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Implicit Learning and Stress Hormones

Autor:

Sonja Römer, Dipl.-Psych.

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Gutachter:

Prof. Dr. med. Hartmut Schächinger

Prof. Dr. Jobst Meyer

This dissertation thesis and the presented research were performed at the

Division of Clinical Physiology

Institute of Psychobiology

University of Trier.

Affiliation of the Supervisors:

Prof. Dr. med. Hartmut Schächinger

Division of Clinical Physiology

Institute of Psychobiology

University of Trier

Prof. Dr. Jobst Meyer

Division of Neurobehavioral Genetics

Institute of Psychobiology

University of Trier

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[1] Adams, D. (1979). The hitchhiker's guide to the galaxy: Harmony Books.

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This doctoral thesis consists in general of four chapters (and one additional chapter that represents a general introduction and overview), which are published as 'Original Research Articles' in international peer-reviewed journals. 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
Chapter II	Roemer, S., Nees, F., Richter, S., Blumenthal, T. D., & Schachinger, H. (2009). Endogenous cortisol suppression with metyrapone enhances acoustic startle in healthy subjects. *Horm Behav, 55*(2), 314-318. (Impact Factor 2009: 3.770).
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Index of Abbreviations

11-β-HSD-1	11-β-hydroxysteroiddehydrogenase type-1
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
CES-D	Center for Epidemiologic Studies Depression-Scale
cm	Centimeters
CNS	Central nervous system
CR	Conditioned response
CRH	Corticotropin releasing hormone
CS	Conditioned stimulus
dB(A)	Decibels (A-scale)
DHEA	Dehydroepiandrosterone
e.g.	For example
FMS	Fibromyalgia syndrome
FSM	Flying Spaghetti Monster
EMG	Electromyogram
GBB	Gießener Beschwerdebogen
GR	Glucocorticoid receptor
HPA	Hypothalamus-pituitary-adrenal
ISI	Inter-stimulus interval
ITI	Inter-trial interval
kg/m^2	Kilograms per square meter
LED	Light emitting diode
lm	Lumen
LTD	Long-term depression
lx	Lux
mcd	Millicandela
mg	Milligrams
min	Minutes
ml	Milliliters
mm	Millimeters

MR	Mineralocorticoid receptors
ms	Milliseconds
µV	Microvolts
nmol/l	Nanomoles per liter
%	Percent
PC	Personal Computer
Psi	Pounds per square inch
PTSD	Post-traumatic stress disorder
SCID	Structured Clinical Interview for DSM-IV
SCL	Symptom Check List
SD	Standard deviation
SE	Standard error
SOC	Second order conditional
SRTT	Serial reaction time task
UR	Unconditioned response
STAI	State-Trait-Anxiety Inventory
US	Unconditioned stimulus

General Abstract

There is a lot of evidence for the impact of acute glucocorticoid treatment on hippocampus-dependent explicit learning and memory (memory for facts and events). But there have been few studies, investigating the effect of glucocorticoids on implicit learning and memory.

We conducted three studies with different methodology to investigate the effect of glucocorticoids on different forms of implicit learning.

In *Study 1*, we investigated the effect of cortisol depletion on short-term habituation in 49 healthy subjects. 25 participants received oral metyrapone (1500 mg) to suppress endogenous cortisol production, while 24 controls received oral placebo. Eye blink electromyogram (EMG) responses to 105 dB acoustic startle stimuli were assessed. Effective endogenous cortisol suppression had no effect on short-term habituation of the startle reflex, but startle eye blink responses were significantly increased in the metyrapone group. The latter findings are in line with previous human studies, which have shown that excess cortisol, sufficient to fully occupy central nervous system (CNS) corticosteroid receptors, may reduce startle eye blink. This effect may be mediated by CNS mechanisms controlling cortisol feedback.

In *Study 2*, we investigated delay or trace eyeblink conditioning in a patient group with a relative hypocortisolism (30 patients with fibromyaligia syndrome/FMS) compared to 20 healthy control subjects. Conditioned eyeblink response probability was assessed by EMG. Morning cortisol levels, ratings of depression, anxiety and psychosomatic complaints as well as general symptomatology and psychological distress were assessed. As compared to healthy controls FMS patients showed lower morning cortisol levels, and trace eyeblink conditioning was facilitated whereas delay eyeblink conditioning was reduced. Cortisol measures correlate significantly only with trace eyeblink conditioning. Our results are in line with studies of pharmacologically induced hyper- and hypocortisolism, which affected trace eyeblink conditioning. We suggest that endocrine mechanisms affecting hippocampus-mediated forms of associative learning may play a role in the

generation of symptoms in these patients.

In *Study 3*, we investigated the effect of excess cortisol on implicit sequence learning in healthy subjects. Oral cortisol (30 mg) was given to 29 participants, whereas 31 control subjects received placebo. All volunteers performed a 5-choice serial reaction time task (SRTT). The reaction speed of every button-press was determined and difference-scores were calculated as a proof of learning. Compared to the control group, we found a delayed learning in the cortisol group at the very beginning of the task. This study is the first human investigation, indicating impaired implicit memory function after exogenous administration of the stress hormone cortisol. Our findings support a previous neuroimaging study, which suggested that the medial temporal lobe (including the hippocampus) is also active in implicit sequence learning, but our results may also depend on the engagement of other brain structures.

Chapter I: General Rationale

Everyday experiences during our lives can be both, protective, when they help to avoid negative events in future or rewarding, when positive events can be approached. Learning and memory is a very important prerequisite to anticipate future events. Not only information, which is learned consciously, can be important but also implicit knowledge, which is acquired without consciousness. Explicit learning and memory is very vulnerable to stress or stress hormones (for review see Het, Ramlow, & Wolf, 2005), while implicit processes are assumed to be more resistant to stress (e.g. Keenan, et al., 1996; Kirschbaum, Wolf, May, Wippich, & Hellhammer, 1996; Lupien, et al., 1997). The latter may vary with the complexity of the implicit process, which is observed. With the present work, we aimed to extend research in this field. We conducted three studies to examine the impact of the stress hormone cortisol on different forms of implicit learning with growing complexity.

1.1 Implicit Learning

One popular definition of implicit (nondeclarative) learning is "the acquisition of knowledge that takes place largely independent of conscious attempts to learn and largely in the absence of explicit knowledge about what was acquired" (Reber, 1993, p. 5). This kind of learning includes associative as well as non-associative learning processes (L.R. Squire, Kandel, & Niehaus-Osterloh, 2009).

An important prerequisite of implicit learning "is the ability to gradually extract the common elements from a series of separate events" (L. R. Squire, 2004, p. 174), and the success of the acquisition of implicit knowledge is typically demonstrated through performance rather than recollection (L. R. Squire, Stark, & Clark, 2004). Skills and habits, priming and perceptual learning, simple forms of conditioning and non-associative learning are referred to be implicit. These different forms of implicit memory have been commonly suggested to be independent of the hippocampus (Larry R. Squire, 1992), but to relay on other specific brain systems, such as the striatum, the neocortex, the amygdala, the cerebellum, or reflex pathways (compare,

L. R. Squire, 2004).

In the present work we focus on different forms of implicit learning with growing complexity – habituation, classical conditioning and implicit sequence learning – with respect to consciousness and hippocampus-engagement.

We used paradigms in which successful implicit learning is demonstrated through performance, which itself relays on the retrieval of implicit knowledge (memory). Thus, in the present work, the processes of learning and memory can sometimes hardly be disentangled. In this case, the terms learning and memory can be used equivalent.

1.1.1 Habituation

Habituation is the simplest form of implicit learning and an example of non-associative learning (L.R. Squire, et al., 2009). Non-associative learning takes place when an individual is exposed once or repeatedly to a single type of stimulus. In habituation, the organism learns to ignore a novel stimulus after repeated exposure, if it is neither beneficial nor harmful. Depending on the quantity of repetitions, habituation can have a short- or a long-term form. Short-term memory for habituation is mediated by enduring synaptic depression of the connections made by sensory neurons, interneurons, or both at several sites in the reflex circuit. This mechanism explains e.g. habituation of the startle reflex of vertebrates (Kandel, 2000).

1.1.2 Classical Conditioning

Classical conditioning is an example of simple associative learning, which is a more complex form of learning than habituation. This kind of learning is also mediated by changes of the effectiveness of the synaptic connections that make up the pathway mediating behavior, but here the organism learns to associate one type of stimulus with another. In this way, an initially weak conditioned stimulus (CS) can become highly effective in producing a response when paired with a strong unconditioned stimulus (US). Usually the CS precedes the US and the time interval between the CS and US is critical (Kandel, 2000). Delay and trace conditioning are two frequently

used paradigms of classical conditioning.

In delay conditioning the CS overlaps the US, which induces an unconditioned response (UR). CS and US terminate together and after repeated CS-US pairings, the CS is able to elicit a conditioned response (CR) without the application of the US. Delay conditioning is 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, CS and US are separated by an empty interval and an awareness of CS-US contingency is essential (Clark & Squire, 1998). Therefore, trace eyeblink conditioning requires both the cerebellum and the hippocampus (Berger & Thompson, 1978; Clark & Squire, 1998; Moyer, Deyo, & Disterhoft, 1990; Woodruff-Pak & Papka, 1996). For this reason trace conditioning can be seen as a special case of implicit learning: The acquisition of the CS-US contingency takes place independent of conscious attempts to learn, but the development of explicit or conscious knowledge (declarative memory) about this contingency is necessary for its retrieval (compare Clark & Squire, 1998).

1.1.3 Implicit Sequence Learning

Like conditioning, implicit sequence learning is based on the detection of covariation between events. But unlike the simple association in classical conditioning, implicit sequence learning involves abstract induction (Reber, 1993), i.e. a conclusion from empirical phenomena to a more general knowledge. For a typical paradigm, like a SRTT, it is assumed that subjects learn to anticipate forthcoming stimuli on the basis of sequential constraints (Cleeremans, 1993; Cleeremans & McClelland, 1991). One sequential constraint for example is a sequence of two adjacent stimuli, predicting the appearance of a third stimulus. This is a so-called second order conditional (SOC; Reed & Johnson, 1994). In contrast to a simple contingency between two stimuli, learning a SOC sequence requires higher-order associations between more than two successive stimuli.

Recent evidence suggests the medial temporal lobe to be involved in implicit

learning of complex contingencies (Chun & Phelps, 1999; Clark & Squire, 1998; Curran, 1997; Poldrack, et al., 2001; Rose, Haider, Weiller, & Buchel, 2002). In line with this, Schendan et al. (2003a) could show in functional imaging studies that the medial temporal lobe is not only active in explicit but also in implicit sequence learning during a SRTT (Poldrack & Rodriguez, 2003; Schendan, et al., 2003a; Schendan, Searl, Melrose, & Stern, 2003b).

1.2 The Influence of Stress Hormones on Learning

Stress induces the release of corticosteroids, known to modulate cognitive performance (Lupien & McEwen, 1997). Animal and human studies show that acute high levels of glucocorticoids may enhance memory consolidation and impair memory retrieval processes (Roozendaal, 2002). These effects are suggested to be mediated by the hippocampus, a brain structure in the medial temporal lobe which is involved in learning and memory (Lupien & Lepage, 2001; Larry R. Squire, 1992), and which contains a high density of glucocorticoid receptors (GRs; McEwen, Weiss, & Schwartz, 1968). There is a lot of evidence for the impact of glucocorticoids on hippocampus-dependent explicit learning and memory, i.e. memory for facts and events (for review see de Quervain, Aerni, Schelling, & Roozendaal, 2009; Het, et al., 2005; Lupien, Maheu, Tu, Fiocco, & Schramek, 2007). But there have been few studies, investigating the effect of glucocorticoids on implicit learning and memory (e.g. Kirschbaum, et al., 1996). Here, we focus on the most important findings of glucocorticoid-effects on habituation, classical conditioning and implicit sequence learning.

1.2.1 Glucocorticoids and Habituation

On the one hand, studies in rodents show that adrenalectomy (Davis & Zolovick, 1972) or treatment with oral corticosterone (Ardayfio & Kim, 2006) does not affect startle habituation. On the other hand, there is evidence of nonhabituated exaggerated startle reactions in post-traumatic stress disorder (PTSD; Garrick, Morrow, Shalev, & Eth, 2001; Ladwig, et al., 2002; Shalev, et al., 2000). PTSD is a specific type of anxiety disorder that is characterized by altered hypothalamus-

pituitary-adrenal (HPA) axis activity with low levels of peripheral cortisol (Kellner & Yehuda, 1999; Oquendo, et al., 2003) and high levels of corticotropin releasing hormone (CRH; Bremner, et al., 1997; de Kloet, et al., 2008). The common physiological background of these findings in PTSD patients may be excessive central release of CRH, acting on the bed nucleus of the stria terminalis, to promote anxiety, initiated by non-specific stimuli (Marshall & Garakani, 2002).

1.2.2 Glucocorticoids and Classical Conditioning

Glucocorticoids have been shown to modulate classical conditioning (Grillon, Smith, Haynos, & Nieman, 2004), and 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). In line with these findings, human studies demonstrated an impairment of trace but not delay conditioning in persons with endogenous hypercortisolism and during pharmacologically induced mild hypercortisolism (Grillon, et al., 2004; Vythilingam, et al., 2006) as well as a facilitation of trace but not delay conditioning processes during pharmacologically induced endogenous mild hypocortisolism (Nees, Richter, Lass-Hennemann, Blumenthal, & Schachinger, 2008).

1.2.3 Glucocorticoids and Implicit Sequence Learning

Functional imaging studies demonstrated that the medial temporal lobe is active during implicit sequence learning of SOC sequences within a SRTT (Schendan, et al., 2003a, 2003b). Even though these findings suggest that performance on an implicit learning task could be affected by glucocorticoids, to our knowledge there exists no study which investigated the influence of glucocorticoids or stress on implicit sequence learning.

1.3 Further investigation of the influence of cortisol on implicit learning in the present work

Since implicit learning can occur independent of consciousness and explicit knowledge, it is assumed that those implicit processes and corresponding structures

are phylogenetically older and more basic and primitive compared to explicit processes of learning and memory, which require conscious control and therefore must have developed later in evolution (Reber, 1993). Furthermore, older structures "tend to be more robust and resilient, less prone to disruption of function than the newer" and implicit cognitive processes are expected to "show greater resistance to interference from neurological insult and clinical disorder than the explicit processes "(Reber, 1993, p. 7).

Similar, implicit learning and memory is assumed to be less vulnerable to stress or stress hormones (e.g. Keenan, et al., 1996; Kirschbaum, et al., 1996; Lupien, et al., 1997). This supposed resistance to stress may vary with the complexity of the implicit process.

In the present work, we conducted three studies to examine the impact of the stress hormone cortisol on different forms of implicit learning with growing complexity. The next chapters give a short overview of the different methods we used and the results we found.

1.3.1 Endogenous cortisol suppression and short-term habituation in healthy subjects

In the first study, we aimed to investigate, whether pharmacologically induced low levels of cortisol have an impact on the short-term habituation in healthy subjects. One group of subjects received oral metyrapone to suppress endogenous cortisol production, while a control group received oral placebo. EMG responses to acoustic startle stimuli were assessed to observe habituation.

As expected, metyrapone significantly reduced saliva cortisol, indicating effective endogenous cortisol suppression. Startle eyeblink responses were significantly increased in the metyrapone group, but short-term habituation of the startle reflex was not different between groups.

1.3.2 Delay and trace eyeblink conditioning in a patient group with relative hypocortisolism

In the second study, we aimed to investigate, whether a clinical state of low cortisol levels has an impact on to forms of classical conditioning. Patients with FMS, characterized by decreased cortisol levels, and healthy control subjects were randomly assigned to a delay or trace eyeblink conditioning protocol. Conditioned eyeblink response probability was assessed by EMG, and morning cortisol levels were assessed via saliva samples. In both conditioning protocols, the CS was a pure tone and the US was an air puff to the left cornea. Both protocols consisted of an initial air puff familiarization period, an acquisition period and an extinction period. In trace conditioning, there was a free interval between CS offset and US onset.

Compared to healthy controls, FMS patients showed lower morning cortisol levels, corroborating disturbances in neuroendocrine regulation of the HPA axis in these patients. Trace eyeblink conditioning was facilitated in FMS patients whereas delay eyeblink conditioning was reduced. Cortisol measures correlated significantly only with trace eyeblink conditioning.

1.3.3 Oral cortisol and implicit sequence learning in healthy subjects

In the third study, we aimed to investigate, whether pharmacologically induced high levels of cortisol have an impact on implicit sequence learning in healthy subjects. Oral cortisol was given to one group of subjects, whereas control subjects received placebo. One hour after treatment all volunteers performed a 5-choice SRTT. The subjects responded without knowing to a quasi-randomized stimulus sequence, including higher-order sequential regularities. Reaction times were assessed as a proof of learning.

Both groups showed significant implicit sequence learning throughout the experiment. However, we found an impaired learning performance of the cortisol group compared to the placebo group. Further analysis revealed that a delayed learning in the cortisol group occurred at the very beginning of the task.

1.4 Conclusion

The present work includes three studies, conducted with various methods to examine the impact of the stress hormone cortisol on different forms of implicit learning with growing complexity. We investigated short-term habituation in healthy subjects, treated with oral metyrapone, delay and trace eyeblink conditioning in a patient group with a relative hypocortisolism and implicit sequence learning in healthy subjects, treated with oral cortisol.

First, our results confirm that suppression of endogenous cortisol production by metyrapone does not affect short-term startle reflex habituation. Even though metyrapone induces an HPA activity pattern, similar to the pattern found in PTSD patients with impaired startle reflex habituation (Shalev, et al., 2000), we only temporarily reduced cortisol levels. This six hour treatment with metyrapone may be too short to affect the brain mechanisms that determine startle reflex habituation. If the hippocampus would have been involved in short-term habituation, this very basic form of learning was supposed to be affected by the relatively short metyrapone treatment in the present study.

Second, we could show for the first time that FMS patients, characterized by decreased cortisol levels, show not only facilitated trace eyeblink conditioning but also impaired delay eyeblink conditioning, when compared to healthy controls. Lower cortisol levels in the FMS patients were significantly correlated with increased trace eyeblink conditioning, suggesting that hippocampal function is supported by relatively decreased cortisol levels. As cortisol levels in the FMS patients did not significantly correlate with delay eyeblink conditioning, the difference in delay conditioning between FMS patients and healthy controls seems not to be based on the cortisol levels, but might be mediated by other factors differing for people with FMS compared to healthy controls.

Third, we present the first human investigation indicating impaired implicit memory function after exogenous administration of cortisol. This effect may depend on hippocampus-engagement in implicit sequence learning. Another study, which

tested the impact of exogenous cortisol on implicit memory, found no effect of oral cortisol on implicit memory (Kirschbaum, et al., 1996). To our knowledge, this is the only study, which investigated the impact of exogenous cortisol on implicit memory in healthy subjects. However, the results are based on priming, which may not to be generalized to other forms of implicit learning and memory, respectively.

In fact, the results of the present studies suggest that the idea of hippocampus-independent implicit processes and hippocampus-dependent explicit processes is not as simple as it seems. We rather suggest that the hippocampus is also involved in specific kinds of implicit learning, like trace conditioning and implicit sequence learning, which have the detection of co-variation between presented stimuli in common. In contrast to these forms of learning, priming improves the unconscious ability to detect or identify words or objects a short time after prior experience with those (L.R. Squire, et al., 2009). The representation of this knowledge seems to be independent of structures which are vulnerable to cortisol treatment (Kirschbaum, et al., 1996). The same seems to apply to processes which underlie habituation and delay conditioning. Both, trace conditioning and implicit sequence learning in the present task, have also in common, that the to-be-associated stimuli are separated by an empty time interval. Because of the high temporal contiguity between CS and US, delay conditioning is less abstract and challenging than trace conditioning, and therefore may not require engagement of the hippocampus (Kirschbaum, et al., 1996).

While classical conditioning only involves the association between the CS and the US, implicit sequence learning in the present task involved the association of three stimuli, e.g. a certain combination of two adjacent stimuli, which predicted a third stimulus. Furthermore, in the SRTT, the time which had to be bridged, was twice as long as in the trace conditioning paradigm.

Another conclusion of the present work is that hippocampus-engagement may not necessarily be attended by awareness. Evidence suggests that conscious awareness of a contingency is dependent on conditioned associations (Allan, 1993; Price & Yates, 1995), and a contingency-awareness is essential in trace but not delay conditioning (Clark & Squire, 1998).

However, sequence knowledge can be acquired without being aware of it, particularly if higher-order contingencies like SOC sequences are presented. The more abstract the rule, after which the presented stimuli are organized, the more likely it is, that the acquired knowledge remains unconscious.

In our study of implicit sequence learning none of the participants was able to recognize the underlying sequence rule, implying that the hippocampus may not be necessary for acquiring implicit sequence knowledge during this task. In contrast, the vulnerability to cortisol treatment might nevertheless be a sign of hippocampus-involvement. The results suggest that the hippocampus can be engaged in learning processes without developing a consciousness about what is learned or that implicit knowledge is retrieved at all.

The anticipation of positive and especially negative events can be rewarding as well as protective. For this reason it is highly adaptive to learn about those events to explore or avoid them in future. The release of glucocorticoids during stressful events affects explicit learning and memory, but implicit forms of learning and memory, which are supposed to be more robust than explicit processes, may also be more resilient against stress.

The aim of the present work was to investigate the impact of the stress hormone cortisol on different forms of implicit learning with growing complexity. We could confirm that short-term habitation as well as delay conditioning is not affected by low levels of cortisol. Furthermore, we could confirm that the hippocampus-dependent trace conditioning was facilitated with low levels of cortisol. The new finding of impaired implicit sequence learning under high levels of cortisol suggests that the hippocampus may be involved in this kind of learning and that hippocampus-engagement may not necessarily be attended by awareness of what was learned.

The results imply that even without the development of explicit knowledge, implicit processes may involve the hippocampus and can therefore become prone to stress effects. The present work gives an example for the development of higher cognitive

abilities at the expense of stress vulnerability.

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Chapter II: Endogenous cortisol suppression with metyrapone enhances acoustic startle in healthy subjects

Roemer et al. (2009)

Co-Authors: Frauke Nees, Steffen Richter, Terry D. Blumenthal & Hartmut Schächinger

2.0 Abstract

Previous human studies have shown that excess cortisol sufficient to fully occupy CNS corticosteroid receptors may reduce startle eye blink. The present study tested whether cortisol depletion and the resulting reduction in activity of CNS corticosteroid receptors has the opposite effect.

In a single-blind, placebo-controlled, randomized study, eye blink EMG responses to 105 dB acoustic startle stimuli were assessed in 25 healthy subjects who received oral metyrapone (1500 mg) to suppress endogenous cortisol production, while 24 controls received oral placebo.

As expected, metyrapone significantly reduced saliva cortisol, indicating effective endogenous cortisol suppression. Startle eye blink responses were significantly increased in the metyrapone group. Short-term habituation of the startle reflex was not different between groups.

Our results suggest that startle is enhanced during depletion of cortisol. This effect may be mediated by CNS mechanisms controlling cortisol feedback.

Keywords: cortisol, metyrapone, acoustic startle, short-term habituation

2.1 Introduction

Stress-induced release of HPA hormones plays an important role in human affective

disorders such as depression (e.g. Varghese & Brown, 2001) and anxiety disorders (e.g. Yehuda, Giller, Southwick, Lowy, & Mason, 1991), has an anxiogenic effect (Grillon, Duncko, Covington, Kopperman, & Kling, 2007; Shepard, Barron, & Myers, 2000), and facilitates fear conditioning (Jackson, Payne, Nadel, & Jacobs, 2006). HPA derived stress hormones are known to impact startle (Davis, 2006), and a recent review (Risbrough & Stein, 2006) suggested startle methodology as a translational tool for investigating the association between stress-induced HPA system changes and affective disorders.

The startle response, a protective response shown by many species to an abrupt and intense stimulation, is potentiated during exposure to aversive, threatening stimuli and is diminished by appetitive, pleasant stimuli (Bradley, Cuthbert, & Lang, 1999; Grillon & Baas, 2003). Further, evidence exists (mostly animal-based) for an impact of stress hormones on startle responsiveness. Studies in rats revealed a dose-dependent increase in acoustic startle response magnitude after intracerebroventricular infusions of CRH (Liang, Melia, Miserendino, et al., 1992; Swerdlow, Geyer, Vale, & Koob, 1986). This effect was blocked by pretreatment with a CRH antagonist (Liang, Melia, Miserendino, et al., 1992; Swerdlow, Britton, & Koob, 1989; Swerdlow, et al., 1986). In a similar fashion, blockade of negative feedback via GR antagonists increased acoustic startle responding in rats (Korte, Korte-Bouws, Koob, De Kloet, & Bohus, 1996). After neonatal treatment with high doses of the peptide fragment $ACTH_{4\text{-}10}$ adult rats showed an increase in acoustic startle responding (McGivern, et al., 1987), whereas peripheral injections of corticosterone led to a decrease in startle responding (Sandi, Venero, & Guaza, 1996). The latter effect is compatible with an inverse relationship between peripheral cortisol levels and startle, as suggested by human studies showing larger startle eye blinks during evening than morning, in contrast to diurnal cortisol levels being highest in the morning and lowest in the evening (Miller & Gronfier, 2006).

There is preliminary evidence to suggest that not only startle response magnitude, but also habituation of the startle reflex, is modulated by stress hormones. Startle habituation is the normal phenomenon of startle response attenuation following

repetitive stimulation (Koch, 1999; Pilz & Schnitzler, 1996), and represents a very simple form of implicit learning (Kandel, 2000). PTSD is a specific type of anxiety disorder that is characterized by altered HPA axis activity with low levels of peripheral cortisol (Kellner & Yehuda, 1999; Oquendo, et al., 2003), high levels of CRH (Bremner, et al., 1997; de Kloet, et al., 2008), and evidence of nonhabituated exaggerated startle reactions (Garrick, et al., 2001; Ladwig, et al., 2002; Shalev, et al., 2000). The common physiological background of these findings may be excessive central release of CRH, acting on the bed nucleus of the stria terminalis, to promote anxiety initiated by non-specific stimuli (Marshall & Garakani, 2002).

There are only a few human studies published that have investigated the effect of stress hormones on startle. A biphasic effect of escalating doses of oral hydrocortisone (cortisol) was found, with significantly diminished startle eye blink magnitudes after 20 mg compared to 5 mg cortisol (Buchanan, Brechtel, Sollers, & Lovallo, 2001). An oral 20 mg cortisol dose will be readily absorbed in the gastrointestinal tract, and is higher than the normal human average total daily endogenous cortisol production (Weitzman, et al., 1971). Thus, the diminished startle eye blink reactivity found by Buchanan et al. (2001) occurred when central GR and mineralocorticoid receptors (MR) were likely to be completely occupied with their natural active ligand, cortisol. Based on this assumption, it may be expected that the opposite condition, that is cortisol depletion and the resulting reduction in activity of central GR and MR receptors, will result in the opposite effect, namely an enhancement of startle. This, however, has never been reported.

Thus, the current study was undertaken to investigate the effect of pharmacological suppression of endogenous cortisol by metyrapone on human startle responsiveness. Metyrapone inhibits the conversion of the inactive precursor 11-deoxycortisol to cortisol by the adrenal enzyme 11-beta-hydroxylase (Haynes Jr, 1990), and leads to reduced circulating cortisol, increased CRH and adrenocorticotropic hormone (ACTH), and an accumulation of 11-deoxycortisol (Fiad, Kirby, Cunningham, & McKenna, 1994; Hagendorf, et al., 2005; Otte, et al., 2007; Rotllant, Ons, Carrasco, & Armario, 2002). This substance not only exerts effects upon adrenocortical steroid

synthesis, but also inhibits the 11-β-hydroxysteroiddehydrogenase type-1 (11-β-HSD-1) enzyme that regenerates active cortisol from inactive cortisone in the CNS (Raven, Checkley, & Taylor, 1995). This induces an increase of steroids proximal to the enzyme block, accompanied by cortisol depletion and a reduction of MR and GR receptor activity that is followed by an increase of CRH and ACTH due to the impaired negative cortisol feedback (Jahn, et al., 2003). Based on previous human (Buchanan, et al., 2001) and animal (Sandi, et al., 1996) data on exogenous excess cortisol, which showed a decrease in startle magnitude with higher cortisol levels, we expected endogenous cortisol suppression with metyrapone to result in enhanced startle reactivity. Since HPA hormones may have an effect on startle habituation, we chose a between-subject design which is less sensitive to the effects of treatment order (e.g. treatment vs. placebo first) than the more powerful cross-over design, but which allowed us to address whether short-term habituation of startle is affected by metyrapone.

2.2 Methods

2.2.1 Participants

Fifty-four healthy volunteers were recruited at Trier University by announcements posted on a web page, with follow-up emails. Twenty-seven subjects were randomly assigned to either a metyrapone group (20 females and 7 males, mean age: 24, range: 20-30 years) or a placebo group (17 females and 10 males, mean age: 26, range: 19-40 years). Exclusion criteria were chronic physical or mental disease, intolerance to lacteal products, allergies to any pharmaceutical product, use of any pharmaceuticals, use of nicotine or tobacco on a regular basis, a body-mass-index above 30 or below 18 kg/m^2, pathologic laboratory findings (hemogram, renal values, liver values), illicit substance use within the last two years, acute medical or psychiatric symptoms, or participation in a pharmaceutical study within the last three months. All female participants reported the regular use of oral contraceptives, but were still tested for pregnancy with a commercial urine kit. Ethical permission was obtained from the local ethics committee (in accordance with the Declaration of

Helsinki) and volunteers gave informed consent before attending the trial for moderate monetary incentive.

2.2.2 Manipulation of the HPA-axis

Participants entered the laboratory at 8.00 a.m.. At 8.30 a.m. they received either 750 mg metyrapone or placebo together with a snack which included dairy products, to reduce the likelihood of gastrointestinal metyrapone side effects. A further dose of 750 mg was administered together with a sandwich lunch at 12.00 p.m.. Previous studies have shown significantly reduced plasma cortisol levels at about 6 hours after intake of the initial metyrapone dose (Broadley, et al., 2005; Young, Lopez, Murphy-Weinberg, Watson, & Akil, 1997). Subjects were not allowed to leave the laboratory until the end of the experiment, but were free to engage in low arousing activities like reading. They refrained from smoking and consuming caffeinated beverages during the session. All subjects tolerated the metyrapone treatment well, and reported no side effects.

2.2.3 Collection and Determination of Salivary Cortisol

Saliva samples for a diurnal cortisol profile (6.30 a.m., 6.45 a.m., 7.00 a.m. 7.15 a.m., 7.30 a.m., 8.00 a.m., 11.00 a.m., 13.00 p.m., 15.00 p.m. and 20.00 p.m.) were collected on two consecutive days one week before the test day, and an additional two samples were taken on the test day immediately before and after the eye blink protocol (see below). Saliva was collected by the subjects themselves using standard Eppendorf tubes (1.5 ml, Eppendorf, Hamburg; Germany). Cortisol data of one participant was lost due to incomplete saliva sampling. Saliva samples were stored at -20°C and analyzed for cortisol with a time-resolved fluorescence immunoassay as repeat determination (Dressendörfer, Kirschbaum, Rohde, Stahl, & Strasburger, 1992). Intra- and interassay variability were below 5 % and 12 %, respectively.

2.2.4 Procedure

At 13:30 p.m., participants completed the German version of the state-trait-anxiety-inventory (STAI, \L. Laux, P. Glanzmann, P. Schaffner, & C.D. Spielberger, 1981) to test for differences in anxiety between the groups. At 14:00 p.m., 2 h after administration of the second dose of metyrapone or placebo, the subjects were

prepared for electromyographic eye blink recording (electrode attachment and headphone placement). They were told that they would hear brief, intense tones, and were instructed to sit quietly during the recording period, and neither speak nor move. They were asked to look in the direction of a fixation cross which was attached to the opposing wall. After collection of a saliva sample, eye blink responses to sudden noise bursts (white noise, 105 dB(A), 50 ms, instantaneous rise time) presented binaurally via headphones (Sennheiser Inc.) were recorded by EMG (see below). The acoustic startle protocol consisted of six startle probes with a fixed inter-trial interval (ITI) of 9 s. The startle session lasted about 1 minute, and was followed by collection of a saliva sample. After the startle experiment, participants attended an implicit learning task that was unrelated to the previous experiment. The results of that experiment are reported elsewhere.

2.2.5 EMG-Data Acquisition and Analysis

The eye blink response was measured by recording EMG activity of the orbicularis oculi muscle beneath the left eye, using standard procedures (Blumenthal, et al., 2005). The raw EMG-signal was recorded with a BIOPAC MP 150 at a sampling rate of 1000 Hz with a notch filter of 50 Hz, and a band-pass filter of 28 to 500 Hz. Data were rectified and integrated with a time constant of 10 ms.

EMG blink responses were identified by means of proprietary computer-assisted scoring software using the largest peak in the time interval of 50-150 ms after the startle stimulus onset, relative to a stable baseline 50 ms before startle-stimulus onset. All trials were analyzed with respect to zero-response (no visible startle response) and artifacts (i.e. excessive background noise and voluntary or spontaneous eye blinks at or near the startle stimulus onset). Startle magnitudes were computed, including zero-responses and excluding artifacts. Five of the 54 participants were excluded because they showed no reliable startle response due to absence of eye blink responses or excessive blinking. The final sample size consisted of 49 subjects, with 25 subjects in the metyrapone group (18 females and 7 males, mean age: 24, range: 20-30 years), and 24 subjects in the placebo group (15 females and 9 males, mean age: 26, range: 19-40 years).

2.2.6 Statistical Analysis

For both, metyrapone and placebo group, cortisol data of the diurnal profiles were averaged at each time over the two consecutive pre-test days (pre-administration) and analyzed with a group (metyrapone vs. placebo) X time of collection analysis of variance (ANOVA). To evaluate the impact of administration of metyrapone on cortisol, data from the two measures on the startle test day were averaged (post-administration), and then compared to cortisol measured at a comparable time on the pre-test days (one week earlier). These four cortisol measures were subjected to a treatment (metyrapone vs. placebo) X time (pre- vs. post-administration) ANOVA to check the effect of drug manipulation. The EMG eye blink magnitudes were analyzed with a treatment (metyrapone vs. placebo) by trial (1-6) repeated measures analysis of covariance (ANCOVA) with sex as a covariate. Differences in anxiety between the groups were evaluated with t-tests for both trait and state anxiety.

For all statistical analyses (performed with SPSS for Windows, Statistical Package of the Social Sciences, Version 11.5), the level of significance (α) was .05 and in case of violation of the assumption of homogeneity of variances the Greenhouse-Geisser-adjustment was applied and adjusted p-values are reported, with uncorrected degrees of freedom and epsilon-values. Significant main effects or interactions were further analyzed with Bonferroni-adjusted paired t-tests and effect sizes (partial eta squared: η_p^2) are reported.

2.3 Results

2.3.1 Saliva Cortisol Levels

As illustrated in Figure 1, the diurnal cortisol profiles collected prior to metyrapone treatment showed the typical pattern, a significant effect of time ($F_{9,468}$ = 108.072, $p < .001$, η_p^2 = .675, ε = .435), with increased cortisol levels during the morning and gradually decreasing levels during the day. There was no main effect of group ($F_{1,52}$ = 1.349, p = .251) and no interaction between group and time ($F_{9,468}$ = .372, p = .824, ε = .435), indicating that the diurnal cortisol profiles did not significantly differ between the placebo and metyrapone groups.

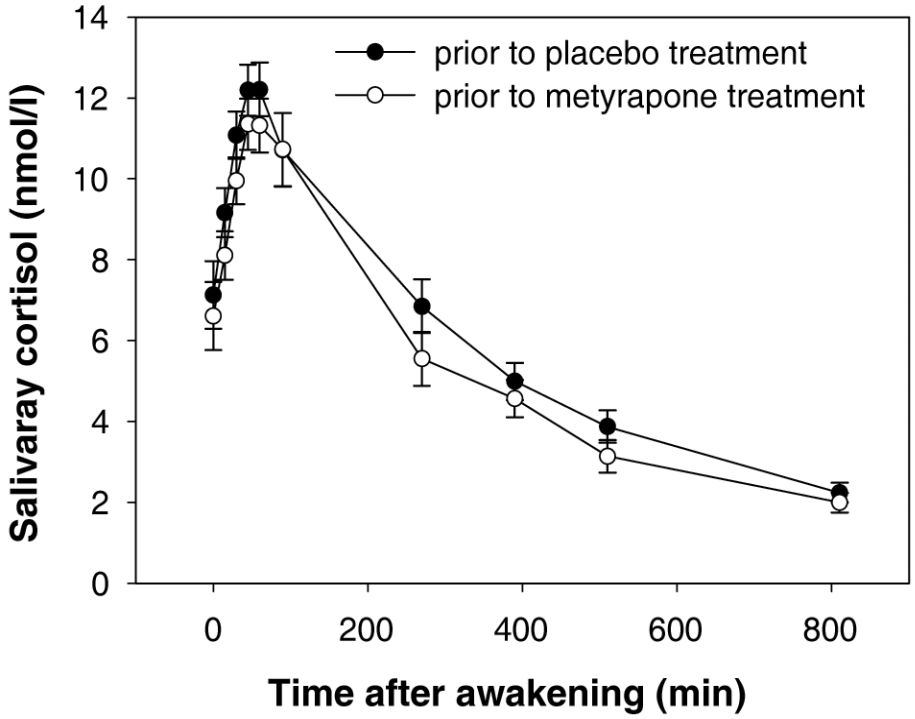

Figure 1 Diurnal saliva cortisol profiles (averaged data over two consecutive days) of placebo and metyrapone group, indicating that groups did not differ in cortisol prior to treatment.

The manipulation check revealed a significant interaction of treatment (placebo vs. metyrapone) and time (pre- vs. post-treatment; $F_{1,51} = 11.451$, $p < .001$, $\eta_p^2 = .183$; placebo-pre = 4.958 (SE = .473), placebo-post = 4.656 (SE = .399), metyrapone-pre = 4.563 (SE = .464), metyrapone-post = 1.780 (SE = .391)). Post-treatment cortisol levels were significantly decreased in the metyrapone group, ($t_{26} = 5.752$, $p < .001$), but not in the control group, ($t_{25} = .545$, $p = .591$).

2.3.2 Startle reflex Magnitude

Startle reactivity was significantly increased in the metyrapone group compared to the placebo group ($F_{1,46} = 4.284$, $p < .05$, $\eta_p^2 = .085$; see Figure 2). The startle reflex habituated for both metyrapone and placebo groups, ($F_{5,230} = 3.458$, $p < .05$, $\eta_p^2 = .07$, $\varepsilon = .716$), but there was no interaction of treatment and time ($F_{5,230} = .212$, $p = .916$, $\varepsilon = .716$), indicating that the decline of startle response magnitude with repeated stimulation was not different between the groups. The ANCOVA revealed neither a sex effect ($F_{1,46} = 2.628$, $p = .112$) nor a treatment X sex interaction ($F_{5,230} = .717$, $p = .566$, $\varepsilon = .716$).

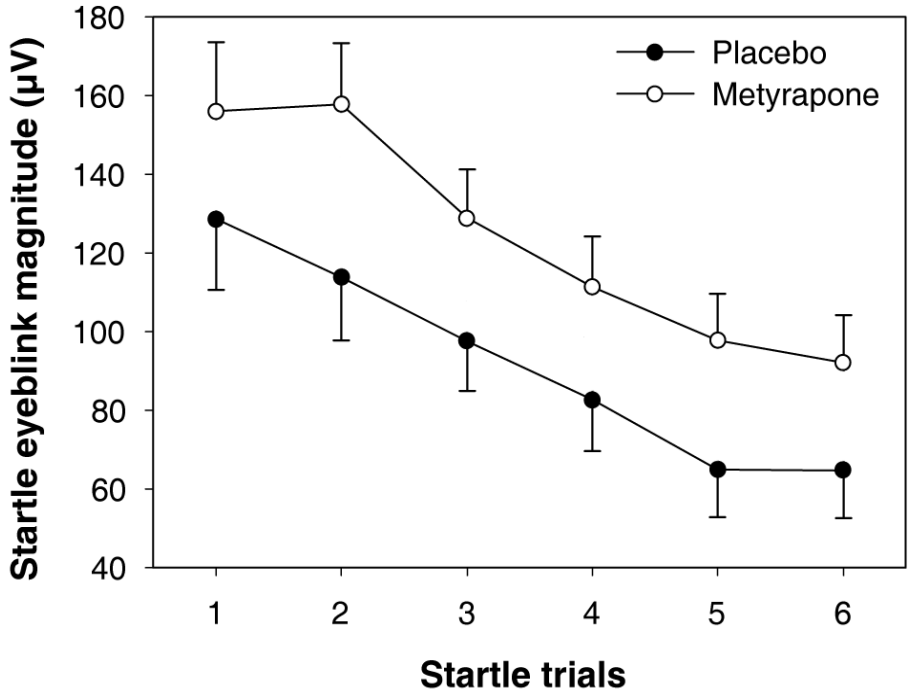

Figure 2 Startle reactivity after treatment with metyrapone or placebo and habituation within 6 startle trials. Startle magnitude is significantly increased in the metyrapone group compared to the placebo group ($F_{1,46} = 4.284$, $p < .05$, $\eta p2 = .085$), and there is a significant decline in startle response magnitudes over the 6 trials for both metyrapone and placebo groups ($F_{5,230} = 3.458$, $p < .05$, $\eta p2 = .07$, $\varepsilon = .716$).

2.3.3 Anxiety scores

Metyrapone had no significant effect on either trait anxiety ($t_{52} = .854$, $p = .397$; placebo = 39.96 (SE = 1.82), metyrapone = 37.67 (SE = 1.98)) or state anxiety ($t_{52} = -.622$, $p = .537$; placebo = 35.19 (SE = 1.44), metyrapone = 36.48 (SE = 1.51)).

2.4 Discussion

This placebo-controlled study was performed to investigate the effects of endogenous cortisol suppression by metyrapone on acoustic startle eye blink responses. As expected, administration of metyrapone effectively suppressed endogenous cortisol production. At the same time, metyrapone treatment enhanced startle eye blink response magnitude significantly, but did not affect the short-term habituation of this response. The enhancement of eye blink responses after reduction of central corticosteroid receptor activity is consistent with previous animal (Sandi, et al., 1996) and human (Buchanan, et al., 2001) research reporting reduced startle

responsiveness following corticosteroid administration at a dose which is sufficient to fully activate central GR and MR receptors with their natural ligand. It has already been demonstrated in previous research that metyrapone treatment and interruption of the negative cortisol feedback loop, increases central CRH activity (Jahn, et al., 2003). Since central CRH enhances startle response magnitude (Liang, Melia, Campeau, et al., 1992), it seems reasonable to assume that increased central CRH mediated the observed metyrapone effect on startle eye blink responsiveness in this study, although we were not able to measure central CRH.

Suppression of endogenous cortisol production by metyrapone did not affect startle reflex habituation in the present study. Metyrapone induces an HPA activity pattern of reduced peripheral cortisol and increased central CRH similar to the pattern found in PTSD. Also, PTSD can involve impaired startle reflex habituation (Shalev, et al., 2000). However, the pathological state of PTSD usually exists for a prolonged time period before a clinical diagnosis is established; time enough to allow for functional or even structural adaptations of the CNS. Thus, a six hour treatment with metyrapone may be too short to affect the brain mechanisms that determine startle reflex habituation. Our findings are in line with previous studies in rodents showing that adrenalectomy (Davis & Zolovick, 1972) or treatment with oral corticosterone (Ardayfio & Kim, 2006) does not affect startle habituation and, therefore suggest that an intact adrenal-pituitary system is not crucial for habituation of the startle reflex.

Another factor that is known to enhance startle reactivity is anxiety (Bradley, et al., 1999; Grillon & Baas, 2003). If metyrapone treatment and/or increased CRH had had an anxiogenic effect in our sample, that may have been visible in increased post-treatment state anxiety scores. However, we did not find differences in anxiety scores between treatment groups, suggesting that an effect of metyrapone treatment on self-reported anxiety in this study sample can be excluded.

There are also other substances affected by metyrapone treatment, such as serotonin, growth hormone, dehydroepiandrosterone, and progesterone and its metabolite 3α-hydroxy-5α-pregnane-20-one (Hirani, Khisti, & Chopde, 2002; Jahn, et al., 2003; Korte-Bouws, Korte, De Kloet, & Bohus, 1996) that we did not control for, which

might have had an influence on startle.

There are two implications of our study: First, our results complement previous studies of reduced startle due to excess cortisol in showing that cortisol depletion has the opposite effect on startle. As such, our results suggest that startle methodology may be a useful tool to index the activity level of central networks affecting HPA regulation over the whole range of relative hypo- to hypercortisolism. Since startle is sensitive to alterations of the HPA axis, it may prove useful in further studies in patient groups characterized by HPA axis changes. Startle methodology is widely available and does not pose a great burden on subjects. Second, our findings suggest that acute metyrapone-treatment induces a startle eye blink pattern similar to the pattern found in PTSD (Shalev, et al., 2000). This interpretation is corroborated by the endocrinological profile of metyrapone, which induces low peripheral cortisol and high central CRH levels. However, this interpretation is in contrast to the anxiolytic effects of metyrapone previously reported (Roozendaal, Bohus, & McGaugh, 1996). Future studies will have to replicate these findings.

Several limitations of the current study have to be mentioned. The experimental between-subjects design chosen is less statistically powerful than a within-subject cross-over design, where placebo control data would have been assessed for all participants. However, the expectation of a treatment effect on habituation over sessions may complicate the interpretation of a cross-over study. Thus, we decided to use a cohort study. Another potential limitation of this study was the time of day when the startle eye blink protocol took place. We assessed the effects of metyrapone for all subjects in the afternoon, when basal cortisol levels are low in comparison to morning cortisol. Although we found a significant suppression of afternoon cortisol levels, the treatment effect might have been greater if data acquisition had been performed in the morning. If there is the capacity to watch the participants overnight, to reschedule the data acquisition to the morning, this could also be considered in a replication study. Also, this study involved healthy young volunteers, and it would be interesting to investigate whether clinical populations, for example adrenalectomized patients, would have shown the same results or

different findings. Finally, plasma ACTH should be assessed in future studies since it may influence CNS processes.

In summary, the present results indicate that acute cortisol depletion is associated with enhanced startle reactivity, but does not seem to affect habituation of the acoustic startle response. Thus, the PTSD-like pattern of cortisol and CRH levels after short-term metyrapone administration results in the same phenotype of enhanced startle responsiveness.

2.5 Author Notes

Please correspond to Dipl.-Psych. Sonja Römer (Department of Clinical Psychology and Psychotherapy, Saarland University, 66041 Saarbrücken, Germany, E-mail: s.roemer@mx.uni-saarland.de) or Prof. Dr. med. Hartmut Schächinger (University of Trier, Institute of Psychobiology, 54290 Trier, Germany, E-Mail: schaechi@uni-trier.de).

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Chapter III: Alteration of delay and trace eyeblink conditioning in fibromyalgia patients

Nees et al. (2010)

Co-Authors: Heinz Rüddel, Lutz Mussgay, Linn K. Kuehl, Sonja Römer & Hartmut Schächinger

3.0 Abstract

Classical conditioning processes are important for the generation and persistence of symptoms in psychosomatic disorders, such as the 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.

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 EMG. Morning cortisol levels, ratings of depression, anxiety as well as psychosomatic complaints as well as general symptomatology and psychological distress were assessed.

As compared to healthy controls FMS patients showed lower morning cortisol levels, corroborating previously described disturbances in neuroendocrine regulation of the 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.

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

3.1 Introduction

FMS is a common clinical syndrome characterized by chronic widespread pain and tenderness (Wolfe, et al., 1990). Elevated levels of depression, anxiety and psychosocial stress are frequently reported in FMS patients (Wolfe, 1989; Wolfe, Ross, Anderson, Russell, & Hebert, 1995). While the precise pathophysiological mechanisms are still poorly understood, recent studies suggested a neurobiological basis for FMS (altered CNS pain processing) (Cook, et al., 2004; Gracely, Petzke, Wolf, & Clauw, 2002), and the 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, et al., 1994; Griep, et al., 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 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 KM, 2003) and humans (Clark & Squire, 1998; Fortier, et al., 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 US, e.g. a weak air puff to the

cornea, induces an eyeblink response that serves as UR. In delay eyeblink conditioning the 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, et al., 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 dependent 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, et al., 1990; Woodruff-Pak & Papka, 1996). Stress hormones, in particular glucocorticoids, have been shown to modulate classical conditioning (Grillon, et al., 2004) and thus may affect the generation and persistence of pain symptoms by influencing learning and memory processes (Het, et al., 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, et al., 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, et al., 2006), findings supported by the high concentration of GRs in the hippocampus. A facilitation of hippocampus-based conditioning could be observed after pharmacologically induced endogenous mild hypocortisolism (Nees, et al., 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.

3.2 Methods

3.2.1 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 (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; Strakowski, Keck, McElroy, Lonczak, & West, 1995; Wittchen & Fydrich, 1997). 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 (L. Laux, P. Glanzmann, P. Schaffner, & C.D. 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.

3.2.2 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, et al., 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.

3.2.3 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 ITI varied between 10 and 14 s, with a mean interval of 12 s.

3.2.4 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.

3.2.5 Data Analysis

In both delay and trace eyeblink conditioning, the 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, CRs were not influenced by alpha responses.

In delay eyeblink conditioning, the 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 CRs (“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, et al., 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.

3.2.6 Statistical Analysis

Cortisol data were analyzed with a group (patients vs. controls) X cortisol awakening profile (1-5) repeated measures 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.

3.3 Results

3.3.1 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 1).

Table 1 Symptom ratings 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 (1.0)	<.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

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

3.3.2 Salivary Cortisol Data

Figure 3 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).

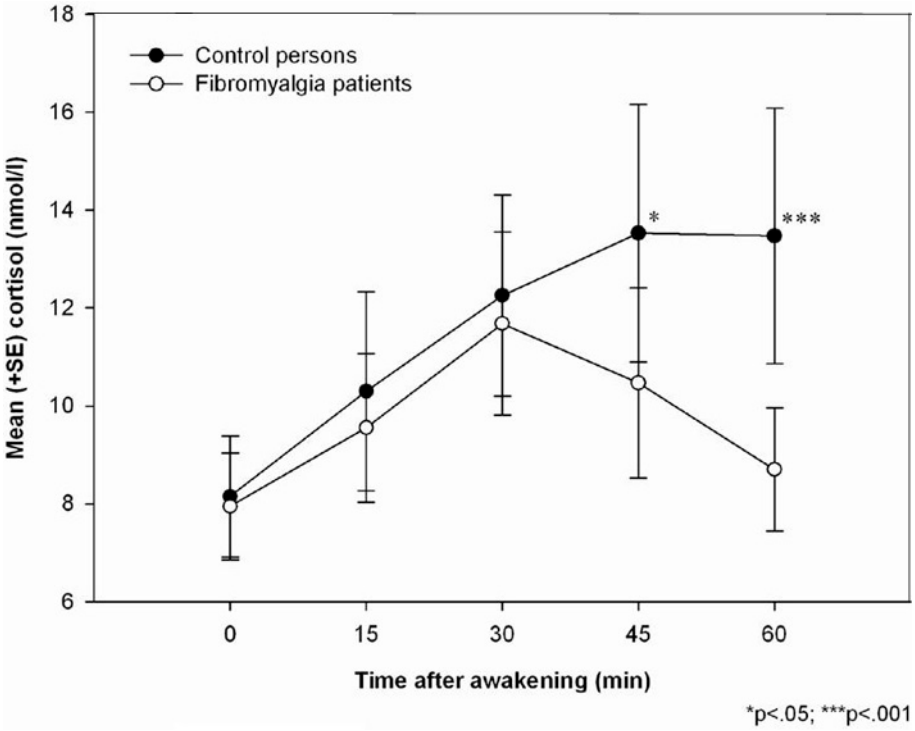

Figure 3 Awakening cortisol profiles (averaged over data of two consecutive days) of control and patient group.

3.3.3 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).

3.3.4 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-CRs as well as slower increase in block by block CR probability during acquisition in patients compared to controls (see Figure 4).

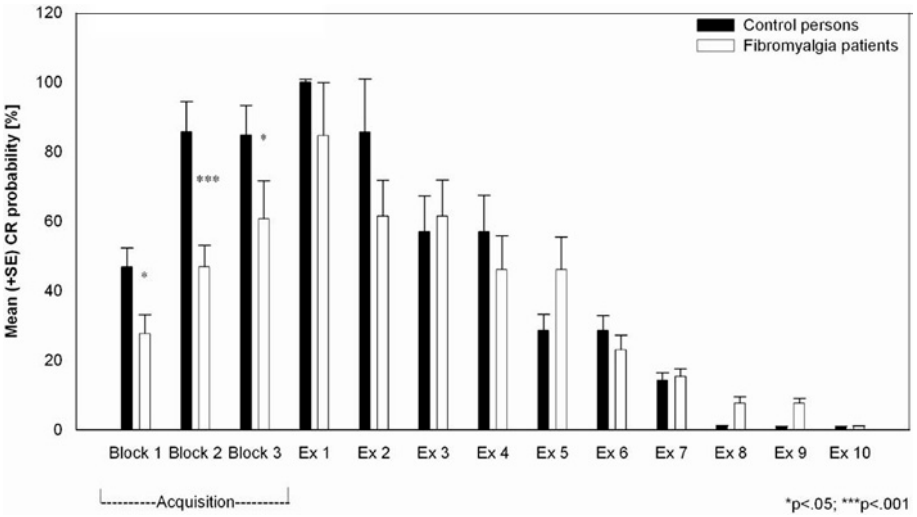

Figure 4 All three acquisition blocks and the extinction block of delay eyeblink conditioning in control and patient group.

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-CRs, with a comparable block by block increase of CR probability to healthy controls (see Figure 5).

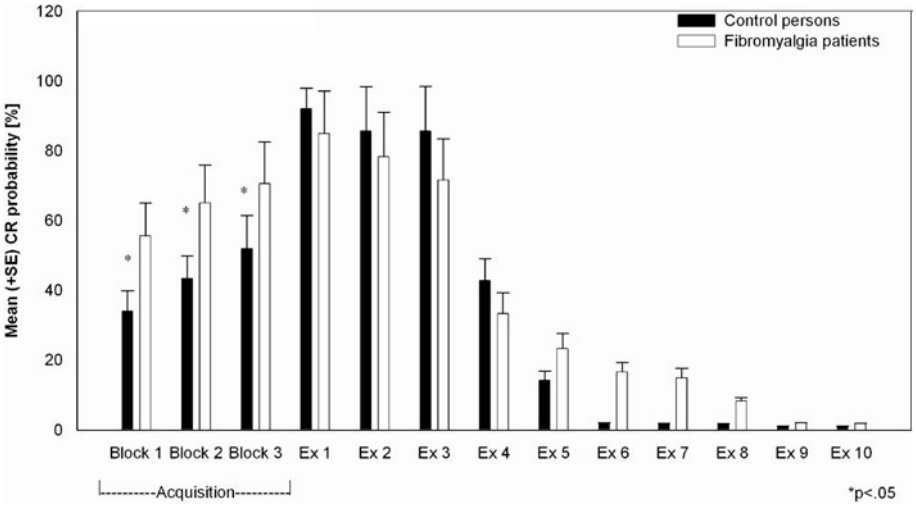

Figure 5 All three acquisition blocks and the extinction block of trace eyeblink conditioning in control and patient group.

3.3.5 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-CRs, 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.

3.3.6 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.

3.4 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-CRs 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, et al., 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, et al., 1990; Griep, et al., 1998; McCain & Tilbe, 1989), suggesting an HPA axis perturbation in terms of a combination of sensitized pituitary with adrenal insufficiency (Griep, et al., 1993; Griep, et al., 1998; 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. J. M. Grisart & Plaghki, 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, & McCain, 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 (J. Grisart, Van der Linden, & Masquelier, 2002). As awareness is important for trace, but not delay eyeblink conditioning, one would suggest an increase in CRs 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.

3.5 Author Notes

Please correspond to Frauke Nees, Ph.D. (Department of Cognitive and Clinical Neuroscience, Central Institute of Mental Health, J 5, Mannheim, Germany, e-mail: frauke.nees@zi-mannheim.de).

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Chapter IV: Oral cortisol impairs implicit sequence learning

Römer et al. (2010)

Co-Authors: André Schulz, Steffen Richter, Johanna Lass-Hennemann, & Hartmut Schächinger

4.0 Abstract

Glucocorticoids have been shown to affect declarative memory, an explicit form of memory for facts and events operated by medial temporal lobe structures. Recent neuroimaging data suggest that the medial temporal lobe (including the hippocampus) is also active in implicit sequence learning.

The aim of the present study was to investigate whether implicit sequence learning may also be affected by glucocorticoid administration.

Oral cortisol (30 mg) was given to 29 healthy subjects, whereas 31 control subjects received placebo. One hour after treatment all volunteers performed 5 consecutive blocks of a 5-choice SRTT by responding to coloured lights by pressing buttons of the same colour. The subjects responded without knowing to a quasi-randomised stimulus sequence, including higher-order sequential regularities (a combination of two colours that predicted the following target colour). The reaction speed of every button-press (100 per block) was determined and difference-scores were calculated as a proof of learning.

Both groups showed significant implicit sequence learning throughout the experiment. However, we found an impaired learning performance of the cortisol group compared to the placebo group. Further analysis revealed that a delayed learning in the cortisol group occurred at the very beginning of the task.

This study is the first human investigation indicating impaired implicit memory function after exogenous administration of the stress hormone cortisol. This effect

may depend on hippocampus engagement in implicit sequence learning, but the involvement of other brain structures is also discussed.

Keywords: serial reaction time task, implicit sequence learning, memory retrieval, stress, HPA-axis, glucocorticoids, cortisol, hydrocortisone, medial temporal lobe, hippocampus

4.1 Introduction

Stress induces the release of corticosteroids, known to modulate cognitive performance (Lupien & McEwen, 1997). Animal and human studies show that acute high levels of glucocorticoids may enhance memory consolidation and impair memory retrieval processes (Roozendaal, 2002). Those effects are suggested to be mediated by the hippocampus, a brain structure in the medial temporal lobe which is involved in learning and memory (Lupien & Lepage, 2001; Larry R. Squire, 1992), and which contains a high density of GRs (McEwen, et al., 1968). There is a lot of evidence for the impact of acute glucocorticoid treatment on hippocampus-dependent explicit learning and memory (memory for facts and events) (for a meta-analysis see Het, et al., 2005). But there have been few studies, investigating the effect of acute glucocorticoid treatment on implicit learning and memory (e.g. Kirschbaum, et al., 1996). An important principle of implicit memory "is the ability to gradually extract the common elements from a series of separate events" (L. R. Squire, 2004, p. 174), and the success of the acquisition of implicit knowledge is typically demonstrated through performance rather than recollection (L. R. Squire, et al., 2004). Skills and habits, priming and perceptual learning, simple forms of conditioning and non-associative learning are referred to be implicit. These different forms of implicit memory have been commonly suggested to be independent of the hippocampus (Larry R. Squire, 1992), but to relay on other specific brain systems, such as the striatum, the neocortex, the amygdala, the cerebellum, or reflex pathways (compare, L. R. Squire, 2004).

However, recent evidence suggests the medial temporal lobe to be involved in implicit learning of complex contingencies (Chun & Phelps, 1999; Clark & Squire,

1998; Curran, 1997; Poldrack, et al., 2001; Rose, et al., 2002). Schendan et al. (2003a) used functional imaging studies to investigate the activation of medial temporal lobe structures during both, explicit and implicit sequence learning (Poldrack & Rodriguez, 2003; Schendan, et al., 2003a, 2003b). They chose a SRTT and measured performance as a speeded reaction to repeatedly presented stimuli which followed a certain rule. Furthermore, they used SOC sequences to be learned. In contrast to a simple contingency between two stimuli, learning a SOC sequence requires higher order associations between more than two successive stimuli. In contrast to explicit learning conditions, participants were not informed about the underlying sequence under implicit learning conditions. The results showed that the medial temporal lobe is not only active in explicit but also in implicit sequence learning (Schendan, et al., 2003a). These findings suggest that performance on an implicit learning task could be affected by glucocorticoids in the same way as explicit learning and memory.

A study, which tested the impact of exogenous cortisol on both, implicit and explicit memory, found impaired explicit memory and spatial thinking after 10 mg of oral cortisol, but no treatment-effect on implicit memory (Kirschbaum, et al., 1996). In this study, all participants received a list of 26 nouns with the instruction to rate these words according to their melodious sounds, and memory was tested one hour after cortisol administration. Implicit memory was tested with a list, containing 52 two-letter wordstems (26 "old" words and 26 "novel" words from a second, parallel wordlist), and the participants were asked to complete those letters to the first noun which came to their mind. In comparison to completion of the novel words, the probability of the completion of the previously presented ('old') words is usually increased. This effect is known as priming. Explicit memory was tested with a list of wordstems with the first two letters of those 26 nouns they had rated earlier. The participants were instructed to complete the wordstems to the exact nouns they had rated earlier (cued recall). The result of an impaired explicit memory in contrast to an intact implicit memory was explained via GR activation in the hippocampus, which should only play a role in explicit retrieval processes (Kirschbaum, et al., 1996). To our knowledge, this is the only study, that investigated the impact of exogenous cortisol on implicit memory (Kirschbaum, et al., 1996), but the results are based on

priming, which may not to be generalized to other forms of implicit learning and memory, respectively.

To test whether glucocorticoids affect implicit learning and memory, we investigate implicit sequence learning and memory after either cortisol (*cortisol group*) or placebo (*placebo group*) treatment. We chose a 5-choice SRTT, including SOC sequences. Furthermore our stimulus series does not only include a predictive sequence but also a control condition that is a non-predictive sequence with a comparable predictive load, but followed by a random stimulus (*control trial*). In this way, learning in the present task is described by a faster response during *target* compared to *control trials* within each block of the SRTT. The advantage of *target* and *control trials* within one series, allows differentiating sequence learning from a more general skill (practice), and the progress in learning can be observed in each block of the task. We expect learning to be impaired in the *cortisol group*, which should be reflected in smaller differences of reaction speed between *target* and *control trials* in the *cortisol group*, compared to the *placebo group*.

4.2 Method

4.2.1 Participants

Sixty healthy volunteers were recruited at the University of Trier (Germany) by announcements posted on a web page, with follow-up emails. These participants were randomly assigned to either a cortisol group (sex: 14 men, 15 women; mean age: 24 years; range: 19-35 years) which received 30 mg oral cortisol (compare e.g. Monk & Nelson, 2002) or a placebo group (sex: 14 men, 17 women; mean age: 24 years; range: 20-27 years) which received a placebo. For the randomization was considered, that cortisol and placebo were administered by turns and that sex was equally distributed across the groups. In a first examination before attending the study, all participants were screened by a physician. Exclusion criteria were chronic physical or mental disease, allergies to any pharmaceutical product, use of any pharmaceuticals, use of nicotine or tobacco on a regular basis (more than five cigarettes a day), a body-mass-index above 30 or below 18 kg/m^2, illicit substance use

within the last two years, acute medical or psychiatric symptoms, or participation in a pharmaceutical study within the last three months. All female participants reported the regular use of oral contraceptives, but they were controlled neither for cycle- nor for pill-phase. Ethical permission was obtained from the local ethics committee (in accordance with the Declaration of Helsinki) and volunteers gave informed consent before attending the trial for moderate monetary incentive or course credit.

4.2.2 Stimuli and apparatus

Stimuli were represented with a Serial Response Box (Psychology Software Tools, Inc.) that features a 0 ms debounce period and connects directly to the PC. The Response Box was positioned on a table in front of the participant, and included an array of five colored LEDs (5 mm; RED, GREEN, BLUE, YELLOW, WHITE; 7000 mcd; angle of beam spread: 20 degrees; viewing distance: 50 cm; luminous flux: 0.7 lm, illuminance: 28 lx) and five buttons with corresponding colors. LEDs were located in the top row and buttons in a distance of 2 cm below, both with a horizontal spacing of 1 cm. LEDs and buttons of same color were located at most distant.

All participants performed a quasi-randomized learning series. This series included predictive and non-predictive sequences. The predictive sequence was a SOC where the sequence of two adjacent stimuli predicted the appearance of a target stimulus. The predictive sequence was RED then WHITE followed by the target stimulus BLUE. The non-predictive sequence was the color sequence of YELLOW then GREEN, which was followed by a variety of colors (except GREEN), each with a similar probability of 0.25 over adjacent 2 blocks. Thus, the non-predictive sequence did not provide valid information about the following control stimulus. Each block of hundred trials comprised ten presentations of predictive and non-predictive sequences, respectively (see Figure 6). In all series each of the five color stimuli appeared with the same probability of 0.2, and the same color never appeared directly in succession. Other studies from our research group have shown that other color sequences are equally effective in producing an implicit sequence learning effect.

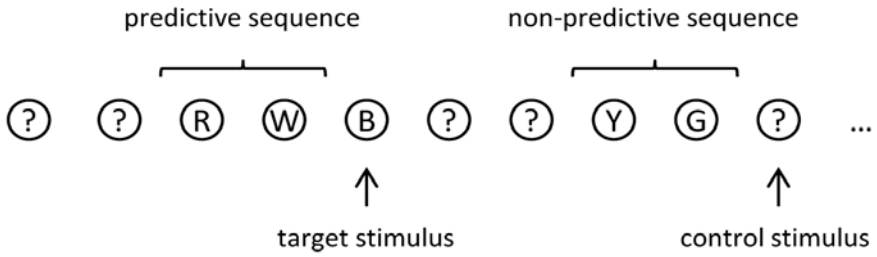

Figure 6 Ten trials of the learning sequence with the target stimulus BLUE (B). Every spot displays one trial. Spots with a question mark are placeholders for random trials (?). The RED (R)-, WHITE (W)- and BLUE (B)-colored spots represent the SOC. The YELLOW (Y)- and GREEN (G)-colored spot, followed by a random trial (?) represent the control condition.

4.2.3 Procedure

Drug administration and Collection and Determination of Salivary Cortisol

All volunteers, blinded to treatment status, received either 30 mg oral cortisol (Hydrocortison, JENAPHARM®) or placebo at 1.00 p.m.. Saliva samples were collected immediately before and one hour after medication (at 2 p.m., immediately before performing the SRTT), when the cortisol concentration is supposed to reach its peak (compare Czock, Keller, Rasche, & Haussler, 2005). A last sample was taken immediately after the SRTT. During the session, participants refrained from smoking and consuming caffeinated beverages. Saliva was collected by the participants themselves using standard Eppendorf tubes (1.5 ml, Eppendorf, Hamburg; Germany). Saliva samples were stored at -20°C and analyzed for cortisol with a time-resolved fluorescence immunoassay as repeat determination (Dressendörfer, et al., 1992). Intra- and interassay variability were below 5 % and 12 %, respectively.

The Serial Reaction Time Task

All participants completed five blocks of the learning task, which is described above. Each block consists of hundred trials (1000 ms ITI). On each trial one of the five colored light stimuli was activated for the first 500 ms, followed by a 500 ms dark period. Participants were instructed to sit comfortable, and to respond as fast and accurate as possible by pushing the same-colored button of the Response Box with always the same finger of the dominant hand. Reaction time was recorded, and incorrect or missing responses were counted as errors that were not included in data analyses. Successive blocks were separated by a short break which was finished by

the participants themselves via pushing one of the buttons.

The whole reaction time task lasted about fifteen minutes. Then, participants were invited to comment on the task. If they did not address the issue of series structure, participants were asked, if they noticed anything special referring to the color series. If they did not name any pattern, they were explicitly asked to suggest a color sequence that might have appeared in a row.

We expected faster responses during *target trials* compared to *control trials* to develop during the course of implicit learning.

Reaction time scoring

There are some problems concerning the analysis of reaction time data (see Whelan, 2008): First, reaction time data usually vary across trials and therefore may include outliers. Second, psychomotor performance, measured by reaction times, varies between subjects. Third, reaction time data are statistically not normal distributed but right-skewed. Fourth, psychomotor performance within subjects usually gets faster with practice, and this effect is not related to implicit sequence learning.

We dealt with these difficulties as follows: First, we used cutoffs to eliminate reactions times that fall below the physiologic limit for realistic motor responses or that go beyond a time limit for a correct response during the reaction time task: For the applied 5-choice-reaction time task with the inter-stimulus interval (ISI) of 1000 s, reaction times faster than 300 ms and slower than 1000 ms were considered as outliers (for an overview see Kosinski, 2010), and excluded from subsequent analyses. Second, we aimed to prevent that differences in psychomotor performance between subjects obscure implicit sequence learning scores: All individual reaction time data were adjusted to the subject's initial reaction performance, which was calculated per color as the median of the very first 30 responses, when implicit sequence learning should not have played a role. Third, we transformed reaction times to reaction speed (1000/reaction time in ms) to normalize the distribution and reduce the effect of slow outliers (compare Whelan, 2008). Furthermore, we used the median, because this parameter of central tendency is more robust to departures

from normality and reflects typical responses better than the arithmetic mean: We determined median reaction speeds of correct responses per subject and block for *target* and *control trials*, respectively. Fourth, we aimed to control for differences in psychomotor performance and practice effects within subjects by calculating the difference between reaction speeds to *target* and *control trials* per block, individually. This was considered to reflect the true implicit sequence learning effect. Block 1 was divided into three phases (response 1-30, 31-60 and 61-100) to track the fast progress in learning at the beginning of the task.

4.2.4 Statistical analysis

Salivary Cortisol

To check the effect of drug manipulation, we analyzed salivary cortisol concentrations with a two-factorial mixed ANOVA with the between factor *Treatment* (*cortisol* vs. *placebo*) and the within factor *Time* (*pre-treatment*, *post-treatment, post-experiment*). Two participants of the placebo group had to be excluded due to incomplete saliva sampling.

Reaction Speed

Gender was not expected to play a role in this study. After a confirmatory ANCOVA, which showed that gender did not explain any variance, either in a main effect or interaction, we decided to report the results of an ANOVA.

Since the increment of learning is the strongest at the very beginning of the task, difference scores were analyzed separately for early effects within Block 1 and late effects within Block 2 to Block 5.

Two two-factorial mixed ANOVAs were calculated – one with the between factor *Treatment* (*cortisol* vs. *placebo*) and the within factor *Trial (1-30, 31-60, 61-100)* for early effects, and a second with the between factor *Treatment* (*cortisol* vs. *placebo*) and the within factor *Block (2-5)* for late effects. Finally, post-hoc tests were calculated for both ANOVAs. Two participants of the cortisol group had to be excluded due to technical problems.

For all statistical analyses (performed with SPSS for Windows, Statistical Package of

the Social Sciences, Version 17.0.1), the level of significance (α) was .05. In case of violation of the assumption of homogeneity of variances the Greenhouse-Geisser-adjustment was applied and adjusted p-values are reported, with uncorrected degrees of freedom and epsilon-values. Furthermore effect sizes (partial eta squared: η_p^2) are reported for significant main effects and interactions.

4.3 Results

4.3.1 Test of consciousness

Only 30 % of the participants suggested repetitive combinations of colors, and only two participants named the predictive or the non-predictive sequence – but it is to emphasize that this could also have happened by chance, with a probability of 5 %. Even if some participants indicated a detection of a pattern in the stimulus series, there was no participant who was able to name the underlying rule, which is: A predictive sequence of WHITE after RED is *always* followed by the target stimulus BLUE.

4.3.2 Salivary cortisol

The manipulation check (see Figure 7) revealed a significant interaction of *Treatment* (*cortisol* vs. *placebo*) and *Time* (*pre-treatment*, *post-treatment*, *post-experiment*); ($F_{2,108}$ = 798.430; p < .001; η_p^2 = .937) and both, a significant main effect of *Treatment* ($F_{1,54}$ = 1397.853; p < .001; η_p^2 = .963) and *Time* ($F_{2,108}$ = 748.623; p < .001; η_p^2 = .933). Before treatment there was no difference in cortisol concentrations between the groups (t_{54} = -.106, p = .916). But one hour after treatment, cortisol concentrations were significantly increased in the cortisol group, compared to the placebo group (t_{54} = -29.572, p < .001). This effect was still existent after the SRTT (t_{54} = -41.690, p < .001). For the placebo group, mean salivary cortisol concentrations (in nnol/l) and standard errors (in brackets) were as follows: pre-treatment = 4.824 (0.586), post-treatment = 3.654 (2.124), post-experiment = 3.022 (1.484). For the cortisol group, mean salivary cortisol concentrations (in nnol/l) and standard errors (in brackets) were as follows: pre-treatment = 4.847 (0.584). Post-treatment, 85 % of the saliva samples reached the detection limit, but all cortisol concentrations were above 37.97

nmol/l. This effect lasted until the end of the experiment (post-experiment), when still 81 % of the saliva samples reached the detection limit, and all cortisol concentrations were above 46.98 nmol/l.

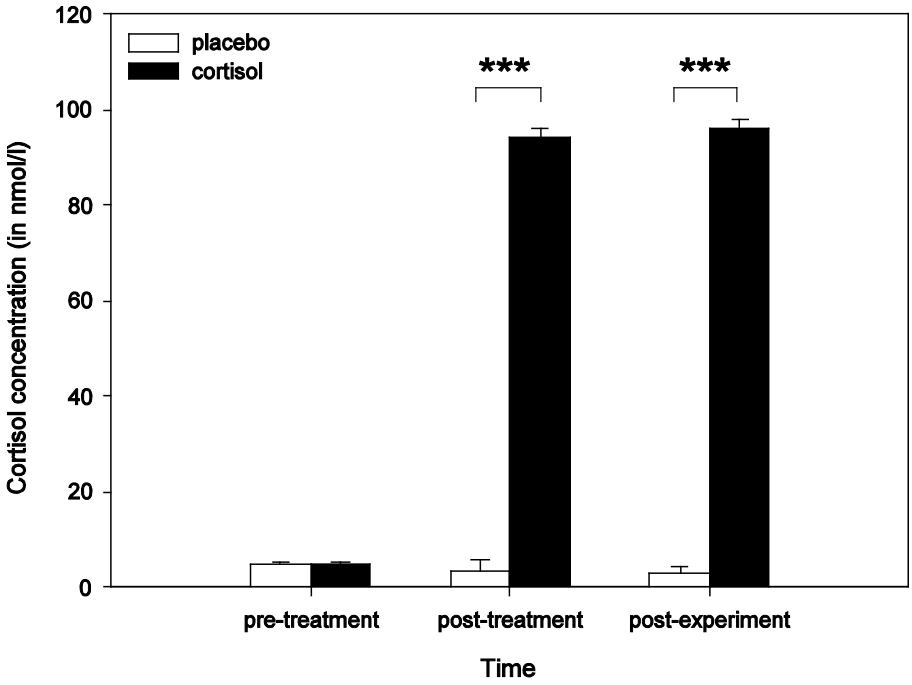

Figure 7 Increased cortisol levels in the cortisol group (black), compared to the placebo group (white), one hour after treatment. This effect lasted until the end of the experiment. Bars represent standard errors; *** $p < .001$.

4.3.3 Reaction Speed

The *Treatment* (*cortisol* vs. *placebo*) X *Trial* ANOVA within Block 1 revealed significant interaction between *Treatment* and *Trial* ($F_{2,108} = 3.401$; $p < .05$; $\eta_p^2 = .059$). There was no significant main effect of *Trial* ($F_{2,108} = 2.509$; $p = .086$), and no significant group main effect ($F_{1,54} = 3.645$; $p = .062$). Post-hoc-t-tests showed a significant treatment-effect for trial 61-100 ($t_{57} = 2.594$; $p < .05$; $\eta_p^2 = .106$), but no effect for Trial 1-30 ($t_{56} = -.652$; $p = .517$) and trial 31-60 ($t_{57} = 1.654$; $p = .104$). For control trials, median reaction times (in ms) were as follows: Trial 1-30 = 674, Trial 31-60 = 657, Trial 61-100 = 614. For target trials, median reaction times (in ms) were as follows: Trial 1-30 = 749, Trial 31-60 = 687, Trial 61-100 = 652.

The *Treatment* (*cortisol* vs. *placebo*) X *Block* ANOVA across Block 2 to Block 5 revealed only a significant main effect of *Block* ($F_{3,162} = 6.731$; $p < .001$; $\eta_p^2 = .111$; $\varepsilon = .797$), but

there was no significant interaction between *Treatment* and *Block* ($F_{3,162}$ = 2.249; p = .085), and no significant group main effect ($F_{1,54}$ = .141; p = .709). Post-hoc-t-tests showed only non-significant results for Block 2 (t_{57} = 1.729; p = .089), Block 3 (t_{57} = -.015; p = .988), Block 4 (t_{57} = .022; p = .983), and Block 5 (t_{57} = -.568; p = .572). For control trials, median reaction times (in ms) were as follows: Block 2 = 610, Block 3 = 593, Block 4 = 583, Block 5 = 571. For target trials, median reaction times (in ms) were as follows: Block 2 = 606, Block 3 = 577, Block 4 = 555, Block 5 = 549.

Figure 8 shows the different time courses of learning in the cortisol group compared to the placebo group.

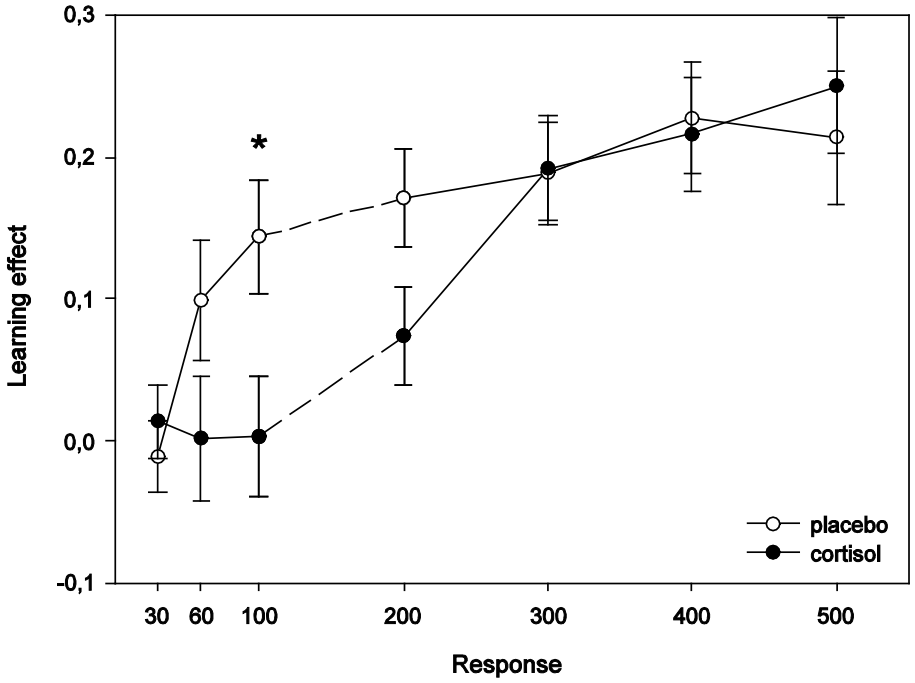

Figure 8 Delayed learning in the cortisol group (black), compared to the placebo group (white), during performance of five blocks of the SRTT. Difference scores (*target - control trials*) of corrected reaction speeds (median of 1000/reaction time in ms) of both groups are shown. Block 1 is divided in response 1-30, 31-60 and 61-100. Bars represent standard errors; * p < .05..

4.4 Discussion

We investigated implicit learning with a 5-choice SRTT in participants who received either cortisol or a placebo one hour before performing the task. The quasi-randomised stimulus series included higher-order contingencies, in which two

certain adjacent stimuli predicted the following stimulus. Learning was described by a faster response during predictable compared to unpredictable trials within each block of the SRTT. We observed effective implicit sequence learning without the participants' being aware of the target, nor the sequence rule. This was true for both groups, but with a different time course of learning. More precisely, we found delayed learning in the cortisol group compared to the placebo group. This impaired performance was most prominent in the very first block of the SRTT, but it finally equaled performance of the placebo group during the last blocks.

In the present study acquisition as well as other as early consolidation and retrieval, took place during effective cortisol treatment. Since the SRTT does not allow disentangling these phases, it is not possible to trace back, whether cortisol affects acquisition, consolidation or retrieval of the implicit knowledge. However, a recent fMRI study found that the hippocampus is not only activated during sequence learning (Schendan, et al., 2003a), but also during the retrieval of sequences (Ross, Brown, & Stern, 2009). Furthermore, it was shown that consequences of glucocorticoids on memory depend on the timing of the glucocorticoid treatment (Het, et al., 2005; Roozendaal, 2002); most studies suggesting impairment of memory function approximately 60 minutes after glucocorticoid administration. Our result of an impaired performance in this SRT-based implicit sequence learning task is in line with many studies demonstrating impaired explicit memory retrieval after cortisol administration (de Quervain, et al., 2003; e.g., de Quervain, Roozendaal, Nitsch, McGaugh, & Hock, 2000; Lupien, et al., 2002; Wolf, et al., 2001), but other studies found enhanced acquisition and consolidation of knowledge during elevated levels of glucocorticoids (Abercrombie, Kalin, Thurow, Rosenkranz, & Davidson, 2003; Beckwith, Petros, Scaglione, & Nelson, 1986; Hsu, Garside, Massey, & McAllister-Williams, 2003; Newcomer, et al., 1999; Rimmele, Domes, Mathiak, & Hautzinger, 2003). Thus, we believe that our results are due to the impairing effects of glucocorticoid administration on memory retrieval.

In the present study, participants were told to perform a simple reaction time task, and they received no explicit instruction to learn the order of the stimuli or to

discover regularity within the stimulus series. However, one may argue that our participants might have recognized the rule, but were not able to express it verbally, but rather spatially, like in the prominent experiment of Nissen and Bullemer (1987). In our study, we gave the participants the time and the chance to validate their judgement about a potential pattern with the color-locations of the response box. Furthermore, our task (compared to the previously mentioned standard-experiment) was much faster (ISI = 1000 ms) and included a more hidden pattern (SOC), which was not as easy to detect as a cycling 10-trial-sequence (compare Nissen & Bullemer, 1987). Some participants recalled colors which appeared in succession, but none of them reported the complete SOC (including not only the predictive sequence but also the target – what could have happened by chance) or was able to name the underlying sequence rule. Therefore, we can exclude that participants applied the rule consciously, and exclude explicit sequence knowledge, which clearly might have involved some sort of hippocampus function. Thus, the task performance achieved in this experiment truly reflects implicit cognitive-motor learning processes. There has been some debate on whether SRTT-based implicit sequence learning reflects motor- or perceptual-related learning. A recent study showed that implicit sequence learning may not solely be attributed to motor learning, but also to perceptual learning (Song, Howard, & Howard, 2008), and the latter being more likely to involve higher cognitive mechanisms. Therefore, the effect of cortisol on implicit sequence learning might reflect a specific engagement of the hippocampus in implicit sequence learning processes. Especially the CA3-region of the hippocampus is involved in the representation of event sequences in time (Poldrack & Rodriguez, 2003).

Furthermore, the 500 ms dark period between successive light stimuli resembles task characteristics of trace classical eyeblink conditioning, in which hippocampus functions are mandatory (Clark & Squire, 1998) and which is sensitive to glucocorticoid modulation (Grillon, et al., 2004; Nees, et al., 2008; Nees, et al., 2010). Interestingly, contingency knowledge is required to produce effective trace eyeblink conditioning (Clark & Squire, 1998), but no such contingency knowledge seems to be necessary for implicit sequence learning to occur.

Nevertheless, since GRs are not only present in the hippocampus, we cannot exclude that other brain areas were responsible for the present results. The hippocampus might also act as an intermediary structure between cortisol treatment and learning (compare Bangasser & Shors, 2010), and cortisol can have a mediating effect on other hormones as well. For example, long-term depression (LTD) in the cerebellum is a cellular mechanism of motor learning, which requires the spontaneous release of CRH from climbing fibers of the olivocerebellar system (Miyata, Okada, Hashimoto, Kano, & Ito, 1999). It might also be possible that exess cortisol inhibits the release of CRH and thereby impairs LTD.

In a previous study of our group (Schwabe, et al., 2009) we did not find effects of stress on the same implicit sequence learning task, but the socially evaluated cold pressor test (Schwabe, Haddad, & Schachinger, 2008), which was used to provoke a cortisol reaction, can also affect other factors to mask or modulate the effects of cortisol. It should be considered, that the present study is a psychopharmacological study, which may not easily be transferred into the stress context. First, other physiological stress mechanisms, such as sympathetic nervous system factors, are active during stress exposure, as well as stress-related changes in cognitive load. Second, timing may play an important role in cognitive effects of stress-induced release of cortisol. Accordingly, fast non-genomic effects of glucocorticoids were described (Joels, 2008) which are processed differently than classical genomic effects of glucocorticoids on gene expression and protein synthesis. Thirdly, dose effects of cortisol may play a role, especially since inverted U-shaped pharmacodynamic relationship have been suggested (Lupien & McEwen, 1997). We have studied cortisol effects in a single dose range frequently employed in human studies. However, future studies need to characterize dose-response relationships in order to better understand the cognitive effects of cortisol. Furthermore, also other HPA axis hormone measures (such as Dehydroepiandrosterone/DHEA, ACTH or CRH) should be taken into account.

To our knowledge the present study is the first study demonstrating an effect of cortisol on implicit sequence learning. Our results are in line with other studies,

which found impaired explicit memory after glucocorticoid treatment and with findings, which suggest that hippocampus depending forms of memory are sensitive to glucocorticoids. For future research, we propose to systematically reinvestigate whether other forms of implicit learning are also sensitive to glucocorticoid administration.

4.5 Author Notes

Please correspond to Dipl.-Psych. Sonja Römer (Department of Clinical Psychology and Psychotherapy, Saarland University, 66041 Saarbrücken, Germany, E-mail: s.roemer@mx.uni-saarland.de).

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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.

(Sonja Römer) Saarbrücken,