UR). No participant had to be excluded because of non-responding to the air puff.

Trials with spontaneous eyeblinks, characterized as eyeblinks that increased at least 15 µV in the time window of 500 ms before CS onset, were rejected.

Eyeblinks that increased at least 15 µV in the first 100 ms after CS onset were defined as alpha responses (unconditioned responses to the CS; Gormezano, 1966). Those were not considered as true responses in the conditioning paradigm, and the response to the CS was not influenced by unconditioned eyeblink responses, as will be described in the "Results" section.

Eyeblinks that increased at least 15 µV in the time interval of 550-950 ms post-CS (period of 400 ms that precede the US) were categorized as conditioned responses ("adaptive", true CRs), because eyeblinks in this time window prevent the airpuff from hitting the eye (Spence & Rose, 1959). Further, eyeblinks that occurred during the interval of 100-550 ms after the CS were considered as "nonadaptive" responses because of their poor timing relative to the US (Grillon et al., 2004; Vythilingam et al., 2006). The probability of the nonadaptive CRs was low and did not differ significantly between the cortisol and placebo groups, as will be described in the "Results" section.

5.2.8 Statistical Analysis

Endocrinological data (saliva cortisol, plasma cortisol, and ACTH) were each analyzed with a treatment (cortisol vs. placebo) x time of collection (saliva: -45, -30, -15, pre, post, +10; plasma cortisol and ACTH: -195, -105, -15) repeated measures analysis of variance (ANOVA). For the eyeblink EMG data during the familiarization period we used a treatment (cortisol vs. placebo) x time repeated measures ANOVA. Magnitude of the eyeblink reactions were taken for the analysis of familiarization data. For the conditioned response data during the acquisition period, a treatment (cortisol vs. placebo) x time (block) repeated measures ANOVA was used. Since the first block was separated into three parts to more sensitively investigate the learning increase, it was analyzed with a treatment (cortisol vs. placebo) x time (trial 1-4, 5-7, 8-10) repeated measures ANOVA, and a second ANOVA was used to test blocks 2 and 3.

For all statistical analyses, α was 0.05, and we applied the Greenhouse-Geisser adjustment in case of violation of the assumption of homogeneity of variances, and adjusted degrees of freedom are reported. In the case of significant main effects or interactions, t-tests for independent groups (cortisol vs. placebo) or paired t-tests with Bonferroni adjustment were

performed. All statistical analyses were computed using the Statistical Package for the Social Sciences for Windows (SPPS Inc., version 17.0).

5.3 Results

Groups did not differ in age (cortisol: 25.9 years, SE 0.7; placebo: 24.8 years, SE 1.1; T(22)= .86, p= .40), body weight (cortisol: 78.0 kg, SE 2.0; placebo: 76.8 kg, SE 2.2; T(22)= .39, p= .70), nor body mass index (BMI; cortisol: 23.8 kg/m^2, SE 0.6; placebo: 23.4 kg/m^2, SE 0.5; T(22)= .44, p= .66).

5.3.1 Endocrinological data

As expected from the metyrapone treatment, a comparison of plasma cortisol levels at three collection times before intravenous administration showed a significant decrease of plasma cortisol (F(2,36)=65.56, p= .000, eta= .79). No significant differences were found between the treatments (cortisol vs. placebo; F(1,18)= .40, p= .53) nor was a significant interaction found (time x treatment; F(2,36)=2.35, p= .11) (see Figure 13A). The same pattern was found for saliva cortisol (see Figure 13C). Four saliva samples were taken in the last 45 min prior to the conditioning test and showed a significant decrease of cortisol (F(3,66)=24.97, p= .000, eta= .53). There was no significant difference between the treatments (F(1,22)= .04, p= .85) nor a significant interaction of time and treatment (F(3,66)= .66, p= .46).

As expected from feedback inhibition of the HPA axis, the opposite pattern was found for ACTH plasma levels. Again, there was a significant effect of time (F(2,38)=15.80, p= .000, eta= .45), but no significant difference between the two treatments (F(1,19)= .82, p= .38) nor a significant interaction (time x treatment; F(2,38)=1.28, p= .29) (see Figure 13B).

Cortisol levels in saliva of the cortisol group differed significantly from those of the placebo group, as the interaction of the treatment x time of collection ANOVA shows (F(2, 33) = 30.6, p= .000, eta= .57). Post-hoc contrasts revealed that after intravenous administration saliva levels of cortisol were significantly enhanced in the cortisol group (2.44 nmol/L, SE 0.24) compared to the placebo group (0.82 nmol/L, SE 0.07) (T(11)= 6.44, p= .000) and when

compared to the collection time point before (T(11)= 7.65, p= .000) cortisol administration (0.88 nmol/L, SE 0.10). Mean saliva cortisol increase was 1.55 nmol/L (see Figure 13C).

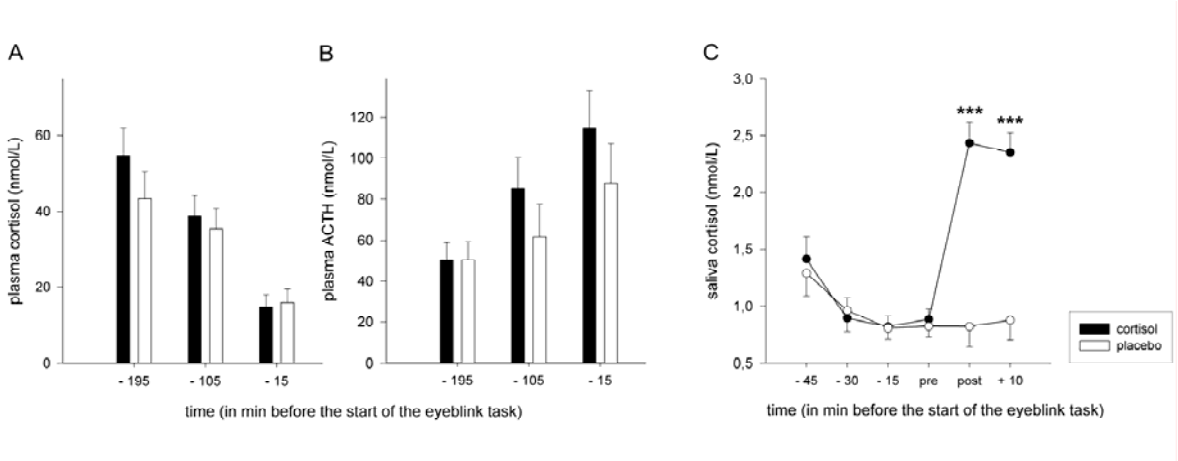

Figure 13. Endocrinological data were used as a manipulation check: plasma cortisol (A) and ACTH (B) data before, and saliva cortisol (C) data before and after the eyeblink conditioning task. Closed bars/symbols=cortisol, open bars/symbols=placebo.

5.3.2 Baseline eyeblinks and air puff familiarization

The eyeblink magnitudes to the air puff during familiarization did not differ significantly between cortisol and placebo treatments (F(1,22)= .19, p= .67). The magnitude was 230.2 µV (SE 14.5) in the cortisol group and 211.5 µV (SE 9.7) in the placebo group (averaged over the six trials).

Probabilities of spontaneous eyeblinks (assessed during the 500 ms time window prior to the CS) did not differ significantly between cortisol and placebo treatments (t(22)=1,14; p= .27) as well.

5.3.3 Alpha responses

Alpha responses were observed for both treatments. Their probability did not differ significantly between the two treatments (t(22)= .37, p= .71; cortisol: 36.8 %, SE 9.14; placebo: 31.6 %, SE 10.7).

5.3.4 Conditioned responses

Nonadaptive responses

The probability of a nonadaptive CR did not differ significantly (t(22)= .12, p= .91) between the cortisol (33.9 %, SE 7.48) and the placebo treatment (32.5 %, SE 9.15).

Acquisition

Figure 14 shows the three acquisition blocks during trace eyeblink conditioning for cortisol and placebo treatment. The first block was separately analyzed with a treatment x time (trial 1-4, 5-7, 8-10) repeated measures ANOVA. The analysis revealed a significant main effect of treatment (F(1,22)=7.06, p= .01, eta= .24), a significant main effect of time (F(2,44)=4.0, p= .03, eta= .15) and a significant treatment x time interaction (F(2,44)=4.3, p= .02, eta= .16). Single comparisons for each group of trials showed significant differences between cortisol and placebo treatments for trial 5-7 (t(22)=2.35, p= .03) and 8-10 (t(22)=2.66, p= .01).

Block 2 and 3 probability for response data were analyzed with a treatment x time (block 2 and 3) repeated measures ANOVA. The probability of CRs did not differ between treatments (F(1,22)=1.76, p= .20). Furthermore, there was no significant main effect of time (F(1,22)= .38, p= .54) nor a significant interaction (F(1,22)= .01, p= .93).

Thus, we observed facilitated learning for cortisol treatment during the first block, but the overall learning performance did not show any differences between the treatments.

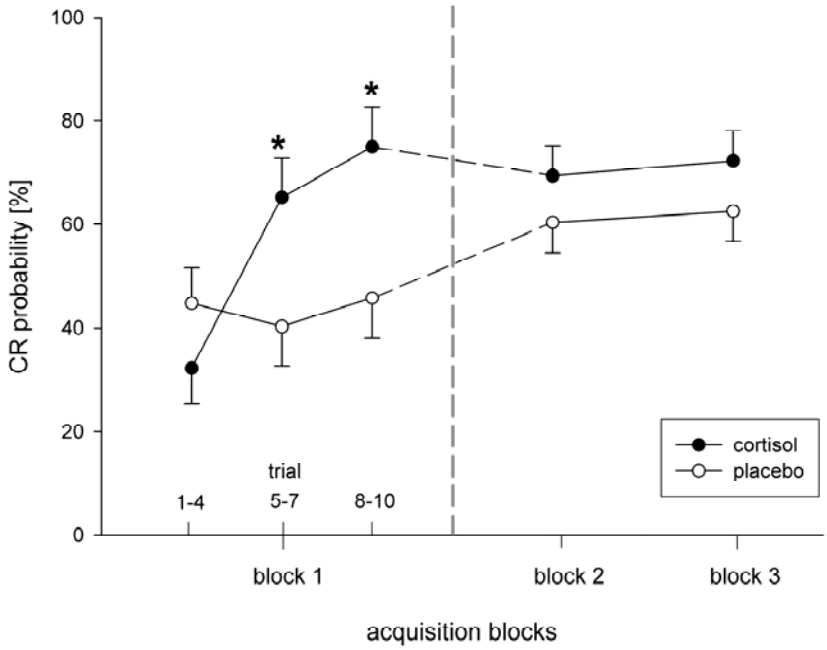

Figure 14. Enhanced CR probability in the cortisol group during the first block of trace eyeblink conditioning, but no difference between cortisol and placebo group during block 2 and 3.

5.4 Discussion

The purpose of the present study was to investigate the effects of rapid cortisol action on eyeblink conditioning in humans. Trace eyeblink conditioning was chosen because its sensitivity for GC influence has been clearly demonstrated (Grillon et al., 2004; Nees et al., 2008; Nees et al., 2010; Vythilingam et al., 2006) and its underlying mechanisms are well understood (Christian & Thompson, 2003). Additionally, eyeblink conditioning requires little cooperation from the subjects and, therefore, results are less likely to be affected by non-specific factors, such as motivation (Grillon et al., 2004).

The main result of the current study is the accelerated learning in a trace eyeblink conditioning paradigm that occurred within minutes after cortisol administration, compared to placebo. The differential learning effect, measured as the probability of adaptive conditioned responses (CRs), occurred very clearly in the first block of the acquisition, even though there was no difference in the total learning performance between cortisol and placebo treatment in the second and third blocks. Such a detailed look at the trials of the first block of an eyeblink conditioning paradigm is important to detect time sensitive effects (Woodruff-Pak, 1999).

Furthermore, the cortisol effects were limited to the conditioned eyeblink responses, and there were no significant differences between the two experimental groups in any other eyeblink measures, such as spontaneous blink rate, alpha responses, non-adaptive CRs, or reactivity during familiarization.

Effects of cortisol on trace eyeblink conditioning have been shown in rodents as well as in humans. Trace eyeblink conditioning is mediated by the hippocampus, and stress-sensitive neurons from the hippocampal CA1 and CA3 regions are involved (McEchron & Disterhoft, 1997). In male rats an acute stressful experience can enhance learning in eyeblink conditioning. The removal of both the adrenal cortex and medulla, but not the medulla alone, prevents an enhancement of trace eyeblink conditioning, highlighting the critical role of glucocorticoids (GCs) in modulating eyeblink conditioning (Beylin, Gandhi, Wood, Talk, Matzel, & Shors, 2001; Beylin & Shors, 2003). Furthermore, male mice showed enhanced corticosterone levels as well as increased excitability of CA1 neurons and increased responses in trace eyeblink conditionings 1 or 24 hours after exposure to an acute stressor, compared to control mice (Weiss, Sametsky, Sasse, Spiess, & Disterhoft, 2005). Interestingly, acute stress and the associated GC release have an opposite effect on eyeblink conditioning in male and female rats. Acute stress facilitated eyeblink conditioning in males, but impaired it in females, and corticosterone correlates with the conditioning performance in males, but not in females (Wood, Beylin, & Shors, 2001).

Studies in humans demonstrate the critical role of GCs in trace eyeblink conditioning modulation as well. Pharmacologically induced mild hypercortisolism and endogenous hypercortisolism in patients with Cushing´s syndrome showed impairing effects on trace eyeblink conditioning (Grillon et al., 2004; Moyer et al., 1990; Vythilingam et al., 2006). Facilitation of trace eyeblink conditioning, on the other hand, has been found for pharmacologically induced hypocortisolism and in fibromyalgia patients with chronic hypocortisolism (Nees et al., 2008; Nees et al., 2010).

Interestingly, the present results are in line with the facilitating effects of stress and corticosterone in rodents, but show a seemingly contrary pattern to the reported effects of cortisol in humans. However, in the present study the conditioning started a few minutes after cortisol administration, in contrast to other studies that have investigated the effects of cortisol on learning for longer time courses, such as 60 min. Therefore, those previously published effects could be explained by well-known “genomic” GC actions on gene expression, effects that are usually expected within hours, but seldom before 10 to 20 minutes. Even the first

step, glucocorticoid dependent transcription, results in detectable mRNA no sooner than 7.5 minutes after addition of the steroid 'in vitro' (Groner et al., 1983).

In the present study, however, the timing of the experimental protocol and the differential learning effect in the very first minutes after cortisol administration suggest a rapid non-genomic mechanism. That rapid GC actions can affect behavior is known from some animal studies: effects on reproductive behavior in male amphibians (Moore & Miller, 1984; Rose et al., 1993), increased locomotion in novelty situations (Sandi et al., 1996), impaired memory retrieval (Khaksari et al., 2007) and increased aggressive behavior in rats (Mikics et al., 2004) have been found.

Several studies have reported that rapid GC effects on neuronal action via non-genomic mechanisms affect glutamatergic mediated neurotransmission (Karst, Berger, Turiault, Tronche, Schutz, & Joels, 2005; Olijslagers, de Kloet, Elgersma, van Woerden, Joels, & Karst, 2008; Venero & Borrell, 1999), involving N-methyl D-aspartate (NMDA) glutamate receptors (McEwen, 1996). Glutamatergic neurotransmission, and especially the NMDA receptor, play an important role in processes of learning and memory (Kim, Foy, & Thompson, 1996; Villarreal, Do, Haddad, & Derrick, 2002; Wigtström & Gustafsson, 1992). Non-genomic enhancement of glutamate transmission (Joels et al., 2009; Karst et al., 2005; Venero & Borrell, 1999) by GCs has been found in the hippocampus. Besides the cerebellum, the hippocampus constitutes the main underlying central structure of trace conditioning (Berger & Thompson, 1978; Clark & Squire, 1998; Moyer et al., 1990; Woodruff-Pak & Papka, 1996), and might therefore be sensitive to changes, including rapid effects, in cortisol levels.

To avoid possible interferences from genomic GC actions the conditioning protocol in the present study was short and lasted less than 10 min. Indeed, the effect of enhanced conditioned responses occurred early, during the first conditioning block. This effect lasted only for a short time and vanished after the first conditioning block. This supports the explanation of rapid non-genomic GC actions and does not conflict with the well established results of longer-lasting GC effects that impair trace conditioning.

To manipulate the cortisol levels of the participants a physiological dosage of 2 mg cortisol was intravenously administered immediately before the eyeblink conditioning protocol started. Endogenous cortisol levels of all participants were suppressed by metyrapone before the session to avoid interference from individual differences in basal levels. Blood samples

that were taken three times before the conditioning protocol revealed a clear decrease of plasma cortisol and, depending on the feedback inhibition of the HPA axis, an increase of plasma ACTH. Prior to the conditioning task, both experimental groups showed comparable levels of saliva and plasma cortisol and plasma ACTH. In contrast, saliva samples that were taken immediately after the task showed a large increase of cortisol in those participants who had received a cortisol administration. The mean increase of 1.55 nmol/L in cortisol levels is comparable to the endogenous increase of a moderate stress reaction. Stress-induced increases of cortisol after laboratory stress tests, e.g. the Trier Social Stress Test, are expected at values of at least 2.0-2.5 nmol/L (Fehm-Wolfsdorf, Soherr, Arndt, Kern, Fehm, & Nagel, 1993; Kirschbaum, Pirke, & Hellhammer, 1993; Schwabe, Haddad, & Schächinger, 2008). Thus, with the chosen dosage of administered cortisol we were able to mimic the cortisol increase of a moderate endogenous stress reaction, increasing the relevance of our results for real life situations.

Trace eyeblink conditioning is a form of simple associative learning. Moreover, it is also a form of fear conditioning because of the potential threat from the stimulus. The air puff constitutes potential harm to the eye, while the closure of the eye by a blink is a protective reflex (Haerich, 1998). In a real life situation it might be very helpful if one were able to adapt faster, by accelerated learning, to a potential threat. Because a fast change in behavior is especially important in a dangerous or stressful situation, fast behavioral effects of GCs, in addition to the well known later effects, would obviously be of advantage.

Via the brief conditioning protocol we were able to focus on rapid non-genomic effects of cortisol. However, the brevity of the protocol also entails some limitations of the study. The acquisition consisted of only three blocks, so that we can only speculate about the maximum learning performance, even though the difference in learning was already small in the second and third block. This might explain why the learning performance was comparable with results from healthy samples (Grillon et al., 2004; Nees et al., 2008; Nees et al., 2010), but still lower than the performance that could be expected for cortisol suppression by metyrapone (Nees et al., 2008). Furthermore, the time limitation did not allow the collection of extinction data. Thus, we cannot make any statement about the stability of the learned performance nor about memory processes. Because of the time limitation, we also could not assess valence and arousal ratings of the CS and US to control the subjective quality of the stimuli. Further limitations result from the specific experimental participants. Because only

young healthy males were tested, no conclusions can be drawn about potential effects of gender and age. Also the sample size of 24 participants was rather small (although sufficiently powerful to uncover several interesting and significant findings). Furthermore, we suppressed the cortisol levels in all participants by metyrapone to avoid interference from endogenous GC basal levels and pulses. Metyrapone blocks the regeneration of cortisol in humans, or corticosteroid in rats, from its inactive 11-keta-derivates via inhibition of the rate-limiting enzyme 11-β-hydroxylase (Sampath-Kumar et al., 1997). The blocked cortisol synthesis results in the removal of negative feedback from the pituitary and hypothalamus, leading to reduced circulating cortisol, and increased adrenocorticotropic (ACTH) and corticotropin-releasing hormone (CRH; Fiad, Kirby, Cunningham, & McKenna, 1994; Hagendorf, Koper, de Jong, Brinkmann, Lamberts, & Feelders, 2005; Otte, Lenoci, Metzler, Yehuda, Marmar, & Neylan, 2007; Rotllant, Ons, Carrasco, & Armario, 2002). Thus, the effects of cortisol on a depleted system may differ from the effects in a natural situation. We cannot exclude interactions from the increased ACTH levels or further endocrinological changes. Because of the IV route of cortisol administration we can be sure that cortisol has entered the blood stream and reached the brain within 1 min of peripheral circulation. However, we cannot be sure about the timing of rapid cortisol effects on CNS functions. Saliva sampling perfectly fulfilled the purpose of a manipulation check in this study, but the time course of cortisol contribution in the brain cannot be exactly reflected by this method. Finally, our protocol of trace eyeblink conditioning allows no conclusion about other forms of classical conditioning or learning processes in general.

5.4.1 Conclusions

We report a remarkably rapid facilitating effect of cortisol on trace eyeblink conditioning in young healthy males. Performance was accelerated during the first conditioning block. Previous studies have suggested that stress and stress hormones affect the quality and amount of learning (Schwabe, Wolf, & Oitzl, 2010). The current study adds the finding that the stress hormone cortisol may also affect the speed of learning. We suggest rapid non-genomic cortisol actions as an underlying mechanism. Faster learning of a protective response might be very helpful in stressful and potentially threatening situations, and could therefore reflect an adaptive strategy of the early stress reaction. Further studies should investigate additional effects of rapid cortisol actions on human behaviors such as learning or memory processes.

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5.ii AUTHOR NOTES

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Erklärung

nach § 9, Abs. 1 der Promotionsordnung des Fachbereichs I der Universität Trier vom 13.11.2008.

Hiermit versichere ich, dass ich die vorliegende Arbeit selber verfasst und keine außer den angegebenen Hilfsmitteln und Referenzen benutzt habe. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

(Linn Kristina Kühl) Trier,