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# Fast and Slow Effects of Cortisol on Several Functions of the Central Nervous System in Humans

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## General Abstract

Cortisol is one of the key substances released during stress to restore homeostasis. Our knowledge of the impact of this glucocorticoid on cognition and behavior in humans is, however, still limited. Two modes of action of cortisol are known, a rapid, nongenomic and a slow, genomic mode. Both mechanisms appear to be involved in mediating the various effects of stress on cognition. Here, three experiments are presented that investigated fast and slow effects of cortisol on several functions of the human brain.

The first experiment investigated the interaction between insulin and slow, genomic cortisol effects on resting regional cerebral blood flow (rCBF) in 48 young men. A bilateral, locally distinct increase in rCBF in the insular cortex was observed 37 to 58 minutes after intranasal insulin admission. Cortisol did not influence rCBF, neither alone nor in interaction with insulin. This finding suggests that cortisol does not influence resting cerebral blood flow within a genomic timeframe.

The second experiment examined fast cortisol effects on memory retrieval. 40 participants (20 of them female) learned associations between neutral male faces and social descriptions and were tested for recall one week later. Cortisol administered intravenously 8 minutes before retrieval influenced recall performance in an inverted U-shaped dose-response relationship. This study demonstrates a rapid, presumably nongenomic cortisol effect on memory retrieval in humans.

The third experiment studied rapid cortisol effects on early multisensory integration. 24 male participants were tested twice in a focused cross-modal choice reaction time paradigm, once after cortisol and once after placebo infusion. Cortisol acutely enhanced the integration of visual targets and startling auditory distractors, when both stimuli appeared in the same sensory hemi-field. The rapidity of effect onset strongly suggests that cortisol changes multisensory integration by a nongenomic mechanism.

The work presented in this thesis highlights the essential role of cortisol as a fast acting agent during the stress response. Both the second and the third experiment provide new evidence of nongenomic cortisol effects on human cognition and behavior. Future studies should continue to investigate the impact of rapid cortisol effects on the functioning of the human brain.

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# Index of Publications

This thesis consists of four chapters. Chapter I is a general introduction. Chapters 2 to 4 have been published or are submitted for publication as "original research articles" in international peer-reviewed journals. The author of this thesis is first author to all articles. However, as usual in psychobiological research, projects were realized with the help of co-authors. In the following paragraph the co-authors of each project are listed. All articles are presented here in the originally published/submitted form except for changes in formatting (i.e., figure/table labeling).

| ***Content*** | ***has been published or is submitted as:*** |
|---|---|
| Chapter II | Schilling, T. M.*, Ferreira de Sá, D. S.*, Westerhausen, R., Strelzyk, F., Larra, M. F., Hallschmid, M., Savaskan, E., Oitzl, M. S., Busch, H-P, Naumann, E., Schächinger, H. (2013). Intranasal Insulin Increases Regional Cerebral Blood Flow in the Insular Cortex in Men Independently of Cortisol Manipulation. *Human Brain Mapping*, doi: 10.1002/hbm.22304. * *both authors contributed equally to this work* (Impact factor 2012: 6.878) |
| Chapter III | Schilling, T. M., Kölsch, M., Larra, M. F., Zech, C. M., Blumenthal, T. D., Frings, C., & Schächinger, H. (2013). For whom the bell (curve) tolls: Cortisol rapidly affects memory retrieval by an inverted U-shaped dose-response relationship. *Psychoneuroendocrinology*, *38*(9), 1565-1572. doi: 10.1016/j.psyneuen.2013.01.001 * *both authors contributed equally to this work* (Impact factor 2012: 5.137) |
| Chapter IV | Schilling, T. M., Larra, M. F., Deuter, C. E., Blumenthal, T. D., Schächinger, H (submitted). Rapid Cortisol Enhancement of Psychomotor and Startle Reactions to Side-Congruent Stimuli in a Focused Cross-Modal Choice Reaction Time Paradigm. |

## Index of Abbreviations

| | |
|---|---|
| ACTH | adrenocorticotrophic hormone |
| Ag/AgCl | silver/silver chloride |
| ANOVA | analysis of variance |
| AUCg | area under the curve with respect to ground |
| BDI | Beck Depression Inventory |
| BMI | body mass index |
| BOLD | blood-oxygen-level dependent |
| *C* | number of comparisons |
| CA1 | cornu ammonis field 1 |
| CASL | continuous arterial spin labeling |
| CBF | cerebral blood flow |
| cf. | compare to |
| cm | centimeter |
| CRH | corticotropin-releasing hormone |
| CSF | cerebrospinal fluid |
| dB(A) | decibels (A-scale) |
| DNA | deoxyribonucleic acid |
| EEG | electroencephalography |
| EMG | electromyography |
| EOG | electrooculography |
| EPI | echoplanar imaging |
| ERK | extracellular signal-regulated kinase |
| FOV | field of view |
| FWE | family-wise-error |
| g | gram |
| GABA | gamma-aminobutric acid |
| GR | glucocorticoid receptor |
| GRE | glucocorticoid response element |
| h | hours |
| HF | Huynh-Feldt |
| HPA | hypothalamic-pituitary-adrenal/adrenocortical |

| | |
|---|---|
| Hz | hertz |
| IV | intravenous |
| kg/m² | kilogram per square meter |
| kHz | kilohertz |
| LCD | liquid-crystal display |
| LF | low-frequency |
| log | logarithm |
| M | mean |
| mg | milligram |
| min | minutes |
| ml | milliliter |
| mm | millimeter |
| ms | milliseconds |
| MNI | Montreal Neurological Institute |
| MR | mineralocorticoid receptor |
| MRI | magnetic resonance imaging |
| MRT | manual reaction time |
| $M_0$ | equilibrium magnetization |
| NaCl | sodium chloride |
| nmol/l | nanomol per liter |
| OOc | orbicularis oculi muscle |
| $p_{corr}$ | corrected *p*-value |
| PPI | prepulse inhibition |
| PET | positron emission tomography |
| PVN | nucleus paraventricularis of the hypothalamus |
| POMC | proopiomelanocortin |
| rCBF | regional cerebral blood flow |
| RNA | ribonucleic acid |
| ROI | region of interest |
| RT | reaction time |
| tEBC | trace eye-blink conditioning |
| SD | standard deviation |
| SEM | standard error of the mean |
| SOA | stimulus-onset-asynchrony |

| | |
|---|---|
| SPM | statistical parametric mapping/map |
| SRT | saccadic reaction time |
| s | seconds |
| T | tesla |
| $T_1$ | longitudinal relaxation time |
| $T_2$ | transversal relaxation time |
| TE | echo time |
| TR | repetition time |
| VAS | visual-analog-scale |

# 1. General Rational

## 1.1 Introduction and Outline

The thesis presented here reports on fast and slow effects of cortisol on several functions of the central nervous system in healthy young humans. Cortisol is a steroid hormone, synthesized and released by the adrenal glands, which is implicated in the regulation of several physiological processes such as immune response or glucose metabolism (see Kino & Chrousos, 2004; Sapolsky, Romero, & Munck, 2000; Stahn & Buttgereit, 2008). However, the focus of this thesis is on yet another important function of cortisol – its prominent role as an active player in the stress response. Thus, the broader scope of the work presented here is on the impact of cortisol on the human brain within the context of stress.

Finding a commonly accepted definition of what constitutes "stress" has been an ever ongoing debate since the term was first introduced by Hans Selye (see Goldstein & Kopin, 2007; McEwen, 2005; Pacak & Palkovits, 2001). I will use two recently suggested definitions that I think nicely capture the concept:

> "Stress can be broadly defined as an actual or anticipated disruption of homeostasis or an anticipated threat to well-being." (Ulrich-Lai & Herman, 2009, p. 397).

> "Stress is (…) a condition where expectations, whether genetically programmed, established by prior learning, or deduced from circumstances, do not match the current or anticipated perceptions of the internal or external environment, and this discrepancy between what is observed or sensed and what is expected or programmed elicits patterned, compensatory responses (…)." (Goldstein & Kopin, 2007, p. 115).

The essence of these two definitions is that stress causes a disruption of a precisely balanced and equilibrated system (homeostasis) and that this disruption will provoke a compensatory response aimed at restoring this equilibrium. Disruptors of homeostasis have been termed *stressors* and the compensatory response *stress response* (e.g., Pacak & Palkovits, 2001). Cortisol is one of the key substances released during stress to restore homeostasis. Consequently, by studying the influence of cortisol on the central nervous system, we also

extend our knowledge of the effects of stress on the human brain. This again seems worth the effort due to the manifold influences of stress on health and disease (McEwen, 2005, 2008).

This thesis consists of four chapters. In the general rationale (chapter I) that starts with this introduction I will give a short overview of the physiology of the stress response and the essential role and modes of action of cortisol within this response. I will try to outline the background necessary to understand the aims, results and implications of the three experimental investigations presented in this thesis. Also, in the second part of this chapter, I will briefly discuss the three experiments and present a general conclusion. The following three chapters (chapter II – IV) contain the reports on the three experiments.

The theoretical outline starts with a short introduction on the physiology of the stress response.

## 1.2 The Stress Response

Adaptation to stress is a joint effort of the whole organism involving physiological, cognitive and behavioral adjustments (de Kloet, Joels, & Holsboer, 2005; Steckler, 2004). The brain is the coordinator of the stress response and controls the appropriate onset and offset of the diverse mediators involved in restoring homeostasis (Ulrich-Lai & Herman, 2009). Several neuronal structures are engaged in this process. Major homeostatic perturbations and *systemic stressors* such as infections or blood loss are predominantly sensed in brainstem centers (Ericsson, Kovacs, & Sawchenko, 1994) and circumventricular organs (Krause et al., 2008). By contrast, *psychological* or *neurogenic stressors*, such as social evaluative threat and a lack of predictability or controllability (Dickerson & Kemeny, 2004), are largely processed in limbic regions like the amygdala (Bhatnagar, Vining, & Denski, 2004; Dayas, Buller, Crane, Xu, & Day, 2001), the hippocampus or the prefrontal cortex (Radley, Arias, & Sawchenko, 2006). The nucleus paraventricularis of the hypothalamus (PVN) is the key integrator of the input from these various neuronal centers and ultimately controls the stress response (for an overview of the central control of the stress response see Herman, Mueller, Figueiredo, & Cullinan, 2004; Ulrich-Lai & Herman, 2009).

The stress response proceeds in several steps (Joels & Baram, 2009). The first reactions are very fast in onset, starting within seconds to minutes and comprise both central and peripheral adjustments. Monoamines and neuropeptides are released in the brain and rapidly alter

different cognitive processes to promote successful coping (Joels & Baram, 2009). For instance, the neuropeptide corticotropin-releasing hormone (CRH) and the monoamine noradrenalin interact in the locus coeruleus to jointly shift attention from a focused processing to a distributed scanning of the environment (Valentino & Van Bockstaele, 2008). Furthermore, CRH secreted from the PVN triggers the endocrinological cascade of the hypothalamic-pituitary-adrenal (HPA) axis, leading to a release of glucocorticoids into the general circulation several minutes later (Fulford & Harbuz, 2004).

While these early central stress effects unfold, control centers in the brainstem rapidly adjust activation of the sympathetic and parasympathetic nervous system in the periphery (Ulrich-Lai & Herman, 2009). Noradrenaline is released from peripheral synapses at the effector organs, such as the heart or the lungs, and adrenalin and noradrenalin are secreted from the adrenal medulla into the bloodstream (Iversen, Iversen, & Saper, 2000). Both substances collectively enhance the sympathetic tone in the body: heart rate, heart contractibility and blood pressure increases, blood flow is redistributed towards skeletal muscles and away from the skin and intestines, bronchia and pupils dilate (Hamill & Shapiro, 2004) and plasma glucose levels are enhanced (Hoeldtke, 2004). In parallel, parasympathetic outflow is adjusted in a manner that optimally supports sympathetic activity (Iversen, et al., 2000). Thus, the body is rapidly prepared for a *fight-or-flight* reaction to efficiently deal with the stressor (Goldstein, 2010).

Shortly after glucocorticoids are released into the general circulation their first effects come into play (Joels & Baram, 2009). These rapid, nongenomic actions of glucocorticoids appear to work in synergism with the effects of monoamines and neuropeptides during acute adaptation to stress. However, their precise role as well as the range and variety of processes regulated by them are still under discussion. Considerably later, usually about one hour after the onset of the stress response, the slow, genomic influences of glucocorticoids become functionally relevant (Joels & Baram, 2009). These later effects are essential for recovery and preparation for future stressful events (de Kloet, et al., 2005).

The effects of glucucorticoids throughout the body are manifold. In the periphery glucocorticoids increase the circulating levels of energy substrates to provide fuel for the ongoing stress response (Kino & Chrousos, 2004), enhance the effects of sympathetic activation, for example on blood pressure and cardiac output (Sapolsky, et al., 2000), and dampen immune and inflammatory responses (Kino & Chrousos, 2004; Sapolsky, et al., 2000;

Stahn & Buttgereit, 2008). Glucocorticoids also readily pass the blood-brain barrier with the help of p-glycoproteins and exert multiple effects on central nervous system activity (for a review see de Kloet, et al., 2005; McEwen, 2007). Among these are the maintenance of structural stability and neuronal excitability in limbic networks under rest as well as the normalization of neuronal excitability in these networks in the aftermath of stress (Joels, 2000). Further, glucocorticoids affect processes of learning and memory (Roozendaal & McGaugh, 2011), for example by enhancing the consolidation of information acquired within the stressful episode (Joels, Pu, Wiegert, Oitzl, & Krugers, 2006). Finally, glucocorticoids play an important role in terminating the HPA axis response to stress via a negative feedback mechanism (Fulford & Harbuz, 2004).

Glucocorticoids play a key role in adaptation to stress and in the restoration of homeostasis. Due to their two modes of action (fast and slow), they are involved in both short and long term adjustments to stress on the cellular, the systemic and the behavioral level (de Kloet, et al., 2005; Joels & Baram, 2009). The next sections will describe in more detail the control of glucocorticoid release by the HPA axis, and the receptors and mechanisms involved in mediating the impact of glucocorticoids in the body.

## 1.3 Glucocorticoids: Release, Receptors and Modes of Action

### 1.3.1 Glucocorticoid Release

Glucocorticoids are the final product of the endocrinological cascade of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis consists of the paraventricular nucleus of the hypothalamus (PVN), the adenohypophysis (anterior part of the pituitary) and the cortex of the adrenal glands. All three anatomical structures and several hormonal messengers are involved in the release of glucocorticoids (Fulford & Harbuz, 2004). The PVN is the apex of the HPA axis and secretes corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the hypophyseal portal blood system, where both neuropeptides are transported to the adenohypophysis. Here, CRH and AVP act in concert to stimulate the release of adrenocorticotrophic hormone (ACTH) into the general circulation. CRH is the more important ACTH secretagogue (Whitnall, 1993) and also induces synthesis of proopiomelanocortin (POMC), the precursor of ACTH, in the adenohypophysis (Eberwine, Jonassen, Evinger, & Roberts, 1987). After release, ACTH travels in the general circulation to the adrenal cortex, where it binds to receptors in the *zona fasciculata*. This induces synthesis

of the glucocorticoid steroid hormones and their subsequent release from the adrenals into the bloodstream. In the general circulation glucocorticoids are transported to various organs throughout the body including the brain. Cortisol is the principal glucocorticoid in humans, whereas in rodents corticosterone prevails (for an overview of the HPA axis see Abel & Majzoub, 2004; Fulford & Harbuz, 2004).

The activity of the HPA axis is regulated by three basic mechanisms. First, glucocorticoids inhibit their own release via negative feedback mechanisms at the level of the pituitary (Keller-Wood & Dallman, 1984), the paraventricular nucleus of the hypothalamus (Swanson & Simmons, 1989) and the hippocampus (Jacobson & Sapolsky, 1991). Second, brainstem and limbic centers, such as those involved in eliciting the stress response, influence HPA axis activity (Ulrich-Lai & Herman, 2009). Third, glucocorticoid secretion follows a distinct circadian rhythm controlled by the suprachiasmatic nucleus (Kalsbeek, van Heerikhuize, Wortel, & Buijs, 1996).

In humans, cortisol levels rise in the morning with the daily peak of cortisol concentration in the body being reached shortly after awakening (Linkowski et al., 1993). Cortisol levels in the blood then slowly decline and drop to a nadir in the evening and during the night. On top of this circadian rhythm glucocorticoids are released in an ultradian rhythm of approximately one pulse per hour (Sarabdjitsingh, Joels, & de Kloet, 2012). The stress-induced surge in glucocorticoids is superimposed on the circadian and ultradian rhythms. As a result, the impact of a stress intervention or of exogenously administered glucocorticoids can differ with the time of day (Haller, Millar, van de Schraaf, de Kloet, & Kruk, 2000) but also with the phase of the ultradian rhythm (Sarabdjitsingh et al., 2010). For this reason, experimental investigations that involve stress interventions or glucocorticoid admission are usually performed during the circadian nadir, when circulating hormone levels are low. In humans, this corresponds to the afternoon (Linkowski, et al., 1993).

### 1.3.2 Glucocorticoid Receptors

Glucocorticoids bind to two different types of receptors, the glucocorticoid (GR) and the mineralocorticoid receptor (MR) (Reul & de Kloet, 1985). Both GR and MR usually reside inside the cell in the cytoplasm or the nucleus. While GRs are found ubiquitously throughout the brain, MRs are mostly restricted to limbic areas (Reul & de Kloet, 1985). A high amount of co-localization of GRs and MRs exists in the hippocampus. The MR has a ten times higher

affinity for glucocorticoids compared to the GR and is abundantly occupied even during the circadian nadir (Reul & de Kloet, 1985) and between ultradian pulses (Conway-Campbell et al., 2007). By contrast, the GR is unbound when circulating glucocorticoid levels are low and becomes activated during glucocorticoid rises, such as at the beginning of the active period (Reul & de Kloet, 1985), following an ultradian pulse (Conway-Campbell, et al., 2007) and during stress (Kitchener, Di Blasi, Borrelli, & Piazza, 2004). Evidence suggests that MRs exert a stable excitatory tone in hippocampal neurons, influence general HPA axis activity (both basal and stress induced) and play a prominent role at the beginning of the stress reaction (Joels, Karst, DeRijk, & de Kloet, 2008). GRs are essentially involved in HPA axis regulation by negative feedback (De Kloet, Schmidt, & Meijer, 2004) and in the normalization of neuronal excitability in the aftermath of stress (Joels, Krugers, Lucassen, & Karst, 2009). In addition to these intracellular GRs and MRs, membrane-based GR and MR variants have recently been discovered, which seem mainly implicated in mediating nongenomic glucocorticoid effects (Groeneweg, Karst, de Kloet, & Joels, 2011). Importantly, the membrane-based MR variant has a ten times lower affinity than the one that resides in the cytoplasm, rendering it responsive to stress induced surges in glucocorticoid levels (Karst et al., 2005).

### 1.3.3 Modes of Action of Glucocorticoids

Glucocorticoids affect target tissue via two different mechanisms: a slow, genomic and a rapid, nongenomic one (Joels & Baram, 2009). Our current knowledge suggests that the genomic mechanism is the principal mode of action of glucocorticoids (de Kloet, et al., 2005). However, it has been known for decades that besides their slow effects, glucocorticoids also exert rapid actions, which do not involve changes in gene expression (Groeneweg, et al., 2011). The role of these fast glucocorticoid effects is increasingly better understood nowadays and the underlying mechanisms are becoming clearer. The following section will describe the two modes of action of glucocorticoids.

#### *1.3.3.1 Genomic Glucocorticoid Effects*

Glucocorticoids exert their effects in the body predominantly by influencing gene expression (Kino & Chrousos, 2004). Indeed, up to 20% of all genes in the human genome are directly or indirectly regulated by glucocorticoids. The molecular cascade that underlies these genomic

effects involves several steps and can either lead to enhanced or to repressed gene transcription (Kino & Chrousos, 2004).

Glucocorticoids are lipophylic and easily cross the plasma membrane to enter the cytoplasm of a cell, where they bind to nuclear receptors of the GR and MR subtype, forming a ligand-receptor complex. Upon binding a glucocorticoid, the receptor dissociates from attached heat-shock proteins, dimerizes with another ligand-bound receptor to form a homodimer or heterodimer (Trapp, Rupprecht, Castren, Reul, & Holsboer, 1994) and then translocates to the nucleus. Here, it connects to specific DNA sequences in the promoter region of glucocorticoid responsive genes, the so called glucocorticoid response elements (GRE). Binding of the dimer complex to a GRE then either enhances (positive GRE) or represses (negative GRE) transcription of the corresponding gene (for an overview of the molecular cascade underlying genomic glucocorticoid effects see Heitzer, Wolf, Sanchez, Witchel, & DeFranco, 2007; Kino & Chrousos, 2004; Stahn & Buttgereit, 2008). Aside from these direct transcriptional regulations, ligand-bound GRs also influence gene expression as monomers by interacting with several transcription factors, for example the nuclear factor-κB and the activator protein 1 (see Gottlicher, Heck, & Herrlich, 1998). These protein-protein interactions allow glucocorticoids to affect genes which do not provide a GRE and usually lead to repressed gene transcription. Due to the various molecular steps involved, glucocorticoids need at least 15 to 20 minutes to alter cell functions via a genomic mechanism, and often even considerably longer (Makara & Haller, 2001).

Genomic effects of glucocorticoids in the central nervous system are multifaceted (for a review see de Kloet, et al., 2005). For example, the negative feedback mechanism of glucocorticoids on HPA axis activity is partly mediated by repression of the CRH and POMC gene in the hypothalamus and the pituitary, respectively (De Kloet, et al., 2004). Besides such specific functions, glucocorticoids also produce more general effects in the brain via genomic mechanisms. In the hippocampal neurons, glucocorticoids influence the expression of genes involved in cellular metabolism, energy production, signal transduction pathways and protein synthesis (Datson, van der Perk, de Kloet, & Vreugdenhil, 2001). Moreover, by affecting transcription of calcium channels (Karst et al., 2002), glucocorticoids may induce long term morphological changes in amygdaloidal neurons (Mitra & Sapolsky, 2008) that ultimately lead to altered and pathological fear behavior (Mitra, Jadhav, McEwen, Vyas, & Chattarji, 2005; Roozendaal, McEwen, & Chattarji, 2009).

#### *1.3.3.2 Nongenomic Glucocorticoid Effects*

Glucocorticoids affect target tissue not only by changing gene expression, but also via genome-independent mechanisms (Evanson, Herman, Sakai, & Krause, 2010; Groeneweg, et al., 2011). This pathway has been termed *nongenomic* in contrast to the above described *genomic* mechanism. The core principle of nongenomic glucocorticoid effects is the independence of any mechanism that involves a genomic activity such as transcription and translation. Consequently, any glucocorticoid-induced effect that is independent of a genomic mechanism is defined to be nongenomic (Makara & Haller, 2001).

Nongenomic glucocorticoid effects are not mediated by a single mechanism but by several different signal cascades that share some general features (for an overview see Groeneweg, et al., 2011; Makara & Haller, 2001; Stahn & Buttgereit, 2008). Common principles are independence of the genome, rapidity in effect onset, and often involvement of membrane based receptors of the MR or GR subtype. Further, in the central nervous system all nongenomic glucocorticoid effects discovered so far are permissive, i.e., glucocorticoids modulate the general excitability of a cell but do not themselves evoke any activity (Groeneweg, et al., 2011).

Glucocorticoids induce both excitatory as well as inhibitory nongenomic effects on neuronal tissue (Groeneweg, et al., 2011). For example corticosterone rapidly (< 5 minutes) and reversibly increases the excitability in hippocampal CA1 neurons in mice by influencing both the pre- (Karst, et al., 2005) and the postsynaptic membrane (Olijslagers et al., 2008). At the presynaptic site, corticosterone binds to membrane based MRs and enhances the spontaneous release of glutamate containing vesicles, which induce miniature excitatory postsynaptic potentials (Karst, et al., 2005). This presynaptic effect is mediated by an extracellular signal-regulated kinase (ERK) 1/2 pathway (Olijslagers, et al., 2008). Further, at the postsynaptic membrane, corticosterone also binds to membrane located MRs and, via a G-protein coupled pathway, inhibits potassium currents in order to support depolarization of the postsynaptic membrane (Olijslagers, et al., 2008). The presynaptic release of the excitatory neurotransmitter glutamate and the inhibited hyperpolarization of the postsynaptic membrane jointly enhance the excitability of the postsynaptic neuron.

These results from *in vitro* experiments match evidence from behavioral studies in rodents. Corticosterone was shown to inhibit memory retrieval in mice via a nongenomic mechanism (Dorey et al., 2011; Sajadi, Samaei, & Rashidy-Pour, 2006). The effect occurred within 15

minutes after application of the hormone and was not blocked by the protein synthesis inhibitor anisomycin (Dorey, et al., 2011). Further, and in line with the results obtained by Karst and colleagues (2005), the deleterious effect of corticosterone depended on a membrane based MR and was not blocked by an MR-antagonist (Dorey, et al., 2011). Thus, both *in vivo* and *in vitro* studies in mice strongly suggest that corticosterone rapidly changes excitability and functions of hippocampal neurons via known receptors and a nongenomic mechanism.

Evidence of nongenomic effects of glucocorticoids have also been found in humans, both *in vivo* (e.g., Richter et al., 2011; Vila et al., 2010) and *in vitro* (e.g., Liu et al., 2005; Yamagata et al., 2012). For example, *in vivo,* it was demonstrated that cortisol acutely increases the first-phase insulin secretion to glucose load (Vila, et al., 2010). Also, cortisol rapidly accelerated trace eye-blink conditioning (Kuehl et al., 2010) and reduced prepulse inhibition (Richter, et al., 2011) in healthy young men. Further, cortisol decreased blood flow in the thalamus and changed the EEG power spectrum within a time-frame incompatible with a genomic mechanism (Strelzyk et al., 2012).

Two criteria are usually applied to prove the involvement of a nongenomic mechanism in a glucocorticoid-induced effect: the independence of the genome and the rapidity in onset (Dorey, et al., 2011; Karst, et al., 2005; Makara & Haller, 2001). The next section will discuss these criteria and their implication for studies on nongenomic cortisol effects in humans.

## 1.4 Criteria for Nongenomic Glucocorticoid Effects

### 1.4.1 Genome Independence Criterion

A glucocorticoid-induced effect is said to be nongenomic if the involvement of a direct genomic activity can be excluded (Makara & Haller, 2001). In both *in vivo* and *in vitro* studies, this is usually demonstrated by suppressing protein synthesis with the help of pharmaceutical inhibitors. If a protein synthesis inhibitor such as anisomycin (Dorey, et al., 2011) or cycloheximide (Karst, et al., 2005) does not block a glucocorticoid-induced effect, enhanced gene transcription cannot be the mode of action underlying the effect. Still, even in the presence of a protein synthesis inhibitor, glucocorticoids can inhibit gene transcription (Makara & Haller, 2001; Webster & Cidlowski, 1999). However, functional changes in cell activity following gene repression are comparatively slow in onset, probably due to the ongoing activity of proteins synthesized before gene repression occurred. Therefore, gene

repression is generally considered an unlikely mechanism if the onset of a glucocorticoid-induced effect is rather fast (Makara & Haller, 2001). Hence, fast-onset glucocorticoid effects not blocked by protein synthesis inhibitors usually provide evidence of the involvement of a nongenomic mechanism.

### 1.4.2 Temporal Criterion

Changes in gene expression need a certain time to develop. Detectable RNA transcription following glucocorticoid admission has been found as early as 7.5 minutes after drug admission (Groner, Hynes, Rahmsdorf, & Ponta, 1983). Still, at least 15 minutes are needed before glucocorticoid-induced RNA is translated into proteins and 15 to 20 minutes before associated changes in cell functioning occur (Hallahan, Young, & Munck, 1973). It seems safe to say, therefore, that any glucocorticoid-induced effect that appears in less than 15 minutes after drug admission must be nongenomic (Makara & Haller, 2001). Effects that occur after 15 minutes can be either genomic or nongenomic and require further clarification for example by showing genome independence (Makara & Haller, 2001). In order to verify that an effect developed within the first 15 minutes after glucocorticoids reached target tissue, the admission of the hormone needs to be precisely timed. *In vivo,* such precision can only be accomplished by injecting glucocorticoids directly into the circulation. An oral application of glucocorticoids will inevitably lead to uncertainties concerning the time passed since the hormone entered the bloodstream.

### 1.4.3 Criteria for Nongenomic Glucocorticoid Effects in Human *in vivo* Studies

Substantial evidence of the involvement of a nongenomic glucocorticoid effect is provided, if the effect occurs within less than 15 minutes and is not blocked by admission of a protein synthesis inhibitor. However, due to potentially adverse side effects, the admission of protein synthesis inhibitors is restricted to cell cultures or studies in animals. In human *in vivo* studies such as behavioral experiments (Richter, et al., 2011) or endocrinological challenges (Vila, et al., 2010) the involvement of a nongenomic mechanism is generally inferred from the time interval between cortisol admission and effect onset. If an effect occurs in less than 15 minutes after cortisol entered the general circulation it is considered to be nongenomic. Timing is therefore crucial and an intravenous (IV) route of cortisol application is mandatory to study nongenomic cortisol effects in humans.

Following this outline of the physiology of the HPA axis and its final product, glucocorticoids, I will now give a short overview of the background, aims, major results and implications of the three experiments that form the presented thesis. A full coverage of each study can be found in the respective chapters II – IV.

## 1.5 Experimental Investigations of Fast and Slow Effects of Cortisol on Several Functions of the Central Nervous System in Humans

Three experimental investigations were conducted to further assess the impact of cortisol on the functioning of the central nervous system in healthy humans. Two of the experiments focused on rapid, nongenomic effects, whereas the other one investigated a slow, genomic effect.

### 1.5.1 Slow, Genomic Effects of Cortisol on Regional Cerebral Blood Flow

*Intranasal Insulin Increases Regional Cerebral Blood Flow in the Insular Cortex in Men Independently of Cortisol Manipulation.*

The first experiment investigated the interaction between genomic cortisol effects and insulin on regional cerebral blood flow (rCBF) in 48 healthy young men under resting conditions.

Glucocorticoids and insulin are key regulators of metabolism that exert largely opposite effects on energy balance and food intake (Dallman, Warne, Foster, & Pecoraro, 2007; Hallschmid & Schultes, 2009; Sapolsky, et al., 2000). Both hormones also cross the blood-brain barrier and affect structures throughout the central nervous system (de Kloet, et al., 2005; Schulingkamp, Pagano, Hung, & Raffa, 2000). For example, cortisol influences the blood-oxygen-level dependent (BOLD) signal in hippocampus and amygdala (Lovallo, Robinson, Glahn, & Fox, 2010) and rCBF in the thalamus (Strelzyk, et al., 2012), while intranasal insulin modulates the BOLD response to food pictures (Guthoff et al., 2010). However, only little is known of possible interactions between the two substances on a central level. Based on the previous finding that insulin facilitates repetitive spike firing in the rat insular cortex (Takei, Fujita, Shirakawa, Koshikawa, & Kobayashi, 2010), a region containing the primary gustatory cortex in man (Small, 2010), we hypothesized that the insula might be a target for a joint central effect of both hormones.

We were interested in the central interaction between slow, genomic cortisol effects and insulin. Therefore, cortisol was given orally in three dosages of 10 mg each in intervals of 15 minutes, starting 45 minutes before the first perfusion measurement. 30 minutes before scanning insulin was administered via an intranasal route, which enables the hormone to reach the cerebrospinal fluid (CSF) without any relevant effect on peripheral insulin or glucose levels (Born et al., 2002). Corresponding placebos were also administered to half of the participants, resulting in a 2(insulin)*2(cortisol) between-subjects design. Changes in blood flow following drug admission were assessed with the help of continuous arterial spin labeling (CASL) sequences that allow the precise quantification of rCBF in milliliter per 100 gram brain tissue per minute (Detre & Alsop, 1999; Wang et al., 2008).

We observed a bilateral, locally distinct increase in rCBF in the insular cortex 37-58 minutes after intranasal insulin admission. Also, a comparable effect in the putamen was found. There was neither an impact of cortisol alone nor an interaction between cortisol and insulin in any brain region investigated.

In this experiment, we demonstrate that insulin enhances blood flow in the insular cortex in men and that this influence is not moderated by genomic cortisol effects. Importantly, rCBF was not affected at all by cortisol, suggesting that this glucocorticoid does not alter brain perfusion via a genomic mechanism. This is of particular interest in the light of the previous reports on rapid, nongenomic cortisol effects on the amygdala, the hippocampus and the thalamus (Lovallo, et al., 2010; Strelzyk, et al., 2012). Taken together, our data and these former results indicate that the effects of cortisol on cerebral hemodynamics are rapid in onset, but also short-lived.

To sum up, we did not observe any genomic cortisol effect on resting regional brain perfusion in healthy young men.

### 1.5.2 Rapid, Nongenomic Effects of Cortisol on Memory Retrieval

*For Whom the Bell (Curve) Tolls: Cortisol Rapidly Affects Memory Retrieval by an Inverted U-shaped Dose-Response Relationship.*

The second experiment investigated nongenomic cortisol effects on memory retrieval in healthy men and women ($N$ = 40; equal number of males and females).

Stress and stress released substances such as glucocorticoids and noradrenalin modulate the retrieval of emotionally arousing material (de Quervain, Aerni, Schelling, & Roozendaal, 2009; Roozendaal & McGaugh, 2011; Wolf, 2009). The impact of cortisol in this process is assumed to follow an inverted U-shaped dose-effect curve (Domes, Rothfischer, Reichwald, & Hautzinger, 2005), but clear experimental evidence for this was lacking. Also, recent experiments in rodents suggest that corticosterone influences memory retrieval via a fast, nongenomic mechanism (Dorey, et al., 2011; Sajadi, et al., 2006). In humans, however, such acute effects of cortisol on memory retrieval were never investigated. Thus, in the current study, we aimed to assess the rapid, presumably nongenomic influence of four escalating doses of cortisol on cued recall of socially relevant information in healthy young participants.

Volunteers came to the laboratory twice, once for learning and once for retrieval, both appointments at an interval of exactly one week. At the first session participants learned associations between male faces with a neutral facial expression and descriptions of positive or negative social behaviors. At the second session participants received an IV infusion of cortisol (dosages: 3, 6, 12, 24 mg) or placebo, 8 minutes before retrieval testing. During testing, the faces which had previously been paired with social descriptions were now presented again without any description, and participants were asked to recall the description as precisely as possible (cued recall). Before and after cortisol infusion, samples of salivary cortisol levels were collected with the help of Salivettes. Post-infusion salivary cortisol levels were log-transformed and used to predict cued recall performance.

We observed an inverted U-shaped relationship between the log-transformed salivary cortisol levels and cued recall performances. Thus, participants exhibiting moderate cortisol levels displayed the best recall performance, whereas participants with high or low salivary cortisol levels both performed worse. This result was not explained by differences in learning on the first session, or by an effect of cortisol on acquisition or consolidation.

Our result suggests that a moderate increase in blood cortisol levels, within a range expected following psychosocial stress (Kirschbaum & Hellhammer, 1994), promotes the retrieval of socially relevant information. Enhanced access to stored memories of other´s positive and negative personality traits might offer specific benefits for successful coping in situations involving social stress. The very short delay between cortisol admission and recall testing suggests that a nongenomic mechanism underlies the observed effect. This seems to guarantee that the cortisol induced modulation of memory retrieval starts as early as possible.

To sum up, this study demonstrates a rapid, presumably nongenomic impact of cortisol on cued memory retrieval in humans.

### 1.5.3 Rapid, Nongenomic Effects of Cortisol on Multisensory Integration

*Rapid Cortisol Enhancement of Psychomotor and Startle Reactions to Side-Congruent Stimuli in a Focused Cross-Modal Choice Reaction Time Paradigm*

The third experiment investigated rapid, nongenomic effects of cortisol on early multisensory integration in 24 healthy young men.

The term multisensory integration refers to the interactions, synergisms and fusions resulting from the combined processing of multiple sensations evoked via different sensory channels (Stein & Stanford, 2008). Stimuli from different modalities that appear simultaneously in time and at the same spatial position are preferentially processed, as reflected in increased neuronal responses (Wallace, Wilkinson, & Stein, 1996) and reduced reaction times (Colonius & Arndt, 2001; Hughes, Reuter-Lorenz, Nozawa, & Fendrich, 1994). This "spatial principle" should be of special relevance during stress, where fast and efficient localization of potential danger sources is crucial for survival. In line with this assumption, a recent study showed that cortisol, presumably via a nongenomic mechanism, reduced blood flow in the thalamus (Strelzyk, et al., 2012), a structure central to multisensory processing (Cappe, Rouiller, & Barone, 2009; Tyll, Budinger, & Noesselt, 2011). We, therefore, hypothesized that cortisol rapidly influences the early multisensory integration of visual targets and acoustic startle noises.

Participants were tested twice on separate days in an adopted focused cross-modal choice reaction time paradigm (Arndt & Colonius, 2003). Visual targets were presented alone and

together with either a bilateral startle noise or with a unilateral startle noise in the same or in the opposite sensory hemi-field. Startle noise was chosen as an accessory because of its significance as a danger cue and its reflex-eliciting impact (*startle reflex*, Koch, 1999; Yeomans, Li, Scott, & Frankland, 2002). Saccadic and manual choice reactions to visual targets as well as EMG-responses to startle noises served as indices of cross-modal cue integration. Shortly after starting the paradigm on each day, 5 mg of IV cortisol or a corresponding placebo solution was infused according to a single blinded, placebo-controlled, and counterbalanced cross-over design.

Following IV cortisol infusion we observed enhanced integration of side-congruent multisensory stimuli. This was reflected in reduced manual choice reaction time as well as in increased EMG-response magnitude to startle noise in trials where visual target and unilateral startle noise were presented in the same sensory hemi-field. The impact of cortisol was visible within less than 10 minutes after IV infusion in manual choice reactions and within less than 20 minutes in EMG-responses to startle. Cortisol did not affect any dependent variable in the other experimental conditions (opposite sensory hemi-field, bilateral startle noise, or no startle noise). Thus, cortisol rapidly and exclusively enhanced the integration of spatially congruent multisensory stimuli.

This experiment provides first evidence of an impact of cortisol on early multisensory integration. The rapidity in effect onset is compatible with a nongenomic mechanism. Cortisol solely enhanced the integration of side-congruent multisensory stimuli, indicating a rapid strengthening of the "spatial principle" by this hormone. Increased processing of same sided multisensory stimuli may accelerate orientation towards potential danger sources and intensify automated reflexes, both processes jointly increasing chances for successful coping during stress.

To sum up, cortisol rapidly and presumably via a nongenomic mechanism enhances the integration of side-congruent multisensory stimuli.

## 1.6 General Conclusion

Three studies were presented that aimed at further extending our knowledge of the impact of fast and slow effects of cortisol on several functions of the central nervous system in humans.

The first experiment investigated slow, genomic effects of cortisol on regional cerebral blood flow. No evidence of a cortisol induced change in hemodynamics was found within the timeframe investigated. The result complements previously published data on fast glucocorticoid effects in the human brain (Lovallo, et al., 2010; Strelzyk, et al., 2012). Collectively, our data and these former reports suggest that the effects of cortisol on cerebral hemodynamics are characterized by rapid onset and short duration. This emphasizes the role of cortisol as a fast acting agent during acute adaptation to stress.

The second experiment examined the impact of cortisol on memory retrieval within a nongenomic timeframe. We demonstrated that cortisol rapidly (< 10 min) affected cued recall in humans by an inverted U-shaped dose-response relationship. So far, effects of cortisol on memory retrieval in humans were only observed one hour after drug admission at the earliest (de Quervain, Roozendaal, Nitsch, McGaugh, & Hock, 2000). Thus, our finding provides evidence that cortisol affects memory retrieval in man much earlier than previously thought, presumably via a nongenomic mechanism.

The third experiment studied fast effects of cortisol on early multisensory integration. Following IV cortisol infusion, we observed enhanced integration of a visual target and a startling auditory accessory, when both stimuli appeared in the same sensory hemi-field. The integration of two stimuli appearing in different sensory modalities is fundamental to perception but had never been investigated following stress or cortisol admission. Our finding suggests that cortisol rapidly enhances the “spatial principle” (Stein & Stanford, 2008) in multisensory integration via a nongenomic mechanism.

The results reported in this thesis provide new evidence regarding fast effects of cortisol on the functioning of the human brain. Both the modulation of memory retrieval and the enhancement of the integration of side-congruent multisensory stimuli demonstrate that cortisol rapidly affects core functions of human cognition. In line with previous studies in humans (Kuehl, et al., 2010; Richter, et al., 2011; Schulz et al., 2013) and rodents (Dorey, et al., 2011; Mikics, Barsy, Barsvari, & Haller, 2005; Mikics, Kruk, & Haller, 2004; Sajadi, et al., 2006) our data emphasizes the importance of early glucocorticoid effects for fast adaptation to stress. Still, the results reported so far may represent only a fraction of the many

cognitive and behavioral processes affected by rapid glucocorticoid action. Future research should, therefore, continue to investigate nongenomic cortisol effects and their role for human cognition and behavior during the stress response.

# SLOW, GENOMIC EFFECTS OF CORTISOL

# 2. Intranasal Insulin Increases Regional Cerebral Blood Flow in the Insular Cortex in Men Independently of Cortisol Manipulation

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## 2.0 Abstract

Insulin and cortisol play a key role in the regulation of energy homeostasis, appetite and satiety. Little is known about the action and interaction of both hormones in brain structures controlling food intake and the processing of neurovisceral signals from the gastrointestinal tract. In the current study, we assessed the impact of single and combined application of insulin and cortisol on resting regional cerebral blood flow (rCBF) in the insular cortex. After standardized periods of food restriction, 48 male volunteers were randomly assigned to receive either 40 IU intranasal insulin, 30 mg oral cortisol, both, or neither (placebo). Continuous arterial spin labeling (CASL) sequences were acquired before and after pharmacological treatment. We observed a bilateral, locally distinct rCBF increase after insulin administration in the insular cortex and the putamen. Insulin effects on rCBF were present regardless of whether participants had received cortisol or not. Our results indicate that insulin, but not cortisol, affects blood flow in human brain structures involved in the regulation of eating behavior.

**Keywords:** Cerebral Cortex, Basal Ganglia, Hippocampus, Glucocorticoids, Pancreatic Hormones, Metabolism

## 2.1 Introduction

Adrenal glucocorticoids and the pancreatic peptide insulin jointly play key roles in energy homeostasis, co-dependably regulating food intake and metabolism (Dallman, Warne, Foster, & Pecoraro, 2007; Peters et al., 2004). Previous research has shown that a disruption in the normal functioning of both these hormones might be crucially involved in the development of obesity and the metabolic syndrome (la Fleur, Akana, Manalo, & Dallman, 2004; Rosmond, 2005). Under normal physiological circumstances, insulin and glucocorticoids have opposite effects on the metabolism of peripheral tissues (Dallman et al., 1993). Additionally, both hormones cross the blood-brain barrier and act on the central nervous system (Joels, 1997; Plum, Schubert, & Bruning, 2005) but the action and interaction of these hormones at the central level still remain issues of investigation.

Insulin is known to affect food-associated behavior in rodents and humans independently of systemic hypoglycemia (Dallman, et al., 2007; Hallschmid, Benedict, Born, Fehm, & Kern, 2004). Peripheral insulin accessing the brain communicates the size of adipose stores to the central nervous system (Figlewicz & Sipols, 2010), acting along with leptin as an "adiposity signal" that exerts negative feedback on brain structures that regulate feeding behavior, and maintaining adipose mass at constant levels (Morton, Cummings, Baskin, Barsh, & Schwartz, 2006). Accordingly, direct infusion of insulin into the brain has an anorexigenic effect, provoking inhibition of food intake, decreased body weight, and improvement in insulin sensitivity in the periphery. In contrast, if insulin signaling is inhibited, there is an increase in food intake and peripheral insulin resistance (Plum, et al., 2005). In line with these observations, it has been shown that intravenous insulin application increases visceral sensitivity without influencing the somatic sensory functions (Softeland et al., 2011) and that central nervous administration of insulin via the intranasal pathway increases satiety and reduces food intake in humans (Hallschmid, Higgs, Thienel, Ott, & Lehnert, 2012). Recently, it has been demonstrated that intranasal insulin increases cerebral high-energy phosphate content which further highlights the crucial importance of insulin in the human brain for metabolic homoeostasis (Jauch-Chara et al., 2012).

Stress and stress hormones, such as adrenal corticosteroids, are also known to influence eating behavior, appetite, and satiety. Humans respond to stress with an increased intake of highly palatable food, i.e., "comfort food" (J. M. Born et al., 2010; la Fleur, 2006). Adam and Epel (2007) proposed a theoretical model of Reward Based Stress Eating, where cortisol effects on central reward circuitry may motivate calorically dense food intake. The effect of "comfort

food" in stress dampening might be mediated by insulin signaling to the brain (Warne, 2009), and it was shown that intranasal insulin administration reduces stress reactivity (Bohringer, Schwabe, Richter, & Schachinger, 2008). Corticosteroid blood levels are positively correlated with insulin levels, and some types of obesity, such as visceral obesity, might be a direct result of elevated corticosteroids, insulin resistance, and increases in insulin (Rosmond, 2005). Low doses of corticosteroids stimulate food and energy intake while high doses inhibit it, and the latter effect might be mediated by high levels of insulin (Dallman, et al., 1993). In general, corticosteroids guarantee energy supply and stimulate food intake (Dallman, et al., 2007). Insulin might interact with this effect by determining the type of nutrients chosen (la Fleur, 2006).

Insulin and glucocorticoid receptors are found throughout the central nervous system, although not uniformly. Receptors for both hormones show high densities in the cerebral cortex (Lee, Herman, & Mattson, 2000; Schulingkamp, Pagano, Hung, & Raffa, 2000; Werther et al., 1987). Recently, it has been demonstrated that insulin facilitates repetitive spike firing in the rat insular cortex (Takei, Fujita, Shirakawa, Koshikawa, & Kobayashi, 2010), a region containing the primary gustatory cortex in humans (Small, 2010). The insular cortex contains a topographically organized visceral sensory representation, integrates information from other sensory modalities to control for feeding behavior (Small, 2010), is sensitive to a large number of food cues such as taste, texture (De Araujo & Rolls, 2004) and visual appearance (Ohla, Toepel, le Coutre, & Hudry, 2012) and has been linked to insulin and satiety (Tataranni et al., 1999). We assumed that the insular cortex might be particularly sensitive to insulin's regulatory impact on eating behavior.

We studied the effects of oral cortisol and intranasal insulin on resting regional cerebral blood flow (rCBF). Hormones were applied separately and combined. The intranasal application of insulin was chosen since it allows the peptide to reach the cerebrospinal fluid without relevant blood absorption, and consequently without peripheral side-effects such as hypoglycemia (J. Born et al., 2002; Kern, Born, Schreiber, & Fehm, 1999). We measured rCBF in the putamen, hippocampus, insular and visual cortex, but we assumed that the insular cortex would be the main target region for the effects of both hormones.

## 2.2 Methods

### 2.2.1 Participants

48 right handed healthy male volunteers (mean age: 23.98, *SD*: 3.4) participated in the study and received a monetary reward for participation. Exclusion criteria were any acute or chronic somatic or psychiatric illness, any history of psychiatric disorders, glaucoma, any family history of epilepsy or aneurysms, a body mass index lower than 20 or greater than 25, any disfavor towards certain kinds of food (including vegetarianism), smoking, caffeine consumption exceeding five cups of coffee per day, or any illicit drug intake in the last six months. In addition participants had to fulfill general magnetic resonance imaging (MRI) safety standards (Kanal et al., 2002) such as lack of any ferromagnetic objects in their body and to be negative of any history of claustrophobia or uneasiness towards medical settings or procedures. Participants gave their informed written consent and were told that they had the right to stop the experiment at any time they wanted. The study was approved by the Ethical Committee of the State's Medical Association (Landesärztekammer Rheinland-Pfalz) and was in accordance with the latest revision of the Declaration of Helsinki.

### 2.2.2 Experimental Design and Pharmaceutical Materials

All participants received oral and intranasal treatment. According to a double-blinded, two by two between subject design, participants were randomly assigned to receive either oral cortisol vs. oral placebo and intranasal insulin vs. intranasal placebo, resulting in four groups ('insulin & cortisol', 'insulin & oral placebo', 'cortisol & intranasal placebo', and 'oral & intranasal placebo'). A total of 30 mg cortisol (Hydroson® Tabl., Dermapharm, Grünwald, Germany) was administered in 3 doses of 10 mg over a period of 30 min (15 min between administrations) to assure heightened "steady-state like" cortisol plasma levels throughout the whole experiment. Participants received 40 units of insulin intranasally (Insulin Human Actrapid Penfill® 100 I.E./ml; Novo Nordisk, Mainz, Germany) or placebo (dilution buffer) by applying two 0.1 ml puffs into each nostril via a high precision medical nose pump (kindly provided by Aero Pump, Hochheim, Germany). Intranasal insulin vs. intranasal placebo was administered together with the second dose of cortisol. Blood glucose was determined by an Accu-Check device (Aviva, Roche Diagnostics Deutschland, Mannheim, Germany).

### 2.2.3 Procedure

Scanning took place between 8 am and 12 pm or 12 pm and 4 pm. Participants of all four groups were distributed to morning or afternoon in a pseudo randomized, balanced fashion. All participants were instructed to have the last meal at 10 pm of the previous day, afternoon participants were instructed to have one extra slice of bread with butter or marmalade at 8 am of the same day. Upon arrival participants had a short medical check-up, filled out some questionnaires, were familiarized with the scanner environment, and then entered the scanner for the first scan. Participants were instructed to move as little as possible, to keep their eyes open and to not fall asleep. The first scan consisted of a functional measurement-block (Baseline; duration 6:50 minutes) which was later used as a baseline reference for the following functional measurement-blocks. At the end of the first scan, participants left the scanner, received the assigned pharmacological treatment and reentered the scanner. At the beginning of the second scan an anatomical $T_1$-image (duration 13:22 minutes) was acquired. This was followed by five independent but subsequent functional measurement-blocks (Block 1-5; duration of 6:50 minutes each, no pause between blocks) to assess the change of regional cerebral blood flow over time. Intranasal insulin is known to show its peak concentration in the CSF around 30 minutes after administration (J. Born, et al., 2002). The functional measurement-blocks therefore covered a time span of approximately 35 minutes, lasting 30 to 65 minutes after intranasal insulin administration. After the last functional measurement-block, a $T_2$-weighted anatomical image of the whole brain was acquired. $T_2$-images of all participants were later checked by a board-certified radiologist for anatomical abnormalities. Last, an $M_0$-measurement (equilibrium magnetization) was acquired. Total duration of the second scan was approximately 55 minutes including entering and leaving the scanner. In the end of the experiment, participants filled out some questionnaires and received a snack. After eating, following directions of the Ethical Committee blood glucose was assessed to reassure normal levels in blood. Also, the monetary reward and a letter disclosing the experimental condition were handed out. See also Figure 1 for an overview on the experimental procedure.

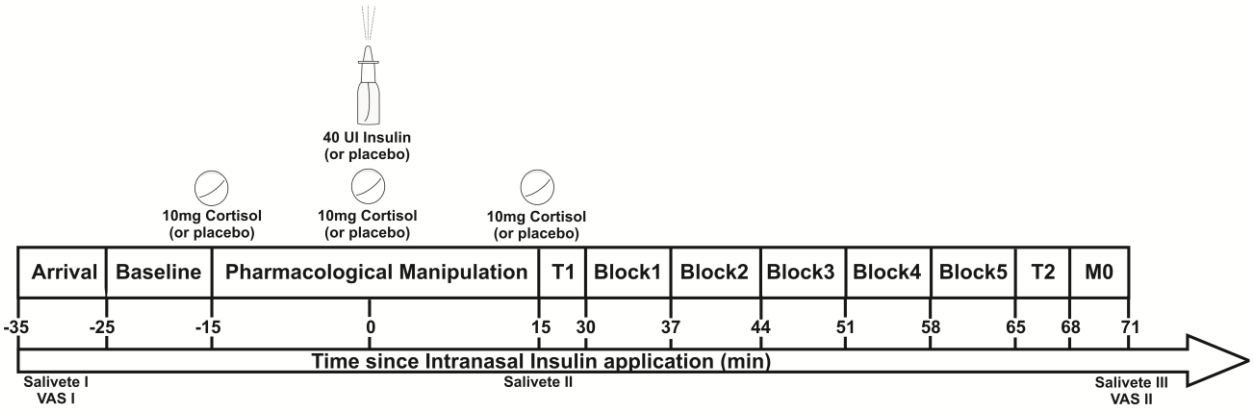


**Figure 1:** Flowchart of the experiment: After arrival participants entered the scanner for scan 1 (Baseline).The pharmacological treatment was given out of the scanner. The second scan started by recording an anatomical $T_1$-scan before five functional-blocks, an anatomical $T_2$-scan and a $M_0$-scan were recorded. Salivary cortisol was assessed three times during the experiment ('Salivette'), mood and hunger ratings were assessed two times via a visual-analog-scale ('VAS'). Also, blood sugar levels were taken at the end of the experiment

### 2.2.4 Measurements

#### *2.2.4.1 Assessment of Salivary Cortisol*

Salivary morning cortisol on the experimental day was assessed for all participants 0, 30, 45 and 60 minutes after awakening with devices for saliva sampling (Salivettes®, Sarstedt, Nümbrecht, Germany). This was done in order to check for preexisting differences in basal cortisol levels between the four groups.

We also measured salivary cortisol levels during the experimental session to check whether the pharmacological cortisol manipulation was successful. Salivary cortisol was acquired three times during the experiment. The first sample was taken immediately after arriving, before any pharmacological manipulation. The second sample was acquired after the pharmacological manipulation, immediately before participants entered the scanner for the second scan. The third sample was taken at the end of the second scan, right after leaving the scanner. Salivary cortisol was analyzed by an immunoassay with fluorescence detection (Dressendorfer, Kirschbaum, Rohde, Stahl, & Strasburger, 1992) with a concentration detection limit of 100 nmol/l. Salivary cortisol levels exceeding 100 nmol/l were marked as above 100 without stating the exact value.

#### *2.2.4.2 Subjective Mood and Hunger Ratings*

Mood and hunger ratings were assessed upon arrival and after the second scan with the help of a visual-analog scale. This was done to check whether participants of all four groups were in a comparable subjective state during the experiment. The mood rating comprised the subscales *stress* and *arousal*.

#### *2.2.4.3 MRI-Measurements*

##### *2.2.4.3.1 Continuous Arterial Spin Labeling (CASL)*

Cerebral blood flow (CBF) was assessed using continuous arterial spin labeling (CASL) sequences. CASL allows the quantification of cerebral blood flow values in absolute physiological values in ml/100g brain tissue/minute. It has a high specificity to tissue, can be repeated many times in short intervals, and shows no sensitivity to baseline drifts which makes it an ideal method to assess the change of blood flow over time following a pharmacological manipulation (Detre & Alsop, 1999; Wang et al., 2008).

##### *2.2.4.3.2 Data Acquisition*

Imaging was performed on a 1.5 T scanner (Intera; Philips Medical Systems) with interleaved label and control images acquisitions, using a single-shot spin echo EPI sequence (Hermes et al., 2007). Thirteen slices covering the whole brain were acquired from inferior to superior (FOV, 230 mm; matrix, 64x64; slice thickness, 8 mm; 1 mm gap; bandwidth, 78.4 kHz, flip angle 90°; TR, 4,125 ms; TE, 42 ms) and reconstructed on a 128x128 matrix. Labeling was achieved with a flow driven adiabatic inversion technique (Alsop & Detre, 1996). In order to control for magnetization transfer effects an amplitude modulated version of the CASL technique was used (Alsop & Detre, 1998).The labeling plane was placed 60 mm beneath the center of the imaging slices (labeling duration, 2.2 s; labeling amplitude, 35 mg; labeling gradient, 0.25 g/cm; post labeling delay, 0.8 –1.8 s). The post labeling delay varied from 0.8 to 1.8 seconds between slices because each slice was acquired at a slightly different time relative to the labeling pulse. CBF was measured in six measurement-blocks. Each of the six measurement-blocks (6:50 min) consisted of 46 pairs of label and control images. A $T_1$-weighted sequence ($T_1$-weighted gradient recalled echo (fast field echo); 160 slices; FOV, 256x192 mm; matrix, 256x256; slice thickness, 1 mm; TR, 11.9 ms; TE, 3.3 ms; duration,

13.22 min) was acquired at the beginning of the second scan. At the end of the second scan a series of 46 $M_0$-images was acquired to determine the equilibrium magnetization (scan duration 3:25 min). Additionally, a $T_2$-weighted sequence was recorded (scan duration 2:57 min). Spatial realignment of data from the first and second scan was facilitated by using the Philips Smart Brain procedure (Intera; Philips Medical Systems) which automatically detects landmarks on the participant's brain anatomy, uses them to realign separately acquired functional or anatomical images and thereby supports comparison of functional imaging data which are acquired in different scans.

*2.2.4.3.3 Data Preprocessing*

Preprocessing of functional CASL-images and structural $T_1$-images was done using SPM8 (Welcome Department of Imaging Neuroscience, London, UK) implemented on a MATLAB System (Version 2011a, Mathworks, Natick, MA, USA) as reported elsewhere (Hermes, et al., 2007; Strelzyk et al., 2012). First, label-, control-, and $M_0$ Images were spatially realigned and coregistered to the anatomical $T_1$-Image and then normalized to the MNI 152 average brain (Montreal Neurological Institute; Mazziotta, Toga, Evans, Fox, & Lancaster, 1995). Next, label- and control-images were averaged separately and separated for each measurement-block. The $M_0$-images were also averaged. We estimated global CBF values separately for each measurement-block using label, control, and $M_0$ images following a formula described by Alsop & Detre (1996), and using the parameter values described in detail by Hermes and colleagues (2007). The blood-brain partition coefficient of water was set to $\lambda = 0.98$ for gray matter quantification (Herscovitch & Raichle, 1985), the $T_1$ for arterial blood to 1.4 seconds and the labeling efficiency to $\alpha = 0.71$. The value for the post-labeling delay in the quantification formula was different for each slice to adjust for slice timing delays during image acquisition (varying between 0.8 to 1.8 seconds). $T_1$-images were segmented and normalized to the MNI 152 average brain and the resulting tissue probability maps converted into dichotomous gray matter mask. These gray matter masks were multiplied with the CBF images resulting in gray matter CBF maps. We defined templates for regions of Interest (ROI) based on published templates (Tzourio-Mazoyer et al., 2002) with the help of the MARINA-toolbox (Walter et al., 2003). Additionally, images for the voxel-based result presentations were generated by spatially smoothing the unsegmented CBF images of each measurement-block with a 6*6*12 mm full width at half maximum kernel.

*2.2.4.3.4 Selection of Regions of Interest*

Receptors for insulin and cortisol have been reported in the entire cortex as well as in subcortical structures (Sanchez, Young, Plotsky, & Insel, 2000; Sara et al., 1982; Schulingkamp, et al., 2000; Unger, Livingston, & Moss, 1991; Werther, et al., 1987). According to our hypotheses we defined the left and right insular cortices to be our primary ROI. This primary ROI was contrasted with the calcarine fissure and surrounding cortex, which forms part of the primary visual cortex. We chose this region as a control ROI because it has recently been shown that intranasal insulin does not affect rCBF in the primary visual cortex (Grichisch et al., 2012). In addition to the primary and control ROI two secondary ROI were included. We selected the hippocampus as a secondary ROI because effects of insulin and cortisol on hippocampus dependent declarative memory systems have frequently been reported (Benedict, Hallschmid, Schultes, Born, & Kern, 2007; Wolf, 2009) and the hippocampus is a region rich in both cortisol and insulin receptors (Sanchez, et al.; Schulingkamp, et al., 2000). Since insulin is implicated in food reward behavior (Figlewicz & Sipols, 2010) and a positive relation between fasting insulin levels and functional connectivity in the putamen has been reported (Kullmann et al., 2012), we also chose the putamen as secondary ROI in this study.

**2.2.5 Statistical Analysis**

All statistical analyses were conducted with SPSS 19 (IBM SPSS statistics). For each model an α-level of $p = 0.05$ was selected and a Greenhouse-Geisser correction for degrees of freedom was applied whenever appropriate. Interactions and main effects were further decomposed using Dunn´s multiple comparison procedure (Dunn, 1961): the critical value (ψ) and the number of comparisons (*C*) are stated. Two participants were excluded after data-acquisition from further statistical analysis. The first exclusion was the result of a technical failure during scanning which led to data loss. The second exclusion was necessary due to an abnormal sleeping pattern in the night before the experiment (participant slept less than 2 ½ hours). A total of 46 datasets were entered in the final statistical analysis (‘insulin & cortisol’ = 11, ‘insulin & oral placebo’=11, ‘cortisol & intranasal placebo’ = 12, and ‘oral placebo & intranasal placebo’ =12).

#### *2.2.5.1 Salivary Morning Cortisol*

Salivary morning cortisol of the experimental day was calculated as the area under the curve with respect to ground (AUCg) following a formula described by Pruessner and colleagues (2003). Groups were compared using a one-way ANOVA.

#### *2.2.5.2 Manipulation Check*

The success of the pharmacological cortisol manipulation was assessed using the three saliva samples acquired during the experiment. Since we did not asses the exact value for salivary cortisol levels exceeding 100 nmol/l only descriptive data are shown.

#### *2.2.5.3 Subjective Mood and Hunger Ratings*

Subjective mood and hunger ratings were analyzed employing a 2('insulin')*2('cortisol') *2('point of measurement') ANOVA model for each of the dependent variables *hunger*, *stress*, and *arousal*.

#### *2.2.5.4 Continuous Arterial Spin Labeling Analysis*

##### *2.2.5.4.1 Primary ROI*

Regional cerebral blood flow values of the primary and control ROI were used for further statistical analysis. Data of both ROI were entered in one model employing a 2('insulin')*2('cortisol')*2('ROI')*2('hemisphere')*6('measurement-block') ANOVA in order to assess whether the impact of the pharmacological manipulation on the change in rCBF over time was different in one ROI from the other.

##### *2.2.5.4.2 Secondary ROI*

For both secondary ROI hippocampus and putamen separate ANOVA models were calculated, entering the same factors as described above except for the factor 'ROI'.

*2.2.5.4.3 Voxel-Based Exploration*

An open, non-hypothesis driven, whole brain voxel-based exploration was conducted within the framework of the general linear model as implemented in SPM8. The unsegmented and smoothed mean CBF-images were employed in a full-factorial 4('group')*6('measurement-block') model and statistical parametric maps (SPMs) were calculated (Friston, Frith, Liddle, & Frackowiak, 1991). We set up normalized contrast vectors for the interactions 'insulin'*'measurement-block', 'cortisol'*'measurement-blocks', and 'insulin'*'cortisol'*' measurement-block', contrasting all five post-intervention measurement blocks with the baseline block. Further, the respective "treatment" group (receiving insulin, cortisol or both) was contrasted with the corresponding placebo group. By including the baseline values only the differences between conditions in CBF changes from the baseline to post-intervention measurement-blocks were contrasted and possible baseline differences, which might have preexisted independently of the pharmacological manipulation, were eliminated. Altogether, six different SPMs were calculated (interaction 'insulin'*'measurement-block'; 'CBF increase/decrease after insulin intake'; interaction 'cortisol'*'measurement-block'; 'CBF increase/decrease after cortisol intake'; interaction 'insulin'*'cortisol'*'measurement-block'; 'CBF increase/decrease after intake of insulin and cortisol'). Each SPM spanned the time interval of 30 to 65 minutes after intranasal insulin intake, showing either the baseline corrected CBF increases or decreases after insulin or cortisol or insulin and cortisol intake. The height threshold at the voxel level was set to a liberal criterion of $p < 0.001$ (uncorrected). Significant clusters (Forman et al., 1995) were identified at a family-wise-error-corrected extent threshold level of $p < 0.05$ (FWE-corrected). No covariates were entered. We applied no predefined template or masking and therefore the statistical parametric maps comprised the whole brain as it was covered during image acquisition. For visualization purposes the SPMs were saved and overlaid on the Ch2bet brain template implemented in MRIcron (http://www.mccauslandcenter.sc.edu/ricro/mricron/).

## 2.3 Results

### 2.3.1 Salivary Morning Cortisol

Participants of the four groups had comparable salivary morning cortisol levels, calculated as AUCg ($F$ [3 42] = .51, $p$ = .68). Mean and standard error of means for each group were as follows: 'insulin & cortisol' $M$= 665 ± 89; 'insulin & oral placebo' $M$= 543 ± 87, 'cortisol & intranasal placebo', $M$=562 ± 51, 'oral & intranasal placebo' $M$= 633 ± 89.

### 2.3.2 Manipulation Check

Participants receiving oral cortisol but not those receiving oral placebo showed increased salivary cortisol levels at measurement 2 (cortisol condition $M$= 77.38 nmol/l, placebo condition $M$= 7.75 nmol/l) and measurement 3 (cortisol condition $M$= 87.53 nmol/l, placebo condition $M$= 10.06 nmol/l). The pharmacological manipulation with cortisol was, therefore, successful.

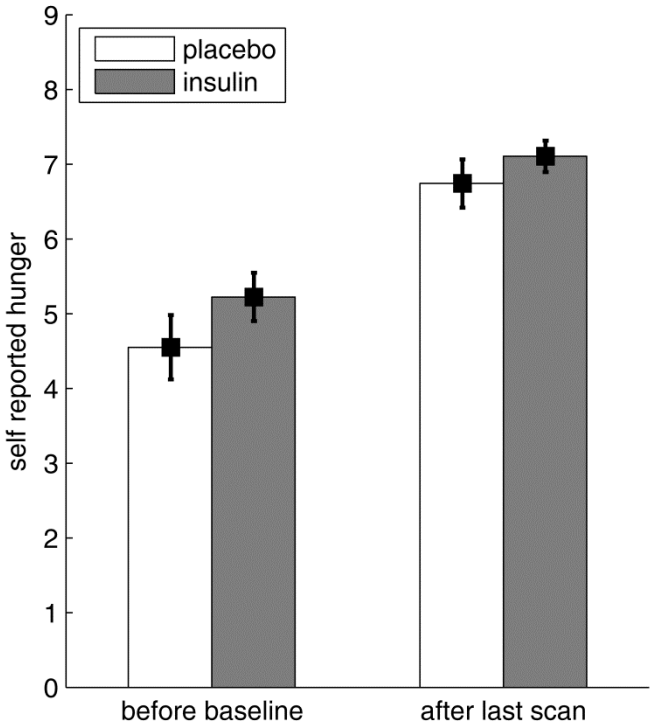


**Figure 2:** Values of self-reported hunger of participants receiving insulin (N = 22) compared to participants receiving placebo (N = 24) before baseline and after the last scan. Data present mean and standard error of the mean.

### 2.3.3 Subjective Mood and Hunger Ratings

Participants did not differ regarding their subjective mood or hunger ratings. We found neither a main effect of 'insulin' or 'cortisol' nor an interaction between the two hormones, nor an interaction with the factor 'point of measurement' in any of the dependent variables (all $p > 0.05$).

### 2.3.4 ROI-Analysis

#### *2.3.4.1 Primary vs. Control ROI*

We observed a three-way interaction 'insulin'*'ROI'*'measurement-block' ($F$ [5, 210] = 2.804, $p$ = .018, $\eta_p^2$ = .063). The influence of insulin on the change in rCBF over time was different in the primary ROI compared to the control ROI. Dunn´s multiple comparison procedure ($\psi_{5\%}$ = 5.00 ml/100g brain tissue/minute, $C$ = 20) showed that this effect was due to an increase in rCBF in the insular cortex relative to baseline in participants receiving insulin. Increased rCBF compared to baseline values was observed in block 2 (mean increase compared to baseline and standard error: 6.68 ± 1.89 ml/100g/minute), block 3 (7.51 ± 2.03 ml/100g/minute) and block 4 (6.79 ± 1.99 ml/100g/minute) after the pharmacological manipulation. This corresponded to a time interval of about 37 to 58 minutes after intranasal insulin administration. No change in rCBF in the insular cortex was observed for participants receiving placebo (all differences below the critical Dunn's value of $\psi_{5\%}$ = 5.00 ml/100g brain tissue/minute). Also, insulin did not have an influence on rCBF in the calcarine fissure and surrounding cortex (see Figure 3). Both higher order interactions including the factors 'insulin', 'cortisol', 'ROI' and 'measurement-block' failed to reach significance ('insulin'*'cortisol'*'ROI'*'measurement-block' ($F$ [5, 210] = .66, $p$ = .65) and 'insulin'*'cortisol'*'ROI'*hemisphere'*'measurement-block' ($F$ [5, 210] = .78, $p$ = .56). Also, no interaction including the factors 'cortisol' and 'measurement-block' was found ('cortisol'*'ROI'*'measurement-block' ($F$ [5, 210] = .55, $p$ = .53); 'cortisol'*'ROI' *'hemisphere'*'measurement-block' ($F$ [5, 210] = .72, $p$ = .61)).

#### *2.3.4.2 Secondary ROI*

We observed a two-way interaction 'insulin'*'measurement-block' in the secondary ROI putamen ($F$ [5, 210] = 4.087, $p$ = .001, $\eta_p^2$ = .089). Dunn`s Post-hoc procedure ($\psi_{5\%}$ = 5.90 ml/100g brain tissue/minute, $C$ = 10) showed that rCBF was elevated compared to baseline

values in the post-intervention measurement-blocks 1 to 4 (mean increase compared to baseline and standard error in block 1: 6.28 ± 1.94, block 2: 6.58 ± 2.09, block 3: 6.76 ± 2.53, block 4: 6.55 ± 2.21, block 5: 5.83 ± 2.32 ml/100g/minute) in participants receiving insulin but not in those receiving placebo. We did not observe any interaction between 'measurement-block' and 'insulin' or 'cortisol' in the secondary ROI hippocampus (all $p > 0.05$).

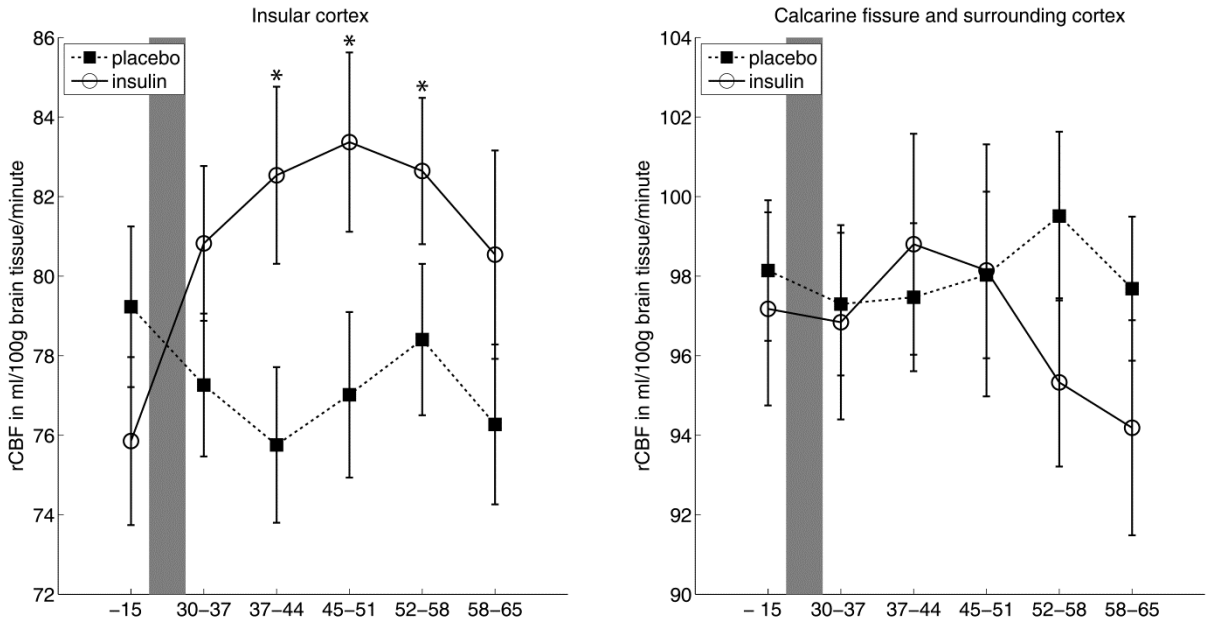


**Figure 3:** Regional cerebral blood flow (rCBF) over time for participants receiving insulin (N = 22) vs. participants receiving placebo (N = 24); Left panel: in the insular cortex; right panel in the calcarine fissure and surrounding cortex. X-axis values are in minutes relative to the application of the intranasal insulin vs. placebo. Grey-shaded area indicates pharmacological treatment. Data present mean and standard error of the mean. Post-intervention measurement-blocks which differ significantly from their corresponding baseline are marked with an '*' (according to Dunn's multiple comparison test, $\psi_{5\%}$ = 5.00 ml/100g brain tissue/minute, $C$ = 20).

### *2.3.4.3 Voxel-Based Exploration*

The voxel-based exploration revealed CBF increases after intranasal insulin intake, lasting throughout all five post-intervention measurement-blocks (interaction 'insulin' *'measurement-block'; SPM 'CBF increases after insulin intake'). Clusters of significant activation were found bilaterally in the insular cortex as well as unilaterally in left putamen and left caudate nucleus (see Figure 4 and Table 1). Moreover, a bilateral CBF increase in the opercular part of the inferior frontal gyrus was observed. No CBF changes were observed in

any of the other SPMs calculated. There were no CBF decreases after insulin intake, neither CBF increases nor decreases after cortisol intake, and no interaction between insulin and cortisol on CBF.

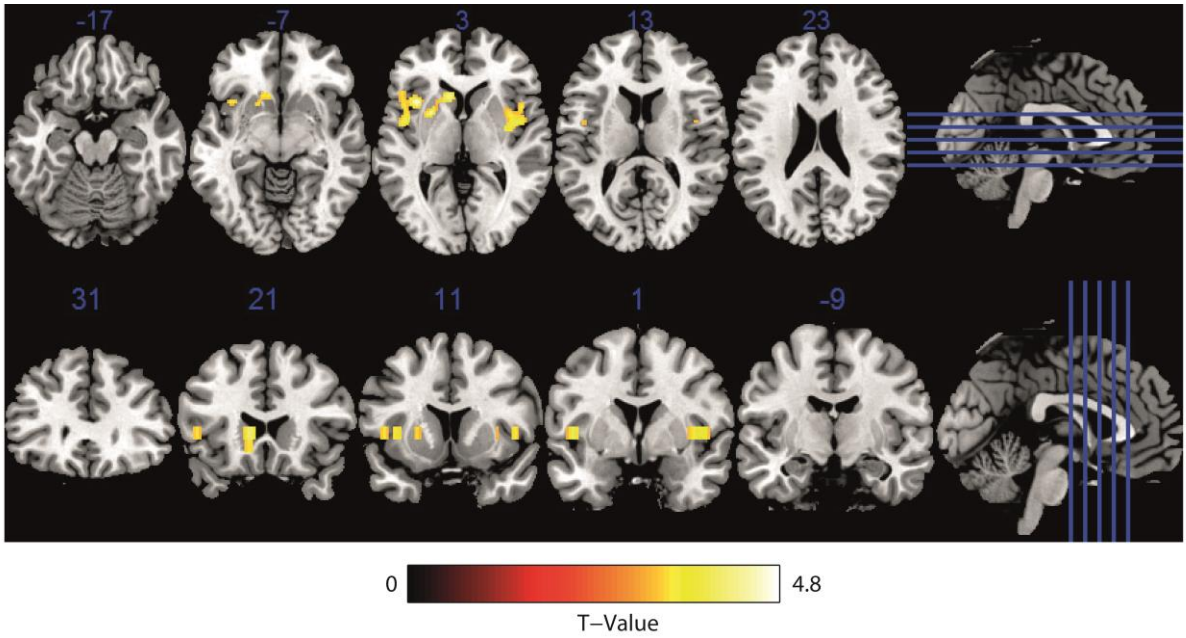


**Figure 4:** Baseline corrected CBF-increases in participants receiving intranasal insulin compared to participants receiving placebo in all five post-intervention measurement-blocks, corresponding to a time interval of 30-65 minutes after the pharmacological manipulation. No CBF-decreases were observed. Functional image overlaid on the Ch2bet template implemented in MRIcron and then multislized in the axial and coronar plane. Statistical parametric mapping of the normalized contrast vector of the interaction 'insulin'*'measurement-block' contrasting the post-intervention measurement-blocks 1 to 5 with the baseline block and participants receiving insulin with participants receiving placebo (full-factorial 4('group')*6('measurement-block') model), peak level $p < 0.001$ (uncorrected), cluster level $p < 0.05$ (FWE-corrected), no covariates). See also Table I for more information on cluster significance values and peak *Z*-scores.

**Table 1:** Baseline corrected CBF-increases in participants receiving intranasal insulin compared to participants receiving placebo in all five post-intervention measurement-blocks, corresponding to a time interval of 30-65 minutes after the pharmacological manipulation. Table shows local maxima more than 8.0 mm apart and corresponds to Figure 4 (normalized contrast vector of the interaction 'insulin'*'measurement-block' contrasting the post-intervention measurement-blocks 1 to 5 with the baseline block and participants receiving insulin with participants receiving placebo, employed in a full-factorial 4('group')*6('measurement-block') model, peak level $p < 0.001$ uncorrected, cluster level $p < 0.05$ (FWE-corrected), no covariates).

| **Anatomical Region**[1] | **Cluster-Level** | | **Peak-Level** | | | |
|---|---|---|---|---|---|---|
| | $P_{FWEcorr}$[2] | $k_E$[3] | Z-Value | MNI[4] Coordinates (mm) | | |
| | | | | X | Y | Z |
| **Insula and opercular part of inferior frontal gyrus, left** | **0.01** | **98** | **4.69** | **-37** | **14** | **4** |
| | | | 3.95 | -46 | 0 | 4 |
| | | | 3.39 | -46 | 10 | 4 |
| **Putamen and caudate nucleus, left** | **0.03** | **69** | **4.30** | **-26** | **7** | **4** |
| | | | 4.20 | -10 | 19 | 4 |
| | | | 3.37 | -17 | 14 | -5 |
| **Insula and putamen, opercular part of inferior frontal gyrus, right** | **0.02** | **81** | **3.85** | **41** | **-2** | **4** |
| | | | 3.51 | 37 | 5 | 4 |
| | | | 3.42 | 46 | 10 | 4 |

[1]based on the AAL template implemented in MRIcron, [2]familywise error corrected value for cluster, [3]number of active voxels per cluster, [4]Montreal Neurological Institute

## 2.4 Discussion

The aim of the present experiment was to assess the effect of single and combined administration of intranasal insulin and oral cortisol on neuronal activity of the brain. Our main purpose was to investigate the impact of both substances on rCBF in the insular cortex, a region containing the primary gustatory cortex in humans and crucially involved in the processing of gustatory information and interoceptive signals from the gastrointestinal tract. Changes in rCBF in the insular cortex were contrasted with blood flow values in the primary visual cortex, a region proven to be unaffected by intranasal insulin in previous research (Grichisch, et al., 2012). We observed a bilateral increase in rCBF in the insular cortex 37 to 58 minutes after intranasal insulin administration. As expected, no effect of insulin in the primary visual cortex was found. Also, we did not observe an interaction of insulin × cortisol in the primary or the control ROI, indicating that central insulin pathways in the insular cortex are not modulated by cortisol at rest.

Cerebral blood flow is linked to neuronal signaling presumably due to increased energy demand of active neurons which in turn leads to vasodilatation around the active area (Attwell & Iadecola, 2002). Hence, by assessing rCBF, an indirect inference of neuronal activity in a certain brain area can be made. In our study we used CASL-sequences, which allow the quantification of rCBF in absolute physiological values in ml/100g brain tissue/minute. In CASL, cerebral perfusion is estimated using magnetically labeled blood as an endogenous tracer by subtracting an image with magnetically labeled blood from an image without labeling (Alsop & Detre, 1996; Hermes, et al., 2007). This is a major advantage compared to positron emission tomography (PET), which exposes participants to radioactively labeled tracer substances. Also, unlike in PET, the endogenous tracer used in CASL has a very short decay rate (it relaxes with the $T_1$ of arterial blood) which allows a fast repetition of the measurement. Therefore, CASL presents a noninvasive, precise quantification of rCBF in short intervals over a long period of time, making it an ideal method to assess changes in cerebral blood flow following a pharmacological manipulation.

In our experiment, insulin was administered intranasally. Compared to the peripheral administration, the intranasal route of insulin application has the advantage of impacting neither peripheral blood glucose levels (J. Born, et al., 2002) nor peripheral insulin levels (Kern, et al., 1999). Intranasal application of insulin is, therefore, the method of choice to investigate insulin effects on the brain. The oral cortisol administration enabled a fast and safe elevation of blood cortisol levels, leading to an elevated steady-state-like cortisol level.

Although the oral route of cortisol administration does not allow distinguishing between fast, nongenomic and slow, genomic cortisol effects, it has, compared to an intravenous (IV) application, the major advantage of not being invasive and stressful by itself.

The current experiment is the first study showing a direct impact of central insulin on rCBF in the insular cortex in a double-blind placebo controlled experiment. The impact of intranasal insulin on rCBF in the insular cortex is functionally plausible. The insular cortex contains the primary gustatory and visceral cortex in humans (Frey & Petrides, 1999; Small, 2010; Veldhuizen et al., 2011). Activity in the insular cortex is known to be modulated by subjective appetite ratings (Porubska, Veit, Preissl, Fritsche, & Birbaumer, 2006) and seems to be strongly related to eating behavior (Stoeckel et al., 2008; Wagner et al., 2008). We are not the first to report an association between insulin and neuronal functioning in the insular cortex. In rats, insulin was found to facilitate repetitive spike firing in the insular cortex, which is in line with our result (Takei, et al., 2010). Moreover, Tataranni and colleagues (1999) found an inverse correlation between rCBF in the insular cortex and blood plasma insulin levels after food intake in humans. Although this result also emphasizes a functional connection between insulin and rCBF in the insular cortex, it contrasts with our finding of increased rCBF after intranasal insulin administration. Since the insular cortex is characterized by high insulin receptor density (Schulingkamp, et al., 2000) it may be speculated that a direct receptor-mediated mechanism is responsible for the observed increase in perfusion. However, the insula has functional connections to other central and peripheral regions and is highly sensitive to visceral feedback. Therefore, something other than a direct receptor mediated mechanism may underlie the observed effect. This could explain the discrepancy with other studies, such as Tataranni (1999), who found a negative association of plasma insulin levels and insular perfusion during a postprandial state. The experiment presented here is neither able to clarify the mechanisms behind the described effect nor its functional implications, and therefore further research is necessary to clarify the opposite results. However, the experimental setting used by Tataranni and colleagues (1999) differs from our in several aspects such as the time of fasting before the experiment. Hence, the results of both studies might not be directly comparable.

Aside from the intranasal application, effects of systemically administered insulin on neuronal functioning have also been investigated. Kennan and colleagues (2005) administered IV insulin in order to reach a controlled hypoglycemic state and reported increased rCBF in the motor cortex after insulin administration. Although the effect was observed in a different

brain area and insulin was administered systemically, it parallels our finding in that insulin increases blood flow in the human cortex. Seaquist and colleagues (2007) examined the effect of IV insulin application on the fMRI blood-oxygen-level dependent (BOLD) signal in the visual cortex and reported a lower BOLD response in the high insulin state, but no effect of insulin on a P100 visually evoked potential. The authors suggest that this effect could be due to an unspecific effect of insulin on blood vessels resulting in a reduced BOLD signal. In the periphery, insulin is known to enhance blood flow in muscle tissue (Baron et al., 1995). Most recently, intranasal insulin has been shown to not affect rCBF or the BOLD signal in the primary visual cortex (Grichisch, et al., 2012) and to increase the cerebral high-energy phosphate content (Jauch-Chara, et al., 2012). We therefore suggest that the increased blood flow in the insular cortex and putamen observed in our study reflect locally distinct flow enhancements due to an increase in neuronal activity.

In addition to the results observed in the insular cortex, effects of insulin on rCBF were also evident in the putamen. Intranasal insulin increased rCBF in the putamen in all but the fifth post-intervention measurement-block, corresponding to the time period of 30 to 58 minutes after insulin administration. Like the insular cortex the putamen shows increased rCBF in a hungry compared to a satiated state (Tataranni, et al., 1999) and is positively modulated by subjective appetite (Porubska, et al., 2006). Although effects of insulin on hippocampus dependent declarative memory have often been reported (Benedict, et al., 2007) we did not observe an effect of insulin on rCBF in the hippocampus. This suggests that either insulin effects on memory are not reflected in a fast change in rCBF in the hippocampus, or that these changes are context dependent and task-specific.

The voxel-based analysis revealed that the insulin induced CBF increases were not restricted to the insular cortex and the putamen, but extended into the opercular part of the inferior frontal gyrus and into the caudate nucleus. Interestingly, like the insular cortex, the frontal operculum is considered being a part of the human gustatory cortex (Veldhuizen, et al., 2011). Additionally, both structures are connected to each other. Therefore, the result of our voxel-based analysis suggests that insulin affects different regions in a cortical network involved in the perception of gustatory information. As the voxel based exploration covered all five post-intervention measurement-blocks in one contrast and CBF increases after insulin intake were visible for the entire period, it is possible that insulin effects on the brain might start as early as 30 minutes after insulin admission and last until 65 minutes after. The timespan of insulin

effects observed in the ROI analysis of 37-58 minutes after insulin admission might, therefore, represent the peak time of insulin effects in the brain.

Since participants were restricted from eating from 10 pm of the previous day if scanned in the morning or from 8 am if scanned in the afternoon, a possible explanation of the increased blood flow in the insular cortex and the putamen could be different levels of hunger or distress. However, participants of all four groups did not differ regarding their hunger or subjective mood feelings. We can therefore exclude different levels of hunger, stress or arousal as a possible explanation for the differences in rCBF observed in our study. The lack of differences in hunger ratings is according to previous research where hunger was assessed after acute application of intranasal insulin (Benedict, Kern, Schultes, Born, & Hallschmid, 2008; Hallschmid et al., 2004; Jauch-Chara, et al., 2012). Although intranasal insulin provokes a decrease in food intake in men, hunger ratings do not seem to reflect this tendency, suggesting that the acute anorexigenic effects of central insulin are mediated by satiation signals but not by motivation to eat or a conscious behavioral process (Benedict, et al., 2008).

Intranasal insulin effects on rCBF in the insular cortex were present regardless of whether participants received oral cortisol or oral placebo. This may emphasize the fact that insulin dependent pathways in the human insular cortex are not influenced by circulating cortisol. In line with this, a recent study showed that while stress modulates orbitofrontal cortex activity in response to highly palatable foods, an insular cortex response to this kind of stimuli was present independent of stress (Rudenga, Sinha, & Small, 2012). Nevertheless, since we administered oral cortisol in order to reach steady-state cortisol plasma levels, we cannot exclude the possibility that fast, nongenomic cortisol effects would not affect insulin dependent signal pathways. Direct IV administration of cortisol into the human blood exerts nongenomic effects on the human brain, as previously described by our group (Strelzyk, et al., 2012), and therefore could also interact with the effects of intranasal insulin.

An interaction between insulin and cortisol is suggested to play a role in the development of the metabolic syndrome and obesity. Intranasal insulin has been shown to reduce the hypothalamic-pituitary-adrenalcortical (HPA) axis response to psychosocial stress in healthy individuals (Bohringer, et al., 2008). The metabolic syndrome is characterized by an increased HPA-axis reactivity to an oral glucose tolerance test and indices of central adiposity are negatively associated with the cortisol and adrenocorticotropic hormone (ACTH) responses to

dexamethasone (Kazakou et al., 2012; Tyrka, Walters, Price, Anderson, & Carpenter, 2012). Also, obese men were found to show reduced cerebral insulin suppression to psychosocial stress (Kubera et al., 2012). Furthermore, there is accumulating evidence that Alzheimer`s disease is accompanied by central insulin resistance (Craft, 2005), and a positive association between blood and CSF cortisol levels and the severity and progression of Alzheimer`s disease has been reported (Czech et al., 2012; Davis et al., 1986; Laske, Stransky, Fritsche, Eschweiler, & Leyhe, 2009; Popp et al., 2009). We did not find any interaction between insulin and cortisol in any ROI investigated. Therefore, on a basic physiological level, insulin dependent signal pathways were not modulated by genomic cortisol effects in the present study. Nevertheless, an interaction between both hormones associated with the development of the above mentioned disorders may not be reflected in changes in rCBF since such an interaction could emerge in a much longer timeframe, on a molecular level, or could be seen only during specific tasks.

We observed increased rCBF in the insular cortex 37 to 58 minutes after intranasal insulin administration. This effect was independent of whether participants received oral cortisol or not. Our results indicate that insulin plays a central role in metabolism by modulating effects in the gustatory centers. However, this impact seems to not be affected by glucocorticoids, which might come into play only during cognitive tasks and through different pathways.

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## 2.ii Author Notes – Chapter II

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# RAPID, NONGENOMIC EFFECTS OF CORTISOL

# 3. For Whom the Bell (Curve) Tolls: Cortisol Rapidly Affects Memory Retrieval by an Inverted U-shaped Dose-Response Relationship

*Schilling, Kölsch, Larra, Zech, Blumenthal, Frings, Schächinger (2013).*

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## 3.0 Abstract

Stress and cortisol are generally considered to impair declarative memory retrieval, although opposite results have also been reported. Dose-dependent effects and differences between genomic and nongenomic cortisol effects are possible reasons for these discrepancies. The aim of the current experiment was to assess the nongenomic effects of escalating doses of intravenous cortisol on cued recall of socially relevant information in humans. 40 participants (age range 20-30 years; 20 females) learned associations between male faces with a neutral facial expression and descriptions of either positive or negative social behaviors and were tested one week later in a cued recall paradigm. Escalating doses of cortisol (0, 3, 6, 12, 24mg) were administered 8 minutes before testing according to a between-subjects design. An inverted U-shaped dose-response relationship between salivary cortisol levels and recall performance was observed, with moderate elevation of salivary cortisol resulting in the best recall performance. This is the first study in humans demonstrating that cortisol rapidly modulates declarative memory retrieval via a dose-dependent, nongenomic mechanism that follows an inverted U-shaped curve. Our result further emphasizes the importance of fast cortisol effects for human cognition.

Keywords: cortisol, nongenomic effects, dose-response design, inverted U-shape, declarative memory retrieval, humans

## 3.1 Introduction

Evolution fostered the emergence of neural mechanisms able to voluntarily retrieve behavior-guiding information from our memory. This is of crucial importance during stress, challenging situations, and attack, when different survival strategies and behavioral options need to be rapidly evaluated on the basis of their previous failure and success. Given that human stress often evolves in social context (Dickerson & Kemeny, 2004), information on the past social behavior of others will become useful for predicting their future behavior in upcoming distress and conflict. Indeed, stress was found to support the discrimination of potential allies and helpers from antagonists and non-helpers (Lass-Hennemann et al., 2010), based on its effects on memory consolidation. However, our current knowledge of the exact influence of stress and stress-hormones on memory retrieval processes is limited.

Acute stress activates the hypothalamus-pituitary-adrenal cortex (HPA) axis, and leads to the release of glucocorticoids (cortisol in humans, corticosterone in rodents) into the blood stream. Cortisol crosses the blood-brain-barrier to act on the central nervous system in order to regulate magnitude and duration of the stress response by negative feedback mechanisms (de Kloet, Joels, & Holsboer, 2005), as well as affecting memory and behavior processes (Schwabe, Schachinger, de Kloet, & Oitzl, 2010).

Cortisol has been shown to impair episodic memory retrieval (de Quervain, Aerni, & Roozendaal, 2007; de Quervain, Roozendaal, & McGaugh, 1998; de Quervain, Roozendaal, Nitsch, McGaugh, & Hock, 2000; Wolf, 2009). However, opposite effects (Domes, Heinrichs, Reichwald, & Hautzinger, 2002; Nater et al., 2007) have also been found, which may in part be due to different dosing and timing (Het, Ramlow, & Wolf, 2005). In particular, time and dose effects of cortisol on memory retrieval may not follow a linear relationship. An inverted U-shaped relationship between salivary cortisol levels and verbal memory performance was observed after oral cortisol administration (Domes, Rothfischer, Reichwald, & Hautzinger, 2005), and would also explain why autobiographic memory retrieval was reduced after a high dose but not after a low dose of IV cortisol (Young, Drevets, Schulkin, & Erickson, 2011). However, more studies are needed to understand the exact dose-response relationship between cortisol and memory retrieval. Such studies should employ dose-escalating designs with more than two different dose steps, to evaluate the possibility of a quadratic relationship between cortisol and retrieval.

Recent findings in rodents show that the influence of cortisol on memory retrieval may be mediated via a fast, nongenomic mechanism in the dorsal hippocampus which does not involve changes in protein expression (Dorey et al., 2011; Schutsky, Ouyang, Castelino, Zhang, & Thomas, 2011). However, the effects of cortisol on memory retrieval in humans have so far only been studied at longer time intervals, making it impossible to distinguish whether the effects have been mediated by a genomic or a nongenomic mechanism. This is very important, because genomic and nongenomic mechanisms may lead to different effects. For example, dissociations between "slow" genomic and "fast" nongenomic cortisol effects have been described in trace eyeblink conditioning (tEBC). High cortisol is associated with reduced tEBC (Grillon, Smith, Haynos, & Nieman, 2004; Vythilingam et al., 2006), and low cortisol is associated with enhanced tEBC (Nees, Richter, Lass-Hennemann, Blumenthal, & Schachinger, 2008; Nees et al., 2010). This dose-response relationship is found hours after cortisol manipulation or in states of chronic hypo- or hypercortisolism, allowing these effects to be mediated by genomic mechanisms. However, enhanced speed of tEBC acquisition was found on a nongenomic timescale (Kuehl et al., 2010), shortly after cortisol administration, suggesting opposite effects of cortisol on tEBC via a genomic vs. a nongenomic mechanism. tEBC requires awareness of the CS-US relationship and, like episodic memory formation, it depends on an intact hippocampus (Clark & Squire, 1998; Squire, 2004). It is therefore possible that such discrepancy between fast and slow cortisol effects might also be present in episodic memory retrieval. It has already been shown that excess cortisol may impair episodic memory retrieval when following a genomic timescale (de Quervain, et al., 2007; de Quervain, et al., 2000). Assuming analogy to the cortisol effects on tEBC, supportive effects of cortisol on episodic memory retrieval might be expected when following a nongenomic timescale, but this has never been tested before.

The current study was designed to explore fast effects of exogenous cortisol administration on memory retrieval. Associations between neutral male faces and descriptions of either positive or negative social behaviors were learned one week before cued recall testing. Cortisol was administered intravenously eight minutes before the retrieval test, to exclude effect mediation by genomic mechanisms. In order to study the shape of the dose-response curve, cortisol was given in escalating doses of 3 mg, 6 mg, 12 mg, and 24 mg, with a 0 mg control condition.

## 3.2 Methods

### 3.2.1 Sample

40 human participants (20 male, 20 female) took part in the experiment and received a monetary reward for participation. Exclusion criteria were determined by a physician during a standard medical examination and interview. Exclusion criteria were any acute or chronic somatic or psychiatric illness, any history of psychiatric, cardiovascular, or stress-related disorders, glaucoma, pregnancy, smoking, increased caffeine consumption or any illicit drug intake within the last six months, or any family history of epilepsy or aneurysms. There was no control of menstrual cycle phase, and women were allowed to take oral contraceptives other than those containing drospirenon, because this substance may interact with mineralocorticoid receptors. The German version of the Beck Depression Inventory (BDI) (Hautzinger, Bailer, Worall, & Keller, 1994) was used to screen for heightened depression ratings (BDI cutoff < 11). Additionally, participants had to be free of uneasiness towards medical settings or procedures. Participants gave their informed written consent and were told that they had the right to stop the experiment at any time. The study was approved by the Ethical Committee of the State's Medical Association (Landesärztekammer Rheinland-Pfalz) and was in accordance with the latest revision of the Declaration of Helsinki.

### 3.2.2 Procedure

Participants came to the laboratory twice, with one week time between visits. The first appointment lasted one hour and took place in the morning. The second lasted four hours and started after 1200 h. On the first appointment participants were seated in a comfortable chair in front of a computer screen. Next, pictures of male faces with a neutral facial expression and a description of the person were presented on a computer screen one at a time. The participant's task was to learn the association between the face and the description of the person. The description could either be a positive social behavior (example: 'he can easily cheer up other people'), a negative social behavior (example: 'he likes to get drunk at parties and then becomes aggressive') or a neutral, non-socially related behavior (example: 'he likes to listen to music). Altogether, 18 faces were presented, six with positive and six with negative social behaviors and six with neutral, non-socially related behaviors. Faces with neutral descriptions were included as fillers to increase the difficulty of the learning procedure but were not included in further statistical analysis. In the first learning cycle, each picture with the corresponding description was presented once with the instruction to remember the

association. From the second learning cycle onwards, each picture was presented alone without the description and participants were asked to recall the corresponding description. Then, irrespective of whether participants answered correctly or not the description was presented again before showing the next picture. Each picture and the corresponding description were presented in as many learning cycles as needed until the participant had correctly recalled the association in two adjacent learning cycles. After that, the picture was not shown again in the following learning cycles. Participants needed a minimum of three learning cycles to learn and recall an association up to the defined criterion.

At the beginning of the second appointment (after one week) a flexible intravenous catheter was inserted in the left arm of the participant. Next, participants were brought back to a waiting room to have a 45 minute resting period. This was done to avoid carry-over stress effects from the insertion of the needle. After the resting period, participants were seated in a comfortable chair in front of a computer screen in the experimental room and the flexible intravenous catheter was connected to a syringe pump. Hereafter, participants received the pharmacological manipulation (see next section for more details). Total time of the infusion was about two minutes. The infusion was followed by a six minute break before starting the recall test, to allow cortisol to diffuse in the whole body and to access the brain. The recall test started 8 minutes after the start of the cortisol infusion and lasted until 14 minutes after. During the recall test the six faces combined with a positive description on the first appointment and the six faces combined with a negative description on the first appointment were presented alone one after the other without their corresponding description. Participants were asked to recall the corresponding description of each face presented.

One hour later, when disconnected from the syringe pump, faces presented during the recall test and six additional new faces were presented without any description and participants were asked to rate the faces on an investigator-constructed sympathy scale (visual-analog scale with the anchors ‘very unsympathetic’ vs. ‘very sympathetic’). This was done to assess whether the valence manipulation of the associations was successful. After that, the experiment was ended, and participants received a monetary compensation. They were shortly debriefed and were told which pharmacological manipulation they had received during the experiment.

### 3.2.3 Materials and Apparatus

#### *3.2.3.1 Pharmaceutical Materials*

All pharmacological manipulations were applied in a single blinded fashion using a flexible intravenous catheter (Vasofix® Braunüle®, Braun, Melsungen, Germany) connected to a syringe pump (Infusomat® fm, Braun, Melsungen, Germany). Participants either received a saline placebo solution (NaCl 0.9%, Braun, Melsungen, Germany) or 3 mg, 6 mg, 12 mg, or 24 mg of hydrocortisone (Hydrocortison 100 mg, Rotexmedia, Trittau, Germany). Participants were randomly assigned to a group, resulting in eight participants per group with an equal number of males and females in each group.

#### *3.2.3.2 Manipulation Check*

Before and after the recall test four saliva samples were taken with devices for saliva sampling (Salivettes®, Sarstedt, Nümbrecht, Germany). Samples were taken at time intervals of -15, 0, +15, and +30 minutes relative to the cortisol application.

#### *3.2.3.3 Stimulus Material and Presentation*

Descriptions of positive and negative social behaviors were taken from a standardized sample of descriptions of social behaviors in the German language (Ehrenberg, Cataldegirmen, & Klauer, 2001). Neutral descriptions that served as fillers during the learning phase were specifically designed for the current study by the authors. Pictures of male faces with neutral expressions were taken from the CIE Biometrics database (https://biometrics.cie.put.poznan.pl/) and were standardized in terms of background color, cloth color and size. E-Prime 2.0 (PST Software, Inc) was used to present pictures and text on the computer screen, and to store verbal answers of participants and ratings of faces to disk.

### 3.2.4 Data Analysis

#### *3.2.4.1 Salivary Cortisol Levels*

Salivary cortisol levels were analyzed by a time resolved immunoassay with fluorescence detection (intra-assay coefficient of variation: 4.0-6.7%; inter-assay coefficients of variation: 7.1-9.0%) (Dressendorfer, Kirschbaum, Rohde, Stahl, & Strasburger, 1992). Cortisol levels were determined twice, and their average was used in further statistical analysis.

#### *3.2.4.2 Learning Performance*

The number of faces with their corresponding descriptions presented during the learning phase was counted separately for each learning cycle and valence (positive. vs. negative descriptions).

#### *3.2.4.3 Recall Performance*

Verbal answers of participants on the recall test were stored and analyzed offline by two independent researchers. Correctly recalled descriptions were scored with one point. The number of correctly recalled descriptions was identified for positive and negative correct descriptions, and their sum.

#### *3.2.4.4 Rating of Sympathy*

Sympathy ratings of faces with positive descriptions and sympathy ratings of faces with negative descriptions were averaged separately according to their valence.

### 3.2.5 Statistical Analysis

Regression models were separately calculated for each dependent variable. Participants were sorted in the between-subjects factor dose ‘group’ with the five factor levels ‘placebo’ (= 0 mg of cortisol), ‘3 mg of cortisol’, ‘6 mg of cortisol’, ’12 mg of cortisol’, and ’24 mg of cortisol’ according to the pharmacological manipulation that they had received. A within-subject factor ‘valence’ with the two factor levels ‘faces with positive descriptions’ (positive) and ‘faces with negative descriptions’ (negative) was entered in the models with the dependent variables ‘learning performance’, ‘recall performance’, and ‘rating of sympathy’. A with-in subject factor ‘learning cycle’ with the eight factor levels ‘learning cycle 3 to 10’ was entered in the model of the dependent variable ‘learning performance’ additionally to the other factors. All statistical analyses were done using SPSS 19 and SAS (Version 9), with a critical α-level of 0.05 for each model.

#### *3.2.5.1 Manipulation Check*

The success of the pharmacological cortisol manipulation was assessed using the two baseline saliva samples acquired before IV cortisol administration, and a saliva sample acquired directly after the recall test (15 minutes after IV cortisol administration). The average of the two baseline values was subtracted from the post-intervention value for each individual, resulting in a cortisol difference score. These scores were then compared between adjacent dose steps by non-parametric *two-sample* Wilcoxon rank-sum test (two-sided).

#### *3.2.5.2 Learning Performance*

In order to control for general performance differences between dose groups during the learning phase, which could potentially bias the results of the recall performance independently of the pharmacological manipulation, a 5 (‘group’) X 2 (‘valence’) X 8 (‘learning cycle’) mixed design ANOVA was conducted for the dependent variable ‘learning performance’.

#### *3.2.5.3 Recall Performance*

A quadratic relationship between log-transformed salivary cortisol concentration and cued memory recall was predicted. A quadratic least squares regression model including linear and quadratic predictors was used to test for the presence of a quadratic trend component. A further regression model including a linear predictor only was used to test for the presence of a linear component. In a first step, this was done for overall memory performance, independent of whether the recalled associations differed in positively vs. negatively valenced descriptions. However, after identifying the model (linear vs. quadratic) explaining most of the variance in cued memory recall, the regression model was extended by including valence as a two-level, repeated, within-subject factor.

#### *3.2.5.4 Rating of Sympathy*

A 5 (‘group’) X 2 (‘valence’) mixed design ANOVA for the dependent variable ‘rating of sympathy’ was calculated to assess whether the amount of cortisol given and the original valence of the description influenced the perceived sympathy of the faces.

## 3.3. Results

### 3.3.1 Sample Characteristics

The age range of the participants was 20-30 years (mean age: 23.1 years). The BMI range was 19.5-26.5 kg/m$^2$ (mean BMI: 21.9 kg/m$^2$). There were no differences between dosing groups in age, BMI or depression (BDI) scores.

### 3.3.2 Manipulation Check

Salivary cortisol data 15 min after IV cortisol administration for each of the dosing groups are illustrated in Figure 5 (lower panel B), indicating an almost linear relationship between cortisol IV dose and resulting salivary cortisol concentration in the log-transformed space. *Two-sample* Wilcoxon rank sum tests revealed significant differences between baseline corrected salivary cortisol measures 15 minutes after infusion for all successive cortisol IV dosing groups (0 vs. 3 mg: $Z = 2.47$, $p = 0.014$; 3 vs. 6 mg: $Z = 3.0$, $p = 0.003$; 6 vs. 12 mg: $Z = 2.78$, $p = 0.005$; 12 vs. 24 mg: $Z = 2.36$, $p = 0.018$).

### 3.3.3 Learning Performance

All participants learned the associations within 10 learning cycles. The percentage of correctly learned descriptions increased with learning cycles ($F$ [7:245] = 339.37; $p < 0.0001$). There was neither a main effect of 'group' ($F$ [4:35] = 1.37; $p = .26$) nor a main effect of 'valence' ($F$ [1:35] = .054; $p = .82$), nor any interaction between 'group' X 'valence' ($F$ [4:35] = 1.083; $p = .380$), 'group' X 'learning cycle' ($F$ [28:245] = 1.080; $p = .36$), or 'group' X 'valence' X 'learning cycle' ($F$ [28:245] = .70; $p = .87$) (see Figure 6).

### 3.3.4 Recall Performance

A quadratic regression model including a quadratic and a linear term explained ($R^2$) 27.8 % of the variance in overall memory recall ($F$ [2:37] = 7.11; $p = 0.0024$). A simple linear regression model including a linear term, only, explained less than 0.6 % of the variance in overall memory recall, and was statistically not significant ($F$ [1:38] = 0.22; $p = 0.64$). Thus, the relationship between log-transformed salivary cortisol concentration and cued memory recall can be described as being quadratic. Parameter estimation [prediction (x) = $-6.523\ x^2 + 40.528\ x - 16.082$; where x = salivary cortisol concentration; see Figure 5, part A, dotted line]

and visual inspection (see Figure 5, upper panel A) reveal a quadratic, inverted U-shaped configuration. Valence of the recalled associations did not play any role, since extending the regression model by a valence within-subject factor did not indicate significant interactions of this factor with any of the polynomial terms ($F$ [1:37] < 0.11; $p$ > 0.75).

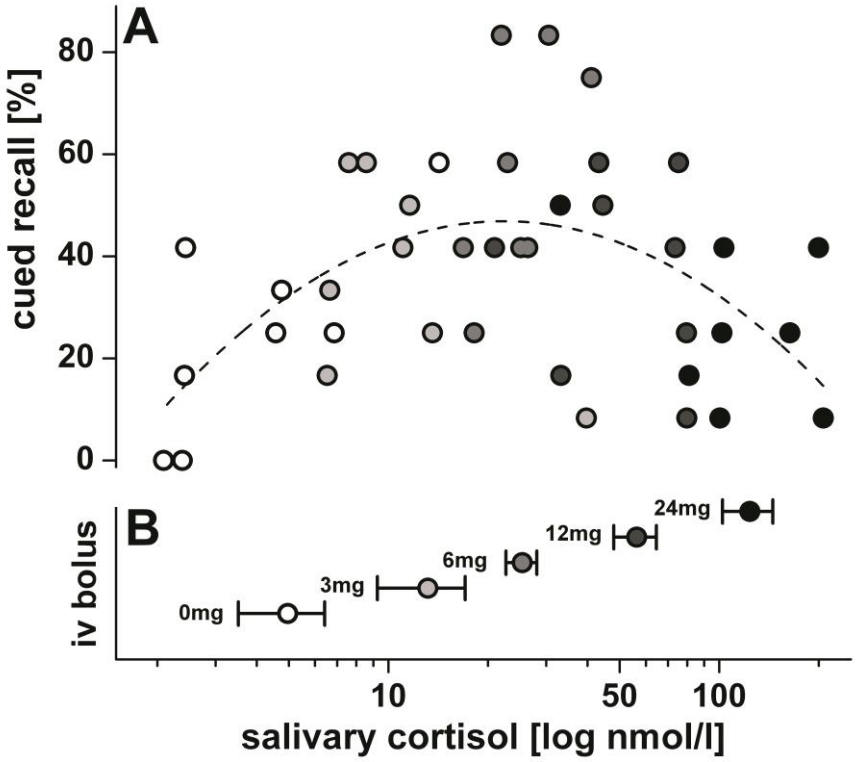


**Figure 5:** Part A represents a plot of log-transformed salivary cortisol (x axis) and cued memory recall performance (y axis), indicating an inverted U-shaped relationship between the two variables. Circles represent data of single individuals, whereby intensity of grey-scale filling of the circles represents increasing IV cortisol dose steps. The dotted line illustrates the quadratic prediction. Part B indicates increasing salivary cortisol concentration after escalating IV cortisol doses in the log-transformed space. Circles represent group mean ± SEM. Grey-scale level of symbols informs about the IV dose.

### 3.3.5 Rating of Sympathy

We neither observed a main effect 'group' ($F$ [4:30] = .735; $p$ = .58) nor an interaction 'group' X 'valence' ($F$ [4:30] = .224; $p$ = .92) for the dependent variable 'rating of sympathy'. However, a main effect of 'valence' was present ($F$ [1:30] = 29.710, $p$ < .0001), indicating that faces formerly associated with positive descriptions were now rated higher on sympathy compared to faces formerly associated with negative descriptions.

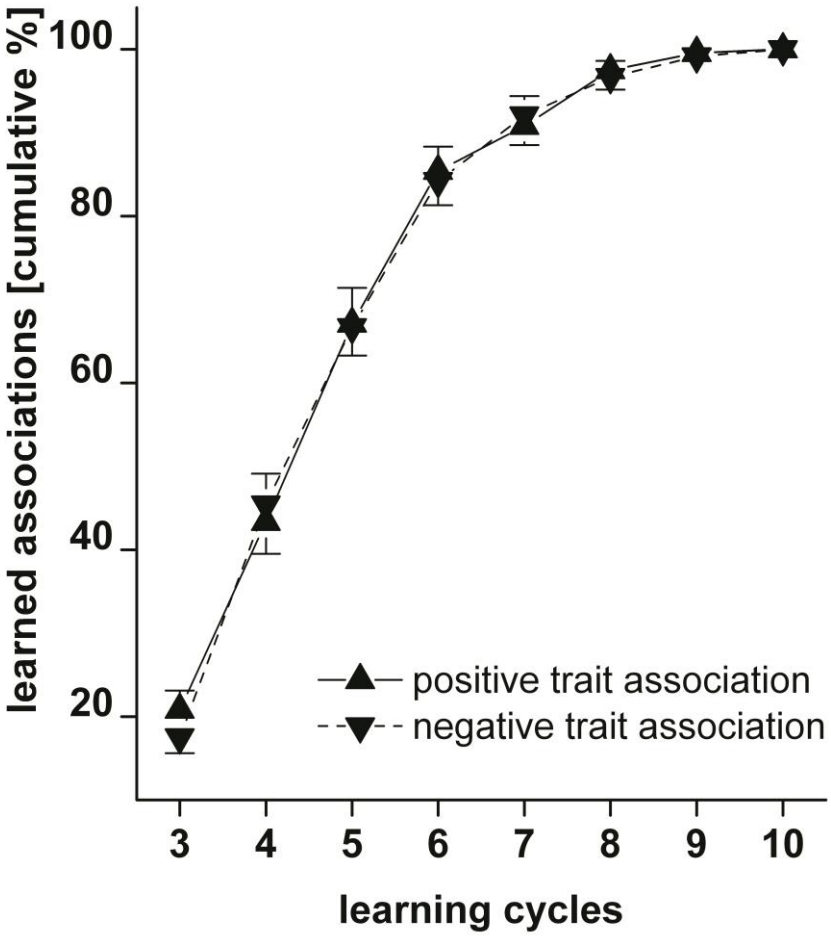


**Figure 6:** Mean ± SEM of the percentage of associations correctly recalled for the second time in adjacent learning cycles during the learning phase separated for learning cycle (cycle 3 - 10) and the valence of the description (positive, negative).

## 3.4. Discussion

The aim of the current study was to analyze rapid effects of escalating cortisol doses on declarative memory retrieval in humans. The time interval between cortisol IV infusion and memory recall testing was shorter than 15 minutes, so any effect cannot be attributed to its genomic actions on gene regulation and protein synthesis. The main finding is that cortisol affected memory retrieval, following an inverted U-shaped function, with the best memory recall in participants exhibiting moderate salivary cortisol levels, in comparison to participants exhibiting lowest and highest salivary cortisol levels. Furthermore, we demonstrate that the cortisol IV infusion was successful, and that participants showed increasing salivary cortisol levels according to the amount of cortisol that they had received.

The results of this experiment cannot be explained by cortisol effects on other memory processes, such as acquisition or memory consolidation, since memory recall testing was scheduled one week after learning. There were no differences in learning between participants of different cortisol dose groups. Hence, we can rule out preexisting memory performance differences between the dose groups. There were also no differences in learning positively and negatively valenced descriptions. Sympathy ratings of the faces at the end of the experiment varied with the original valence of the description, indicating that the emotional valence manipulation of the faces was sustainable; however, again the different dose groups showed no difference with respect to the sympathy ratings.

This is the first study in humans specifically designed to explore fast cortisol effects on memory retrieval. In contrast to earlier studies it involved five cortisol dose steps ranging from 0 to 24 mg. Earlier dose-response studies on slow cortisol effects on memory retrieval only used two or three dosages of cortisol (Domes, et al., 2005; Young, et al., 2011), making it difficult to statistically evaluate a quadratic dose-response relationship. Further, the cortisol dosages used in earlier studies were comparatively high (Young, et al., 2011), sometimes exceeding a physiologically plausible range. Salivary cortisol levels observed in our study in participants receiving 3 or 6 mg of cortisol were similar to those found after a moderate psychosocial stress test or during the cortisol awakening response in the morning (Kirschbaum, Pirke, & Hellhammer, 1993; Nater, et al., 2007; Wust et al., 2000). Participants receiving 12 or 24 mg of cortisol exhibited salivary cortisol levels which would be expected during severe stress or after excessive physical activities such as marathon running (Kirschbaum & Hellhammer, 1994). Cortisol levels in our study were therefore within the physiological plausible range.

Although dose-response studies of cortisol on human cognition are still comparatively rare, the results of our experiment and of other findings strongly suggest an inverted U-shaped relationship between cortisol and different cognitive functions in humans. It was recently demonstrated that participants receiving 10 mg, but not 40 mg, of cortisol 60 minutes before testing showed increased inhibition of angry faces in a negative priming task as evidenced in increased reaction times in negative priming test trials (Taylor, Ellenbogen, Washburn, & Joober, 2011). An inverted U-shaped relationship might also explain the apparent ambiguity in the literature concerning cortisol effects on memory retrieval. The results of our study are supported by another experiment in humans. Domes and colleagues (2005) report on an inverted U-shaped relationship between cortisol and memory retrieval 45 minutes after administration of 30 mg of cortisol. In their study participants with low salivary cortisol levels showed increased memory retrieval compared to participants with high salivary cortisol levels and placebo, while no difference between high salivary cortisol and placebo was observed. Strikingly, the low (30.0 nmol) and high (136.9 nmol) salivary cortisol response levels reported in that study were comparable to those observed in participants of our study receiving 6 mg (25.4 nmol), or 24 mg (123.7 nmol) of cortisol, respectively. The similarity in dose and effect between the study of Domes and colleagues and our results might indicate that cortisol has a similar impact on declarative memory retrieval via fast and slow mechanisms. However, it should be kept in mind that nongenomic effects do not necessarily cease after 15 minutes but might still be in action 45 minutes after cortisol administration, due to the plasma half-life of cortisol of approximately 60 minutes (Weitzman et al., 1971). Hence, while it is possible to exclude genomic effects in the first 15 minutes after cortisol administration, it is impossible to distinguish between the two mechanisms at a later stage.

Fast effects of cortisol on memory retrieval have also been found in rodents (Chauveau et al., 2010; Dorey, et al., 2011; Schutsky, et al., 2011), allowing for the investigation of the underlying mechanisms. Nongenomic corticosterone effects on memory retrieval are most likely mediated via mineralocorticoid receptors (MRs) located at the extracellular side of neuronal cell membranes (Dorey, et al., 2011; Karst et al., 2005). Nongenomic mechanism are responsible for increased glutamatergic signal transmission in hippocampal CA1 neurons (Karst, et al., 2005), at both presynaptic and postsynaptic receptors (Olijslagers et al., 2008). It was recently observed that corticosteroids also rapidly enhance glutamatergic transmission in the basolateral amygdala via membrane based MRs (Karst, Berger, Erdmann, Schutz, & Joels, 2010). Membrane based MRs have a ten-fold lower affinity for glucocorticoids compared to

intracellular MRs, suggesting that, in contrast to the latter, they are not occupied during rest (Joels, Karst, DeRijk, & de Kloet, 2008). It is therefore assumed that, after the release of glucocorticoids during stress, membrane bound MR receptors quickly become activated. However, the exact neurobiological mechanisms mediating the reported effects in humans will be identified when pharmacological methods become available to selectively block or stimulate membrane-bound brain cortisol receptors "in vivo".

Emotional material of positive and negative valence tends to be better remembered than neutral material, due to arousal effects on memory consolidation (Cahill, Gorski, & Le, 2003), but is also more susceptible to the cortisol induced modulations of memory retrieval (Kuhlmann, Kirschbaum, & Wolf, 2005; Kuhlmann, Piel, & Wolf, 2005). It is assumed that simultaneous activity of cortisol and arousal-induced norepinephrine in the amygdala is necessary for stress effects on memory to occur (Roozendaal, McEwen, & Chattarji, 2009). In fact, stress and cortisol effects on memory retrieval of emotional materials can be blocked by administration of the centrally acting β-blocker propranolol (de Quervain, et al., 2007; Schwabe et al., 2009). The stimuli used in our study were emotionally relevant. They consisted of male faces with a neutral facial expression paired with descriptions of positive or negative social behaviors. Faces and socially relevant information are arousing, and have repeatedly been shown to activate brain structures processing emotionally relevant information, such as the amygdala (Adolphs, 2010; Fusar-Poli et al., 2009). It may therefore be assumed that arousal induced by the descriptions and faces supported the consolidation of stimuli used in our study. Thus, our results may not apply to poorly consolidated materials, such as associations between non-arousing, boring materials. Furthermore, we did not find any differences in the cortisol effect on the recall of emotionally positively vs. negatively valenced associations of faces and descriptions. Information about valence was clearly available, and appeared to have been acquired by different memory systems, as indicated by the performance in the "explicit" declarative recall test, as well as the rather "implicit" sympathy ratings of the faces at the very end of the study. Hence, emotional valence may not play a role in the recall effects of cortisol on socially relevant associations between faces and descriptions, suggesting that information about "friend and foe" is equally in favor during stress and danger.

Rapid cortisol effects in humans have been found to extend well beyond memory processes. Cortisol has been shown to rapidly affect brain function and regional hemodynamics (Strelzyk et al., 2012; Symonds, McKie, Elliott, William Deakin, & Anderson, 2012), sensorimotor

gating (Richter et al., 2011), and eye blink conditioning (Kuehl, et al., 2010), as well as peripheral mechanisms, such as initial insulin responses to intravenous glucose (Vila et al., 2010). Our study therefore adds to the growing evidence that cortisol exerts rapid, nongenomic effects on the human brain, presumably in order to swiftly promote adaptive behavior in face of threat and stress. Supported by nongenomic action of cortisol, declarative memory information may be more easily retrievable during the early course of a stressful event. Later on, as well as in higher doses, this cortisol effect may vanish or even reverse. Since consciously retrievable information is processed by cognitive mechanisms rather recently refined in primate evolution, cortisol action seem to guarantee that latest phylogenetic-developed cognitive strategies are recruited to cope with challenge during mild to moderate stress levels.

Several limitations need to be acknowledged. This study does not have enough power to identify potential sex differences, nor can this study rule out that such effects may exist. Future studies should try to explore whether this factor may play a role in rapid cortisol effects on memory retrieval. Furthermore, all subjects studied here were healthy, young and had successfully passed the many tests and examinations offered by the highest level of the German school education system. Different results may be found in older subjects, groups of patients, or subjects less experienced in test situations or school-like examinations. Although the intravenous cannulas were inserted almost one hour before starting the experiment, and subjects were neither aware of the exact time of cortisol/placebo injection nor the dose they received, the fact of the intravenous manipulation itself may have increased subjects' arousal level. We therefore assume that the described findings are limited to situations of mildly to moderately increased arousal levels.

## Conclusion

Cued recall of socially relevant memory is modulated by physiologically plausible cortisol levels via a fast, nongenomic mechanism following an inverted U-shaped curve. Cortisol therefore seems to act dose-dependently and rapidly on human memory retrieval, presumably via a membrane receptor based mechanism. Our result further emphasizes the importance of fast cortisol effects for human cognition.

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## 3.ii Author Notes – Chapter III

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# 4. Rapid Cortisol Enhancement of Psychomotor and Startle Reactions to Side-Congruent Stimuli in a Focused Cross-Modal Choice Reaction Time Paradigm

*Schilling, Larra, Deuter, Blumenthal, Schächinger (submitted).*

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## 4.0 Abstract

The stress hormone cortisol has been shown to affect hemodynamic activity of human brain structures, presumably via a nongenomic mechanism. However, behavioral implications of this finding remain unknown. In a placebo-controlled, blinded, cross-over design the rapid effects of IV cortisol (5 mg) on cross-modal integration of simultaneous, unilateral visual and acoustic signals in a challenging startle and reaction time (RT) paradigm were studied. On two separate days one week apart, twenty-four male volunteers responded by button push to either up- or down-pointing triangles presented in random sequence in the periphery of one of the visual hemi-fields. Visual targets were accompanied by unilateral acoustic startle noise bursts, presented at the same or opposite side. Saccadic latency, manual RT, and startle eye blink responses were recorded. Faster manual reactions and increased startle eye blink responses were observed 11-20 minutes after cortisol administration when visual targets and unilateral acoustic startle noises were presented in the same sensory hemi-field, but not when presented in opposite sensory hemi-fields. Our results suggest that a nongenomic, cortisol-sensitive mechanism enhances psychomotor and startle reactions when stimuli occur in the same sensory hemi-field. Such basic cognitive effects of cortisol may serve rapid adaptation and protection against danger stimuli in stressful contexts.

Keywords: multisensory integration, saccades, reaction time, startle-reaction, hydrocortisone, nongenomic

## 4.1 Introduction

One of the most important functions of sensory-integrating systems is to instantaneously indicate whether multiple input from different senses belongs to the same environmental source or not. This task is usually solved by taking into account the spatial and temporal configuration of cross-modal input (Stein & Stanford, 2008), for example, by preferentially processing stimuli that appear simultaneously and at the same spatial position (Komura, Tamura, Uwano, Nishijo, & Ono, 2005; Wallace, Wilkinson, & Stein, 1996). Given that a normal environment is noisy, full of meaningless signals, a co-appearance of spatially congruent multisensory input may "validate" the existence of a real event and object. This is of special importance in hazardous environments, where danger cues indicate the presence of a predator or invader and urge for immediate escape or defensive reactions. Thus, improving cross-modal cue integration, especially during stress and imminent danger, may foster survival.

Stress, threat, and danger activate physiologic stress systems, such as the hypothalamic-pituitary-adrenocortical (HPA) axis (McEwen, 2008). Subsequently, glucocorticoids (cortisol in humans) are released into the bloodstream. Cortisol crosses the blood-brain barrier and acts on brain neurons both via slow, genomic pathways (de Kloet, Joels, & Holsboer, 2005) as well as by rapid, nongenomic mechanisms (Groeneweg, Karst, de Kloet, & Joels, 2011; Joels & Baram, 2009). Our knowledge about the rapid (nongenomic) impact of cortisol on human cognition is still scarce, coming from only a few studies (e.g., Richter et al., 2011; Schilling et al., 2013; van Ast, Cornelisse, Meeter, Joels, & Kindt, 2013), none of which addressed multisensory integration.

Recently, we showed that IV cortisol administration was associated with a rapid decrease in thalamus perfusion, accompanied by reduced EEG power across different frequency bands (Strelzyk et al., 2012). The thalamus plays a prominent role in multisensory processing (Cappe, Rouiller, & Barone, 2009; Tyll, Budinger, & Noesselt, 2011), as evidenced by its anatomical connections (Cappe, Morel, Barone, & Rouiller, 2009) and its functional involvement in cross-modal task performance (Komura, et al., 2005; Noesselt et al., 2010; van den Brink et al., 2013). Furthermore, and in line with the effect on thalamic blood flow, glucocorticoids are known to increase sensory thresholds (Born, Kern, Fehm-Wolfsdorf, & Fehm, 1987; Henkin & Daly, 1968; Kuehl, Michaux, Richter, Schachinger, & Anton, 2010). Collectively, these findings suggest that cortisol may affect the integration of cross-modal cues.

In the present study we tested the rapid, putatively nongenomic impact of a physiological dose of 5 mg of IV cortisol on cross-modal cue integration in a focused cross-modal choice reaction (see Arndt & Colonius, 2003) and startle paradigm. Non-startling visual targets were presented alone and together with either bilateral acoustic startle noise or with unilateral acoustic startle noise in the same or in the opposite sensory hemi-field. Saccadic latencies and manual reactions to visual targets as well as eye blink responses to startle noises served as indices of cross-modal cue integration. Testing was carried out twice on separate days, once after cortisol and once after placebo administration, according to a single blinded, placebo-controlled, and counterbalanced cross-over design. We expected cortisol to rapidly influence cross-modal cue integration depending on the spatial congruency between visual targets and acoustic startle noises.

## 4.2. Experimental procedures

### 4.2.1 Experimental Design

#### 4.2.1.1 Procedure

The study was approved by the Ethical Committee of the State's Medical Association (Landesärztekammer Rheinland-Pfalz) and was in accordance with the Declaration of Helsinki. 24 male volunteers (age: 19-32 years) gave informed consent and were tested on two afternoon sessions separated by one week, receiving cortisol on the one and placebo on the other day.

Upon arrival a flexible intravenous catheter was inserted followed by a one hour resting period in which the participant was prepared for the experiment (electrodes attached, headphones mounted, adjustment of chin and front support). Then, the participant was habituated to startle stimuli and the experimental task was explained on screen followed by a short practice run. Subsequently, the main experiment started and lasted for about 30 minutes. The pharmacological manipulation was applied 10 minutes after starting the main experiment. At the end catheter and electrodes were removed. After the second session (i.e., second testing day) participants were debriefed and paid 50 Euros. See Figure 7.

#### 4.2.1.2 Experimental Task

A chin and front support system was used to avoid head movements. During a focused cross-modal choice reaction time task participants were asked to push a left or right button as fast and accurately as possible whenever a small up vs. down pointing triangle (diameter 2.5 mm, 0.26° of visual angle) appeared at either the left or right periphery (horizontal eccentricity of 16.76°) of a 19-inch flat LCD monitor (1280 x 800 resolution; distance 60 cm). Half of the participants reacted to up pointing triangles, the other to down pointing triangles (randomized). Due to the small size of the stimuli, participants needed to perform a saccade towards the periphery of the screen. In half of the trials, simultaneous with the appearance of the visual stimulus, bilateral or unilateral startle noise probes (105 dB(A), white noise, instantaneous rise time, duration 50 ms) were presented via headphones (Holmco PD-81, Holmberg GmbH & Co. KG, Germany). The unilateral startle stimulus was presented in the same or in the opposite sensory hemi-field as the visual stimulus (equal frequency per

combination). Startle noise stimuli were also presented alone without any visual targets (control conditions).

Each trial started with a blank screen (1-2 s), followed by a center fixation cross (diameter 5 mm, 0.44° of visual angle) which disappeared after 2-3 s when stimuli were presented. Button push reaction time was scored until 1500 ms after stimulus onset. Inter-trial-interval was 3 - 6.5 s. Every 30 trials a small pause of two minutes duration was included. Total number of trials was 180 per session. All stimulus presentation was controlled by E-Prime 2.0 (PST Software, Inc).

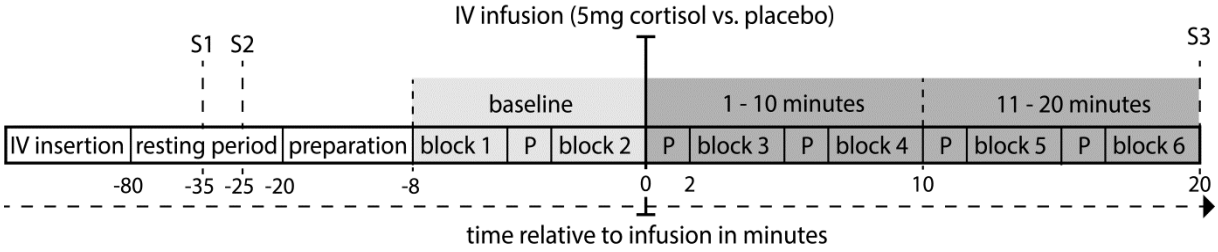


**Figure 7**: Procedure of the experiment. Times are given relative to the onset of the infusion of cortisol or placebo. At the beginning of each session an IV catheter was inserted into the left or right arm of the participant. During a one hour resting period the participant was prepared for the experiment before starting the experimental presentation. The experiment consisted of six blocks which were separated by short pauses ('P'). The first two blocks of the experiment were grouped together for further statistical analysis and served as a 'baseline' ('block 1' and 'block 2') for the subsequent post-infusion blocks. In the second pause 5 mg of IV cortisol or a corresponding amount of placebo solution was infused. Changes in multisensory integration following the pharmacological manipulation were assessed in block 3 to block 6. Post-infusion blocks were grouped into the measurement times '1-10 minutes' ('block 3' and 'block 4') and '11-20 minutes' ('block 5' and 'block 6') after infusion for further statistical analyses. During the waiting period ('S1' and 'S2') as well as after the last experimental block ('S3') saliva samples were taken to assess acute cortisol levels before and after the pharmacological manipulation.

#### 4.2.1.3 Pharmacological Design

In the second pause of the experiment, cortisol or placebo were administered intravenously according to a placebo-controlled, single blinded, counterbalanced crossover design.

### 4.2.2 Materials

#### *4.2.2.1 IV Intervention*

Saline placebo (NaCl 0.9%, Braun, Melsungen, Germany) on one day and 5 mg of cortisol on the other day (Hydrocortison 100 mg, Rotexmedia, Trittau, Germany) were given intravenously as bolus from outside the experimental room, so that participants were not aware of the intervention.

#### *4.2.2.2 Assessment of Salivary Cortisol*

Saliva was sampled via Salivettes® (Sarstedt, Nümbrecht, Germany) on both testing days during the morning at 0, 30, 45, and 60 min after awakening as well as during the experiment at 35 (S1) and 25 min (S2) before (baseline), and 20 min after intervention (S3). Salivary cortisol was analyzed by a time resolved immunoassay with fluorescence detection (Dressendorfer, Kirschbaum, Rohde, Stahl, & Strasburger, 1992). The cortisol awakening response was calculated as the area under the curve with respect to ground (AUCg, Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003).

#### *4.2.2.3 Recording Parameters*

Manual reaction time (PST E-Prime Serial Response Box) and physiological data (Biopac Inc.® MP150 system; 16 bit, 1 kHz) were collected. Horizontal saccades were assessed by electrooculography (EOG; band-pass: 1-15 Hz). Startle eye blink responses were assessed by electromyography (EMG; LF cut-off: 28 Hz) of both orbicularis oculi muscles (OOc), rectified and integrated online (time constant: 10 ms). A customized C++ based semi-automated PC program was used to quantify onset and end of saccades as well as startle eye blinks (see Deuter, Schilling, Kuehl, Blumenthal, & Schachinger, 2013).

### 4.2.3 Data Preprocessing

Saccadic reaction time (SRT) was defined as the interval between the appearance of a visual target on screen and the time the saccade reached the target. SRT faster than 200 ms or slower than 600 ms as well as saccades away instead of towards the target were excluded from analysis (mean exclusion rate: 0.69%). Manual choice reaction time (MRT) was defined as the interval between the appearance of a visual target on screen and button press. MRT faster

than 250 ms or slower than 1250 ms as well as incorrect responses were excluded from analysis (mean exclusion rate: 0.72%).

Startle eye blink responses were quantified in each startle trial by subtracting the baseline (150 to 50 ms before startle stimulus) from the peak (70 to 150 ms after startle stimulus) EMG-activity. Next, responses of both OOc were t-scored and averaged according to the side of startle stimulus presentation ('ipsi-OOc' = OOc ipsilateral to unilateral acoustic startle; 'contra-OOc' = OOc contralateral to unilateral acoustic startle; 'bilateral' = bilateral startle, mean of both OOc) and the side of visual target presentation. Zero-responses were included in the averaging process.

### 4.2.4 Statistical Analysis

One participant failed to show reliable startle eye blink responses and was excluded from analysis. Experimental saliva samples of one participant were missing.

Statistical analyses were calculated using SPSS 20 (IBM SPSS statistics) with a critical α-level of $p = 0.05$ per model. Unless otherwise stated all data were analyzed using within-subjects ANOVAs. A Greenhouse-Geisser correction for degrees of freedom was applied whenever appropriate and the corresponding Huynh-Feldt (*HF*) Epsilon is reported along with the uncorrected degrees of freedom. Higher-order interactions and main effects were followed by simple interaction analyses and simple pairwise comparisons. *p*-values of non-orthogonal, multiple simple pairwise comparisons were adjusted using the sequential Bonferroni-Holm procedure (Holm, 1979) to control for inflation in α-error rate. The number of comparisons (*C*) and the corrected *p*-value ($p_{corr}$) of significant comparisons is stated. Mean ± SEM are reported in text, tables, and figures.

## 4.3. Results

### 4.3.1 Cortisol

Salivary morning cortisol (AUCg) did not differ between experimental days (placebo session: 665.4±55.9; cortisol session: 714.4±56.4; $t$ (22) = .92, $p$ = .36). An interaction *drug*time* ($F$ [1, 21] = 129.2 $p < .001$, $\eta_p^2 = .86$) indicated increased salivary cortisol levels after cortisol infusion as compared to placebo and baseline. See Figure 8.

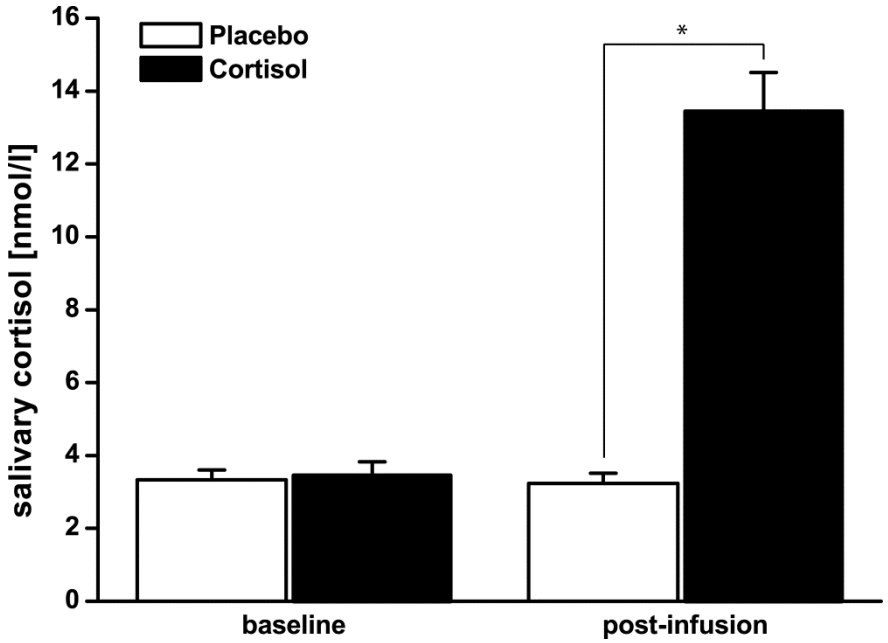


**Figure 8:** Mean and standard error of the mean of acute salivary cortisol levels (nmol/l) before and after the pharmacological manipulation separated by cortisol vs. placebo session. IV cortisol infusion successfully elevated salivary cortisol levels. Asterisk indicates significant difference between cortisol and placebo session ($p_{corr} < 0.01$).

### 4.3.2 Saccadic Reaction Time and Manual Reaction Time

The factors *drug* ('placebo' session vs. 'cortisol' session), *time* ('baseline', '1-10 minutes','11-20 minutes') and *congruency* (unilateral startle and visual target presented in the same sensory hemi-field = 'same', unilateral startle and visual target presented in opposite sensory hemi-fields = 'opposite', bilateral startle and visual target at either left or right side = 'bilateral', visual target only = 'no startle') were entered in 2(*drug*)*3(*time*)*4(*congruency*) factorial designs. Separate models for each dependent variable were tested.

#### *4.3.2.1 Saccadic Reaction Time*

A main effect of *congruency* ($F$ [3, 66] = 3.27, $p$ = .046, $\eta_p^2$ = .13, *HF-Epsilon* = .68) was observed. Simple comparisons ($C$ = 6) showed that saccades were slower in the 'bilateral' condition, compared to both 'same' ($F$ [1, 22] = 17.93, $p_{corr}$ = .002) and 'opposite' conditions ($F$ [1, 22] = 9.60, $p_{corr}$ = .026). All other post-hoc comparisons, including 'same' vs. 'opposite' ($F$ [1, 22] = 0.19, *ns.*), failed to reach significance (see Table 2). No three-way interaction *drug*time*congruency* ($F$ [6, 132] = 1.50, $p$ = .18) was found.

**Table 2:** Mean and standard error of the mean (SEM) of saccadic reaction time (SRT) and manual reaction time (MRT) to visual targets in milliseconds. Data are separated by dependent variable and by factor level of the factor *congruency*. SRT was faster in the unilateral compared to the bilateral startle condition, but not different in same vs. opposite conditions. Also, SRT was not different in startle vs. no startle conditions. By contrast MRT was generally speeded up by acoustic startle noise and specifically enhanced, when visual target and unilateral acoustic startle noise were presented in the same sensory hemi-field. Asterisk (*) indicates significant difference from bilateral (SRT) or from bilateral and opposite (MRT). Hash (#) indicates significant difference from no startle (MRT).

| **Saccadic reaction time (SRT)** | | |
|---|---|---|
| Condition | Mean | SEM |
| same | 392.46* | 9.84 |
| opposite | 394.31* | 9.94 |
| bilateral | 405.90 | 10.04 |
| no startle | 403.23 | 9.33 |

| **Manual reaction time (MRT)** | | |
|---|---|---|
| Condition | Mean | SEM |
| same | 626.45*# | 10.47 |
| opposite | 645.75# | 11.14 |
| bilateral | 640.63# | 9.59 |
| no startle | 667.84 | 12.54 |

#### *4.3.2.2 Manual Reaction Time*

A main effect of *congruency* was found ($F$ [3, 66] = 14.41, $p$ < .001, $\eta_p^2$ = .40, *HF-Epsilon* = .77). Simple comparisons ($C$ = 6) revealed shorter MRT when startle stimuli accompanied targets ('same' vs. 'no startle' = $F$ [1, 22] = 32.34, $p_{corr}$ < .001; 'opposite' vs. 'no startle' = $F$ [1, 22] = 9.50, $p_{corr}$ = .011; 'bilateral' vs. 'no startle' = $F$ [1, 22] = 10.61, $p_{corr}$ = .011). Moreover, MRT was shorter when unilateral startle noises and visual targets appeared in the 'same' visual hemi-field compared to the 'opposite' field ('same' vs. 'opposite' = $F$ [1, 22] = 16.15, $p_{corr}$ = .003), as well as compared to 'bilateral' startle presentation ('same' vs. 'bilateral' = $F$ [1, 22] = 11.77, $p_{corr}$ = .01). MRT did not differ between 'opposite' vs. 'bilateral' ($F$ [1, 22] = 0.83, *ns.*) (see Table 2).

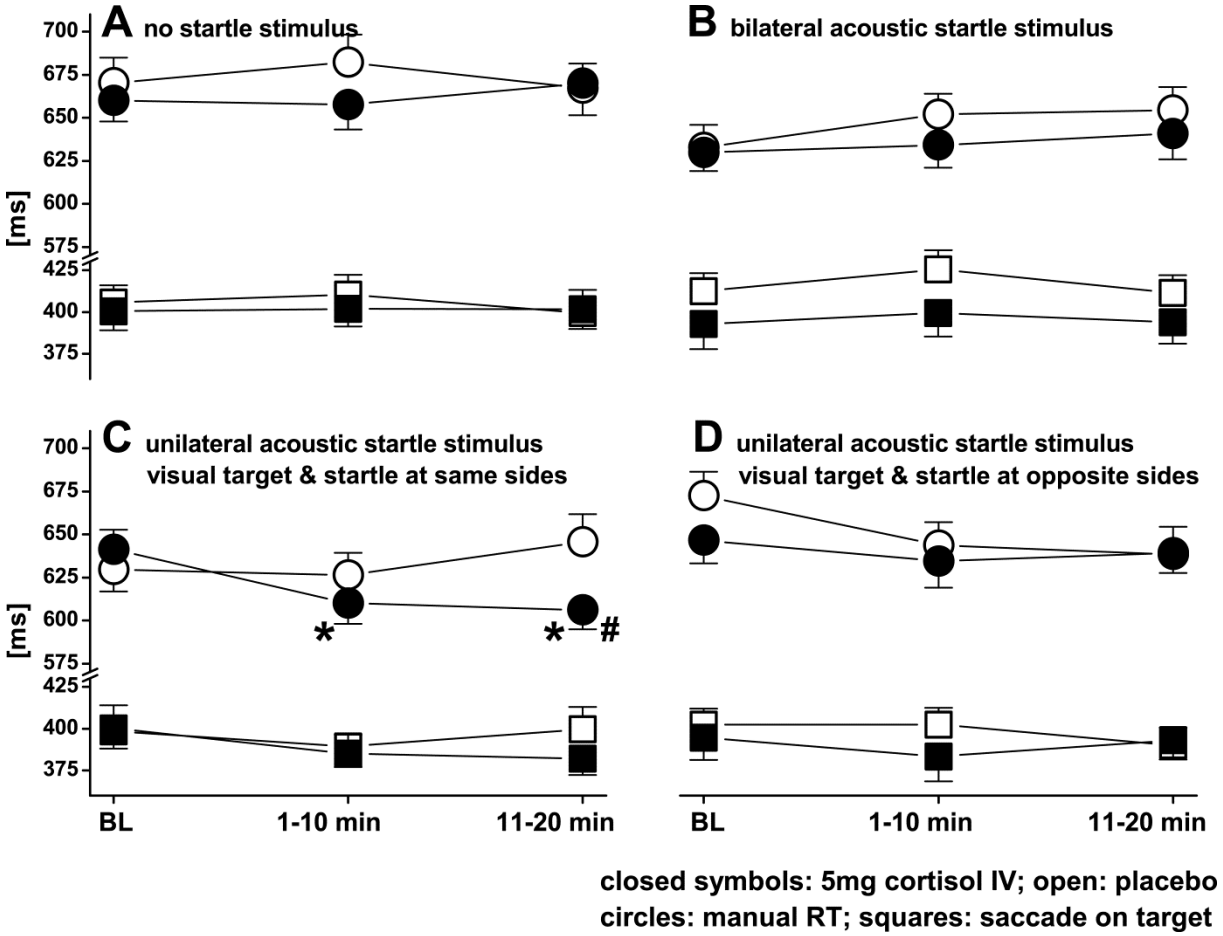


**Figure 9:** Mean and standard error of the mean of saccadic reaction time (saccades on target) and manual choice reaction time to visual targets, separated by visual target only (A), bilateral acoustic startle and visual target (B), unilateral acoustic startle and visual target at same side (C) and unilateral acoustic startle and visual target at opposite sides (D). Circles refer to manual reaction time, squares to saccadic reaction time (saccade on target). Open symbols reflect placebo session, closed symbols cortisol session. Post-hoc simple pairwise comparisons were restricted to dependent variables and factor levels where a *drug*time* interaction was observed (i.e., factor level 'same' in manual reaction time, see results). Manual reaction time was faster following cortisol compared to placebo infusion, when visual target and unilateral acoustic startle noise were presented in the same sensory hemi-field (C). Asterisk indicates significant difference between post-infusion measurement time and corresponding baseline value (BL), hash difference between placebo- and cortisol session.

A three-way interaction *drug*time*congruency* ($F$ [6, 132] = 2.70, $p$ = .017, $\eta_p^2$ = .11) was observed, and subsequently split into four simple *drug*time* interactions, one within each level of the factor *congruency*. A significant *drug*time* interaction was found for the level 'same' ($F$ [2, 44] = 6.11, $p$ = .005, $\eta_p^2$ = .22), but not for other factor levels ('opposite' = $F$ [2, 44] = 1.2, $p$ = .31; 'bilateral' = $F$ [2, 44] = .3; $p$ = .74); 'no startle' = $F$ [2, 44] = 2.5, $p$ = .09). In a final step, the *drug*time* interaction in the factor level 'same' was explored by

calculating simple comparisons (*C* = 7). The post-infusion measurement times '1-10 minutes' and '11-20 minutes' were contrasted with their corresponding 'baseline' separately for both 'placebo' and 'cortisol' session. Also, 'placebo' and 'cortisol' session were directly compared at the different levels of the factor 'time' ('baseline', '1-10 minutes', '11-20 minutes'). The simple comparisons showed that MRT was shorter both '1-10 minutes' ($F$ [1, 22] = 9.70, $p_{corr}$ = .03) as well as '11-20 minutes' ($F$ [1, 22] = 13.68, $p_{corr}$ = .007) after cortisol infusion relative to corresponding baseline values. No difference in MRT was observed after placebo infusion ('baseline vs. '1-10 minutes' = ($F$ [1, 22] = 0.01, *ns.*), 'baseline' vs. '11-20 minutes' = ($F$ [1, 22] = 1.39, *ns.*). Moreover, direct comparison between 'placebo' and 'cortisol' session showed that MRT was faster '11-20 minutes' after cortisol compared to after placebo infusion ($F$ [1, 22] = 9.54, $p_{corr}$ = .025). There was neither a difference between 'placebo' and 'cortisol' at '1-10 minutes' ($F$ [1, 22] = 2.01, *ns.*) nor at 'baseline' ($F$ [1, 22] = 1.22, *ns.*). Therefore, MRT was shortened by cortisol when visual targets were accompanied by unilateral startle noises in the same sensory hemi-field (see Figure 9).

### 4.3.3 Startle Eye Blink (OOc-responses)

EMG changes of both OOc were tested. Thus, during unilateral startle elicitation two responses were scored. For the purpose of this study, the response of the OOc ipsilateral to the stimulated ear ('ipsi-OOc') was considered to be statistically independent from the contralateral OOc ('contra-OOc') response. Two four-factorial models were calculated, an overall,- and a specific model, differing in factor levels.

#### *4.3.3.1 Overall Model*

The factors *drug*, *time*, *startle* ('ipsi-OOc', 'contra-OOc', 'bilateral startle') and *target* (visual target ipsilateral to OOc = 'ipsi-target', visual target contralateral to OOc = 'contra-target' 'no target') were entered in a 2(*drug*)*3(*time*)*3(*startle*)*3(*target*) factorial design. A main effect of *startle* was observed ($F$ [2, 44] = 114.71, $p < .001$, $\eta_p^2$ = .84, *HF-Epsilon* = .64). Simple comparisons *(C = 3)* showed that OOc-responses to startle noise were larger following 'bilateral' stimulation compared to unilateral stimulation at the 'ipsi-OOc' ($F$ [1, 22] = 122.25 $p_{corr} < .001$) and compared to unilateral stimulation at the 'contra-OOc' ($F$ [1, 22] = 125.12, $p_{corr} < .001$). Further, unilateral stimulation at the 'ipsi-OOc' evoked stronger responses compared to unilateral stimulation at the 'contra-OOc' ($F$ [1, 22] = 36.02, $p < .001$). Thus,

bilateral startle stimuli evoked the strongest OOc-responses, followed by ipsilateral and by contralateral unilateral stimulation (see Table 3). Also, a main effect of *target* was found ($F$ [2, 44] = 5.54, $p$ = .007, $\eta_p^2$ = .20). Simple comparisons ($C$ = 3) showed that OOc-responses were stronger in presence of a target compared to startle only stimulation ('no target' vs. 'ipsi-target' = $F$ [1, 22] = 6.78, $p_{corr}$ = .032; 'no target' vs. 'contra-target = $F$ [1, 22] = 7.53, $p_{corr}$ = .036). However, targets 'ipsilateral' to the OOc and targets 'contralateral' to the OOc had a similar impact on response magnitude ($F$ [1, 22] = .14, *ns*.) (see Table 3). Moreover, a main effect of *time* was found ($F$ [2, 44] = 25.06, $p$ < .001, $\eta_p^2$ = .53, *HF-Epsilon* = .81), which was due to a linear decline of OOc-response magnitude during the experiment ($F_{linear}$ [1, 22] = 34.65, $p$ < .001, $\eta_p^2$ = .61), see Figure 10. Importantly, a four-way interaction *drug*time*startle*target* ($F$ [8, 176] = 2.61, $p$ = .029, $\eta_p^2$ = .11, *HF-Epsilon* = .62) was observed, indicating that cortisol affected OOc-responses to startle noise and that this impact of cortisol depended upon both side of target and the side of startle noise.

**Table 3:** Mean and standard error of the mean (SEM) of orbicularis oculi muscle (OOc)-response magnitude to startle noise (t-scored). Data are shown separately for the factor *startle* and for the factor *target*, separated by OOc measured (*startle*) or side of visual stimulus presentation (*target*) (see results). Factor *startle*: OOc-response magnitude was strongest to bilateral startle stimulation, followed by unilateral startle stimulation measured at the ipsilateral OOc and by unilateral startle stimulation measured at the contralateral OOc. Factor *target*: OOc-response magnitude was enhanced in presence of a visual target compared to no target. There was no difference between targets presented ipsilateral vs. contralateral. Asterisk (*) indicates significant difference from 'bilateral' (*startle*) or 'no target' (*target*). Hash (#) indicates significant difference from 'contralateral' (*startle*).

| **Factor *startle*** | | | **Factor *target*** | | |
|---|---|---|---|---|---|
| Condition | Mean | SEM | Condition | Mean | SEM |
| Ipsi-OOc | 48.42*# | 0.192 | Ipsi-target | 50.12* | 0.113 |
| Contra-OOc | 46.41* | 0.333 | Contra-target | 50.18* | 0.117 |
| bilateral | 55.01 | 0.453 | no target | 49.54 | 0.141 |

### *4.3.3.2 Specific Model*

Cortisol did not impact on 'no target' trials (startle noise presented alone without visual target) and 'bilateral startle' trials. Therefore, the factor *startle* was reduced to two levels, and representing unilateral startle, only ('ipsi-OOc', 'contra-OOc'). Furthermore, a factor *congruency* was constructed indicating whether visual targets and unilateral startle appeared on the 'same' vs. 'opposite' side.

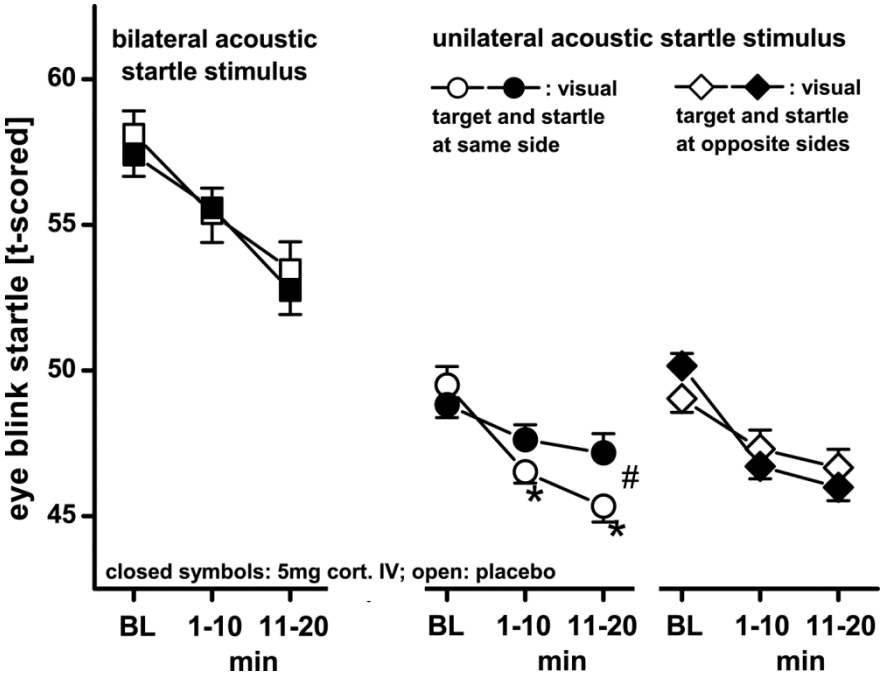


**Figure 10:** Mean and standard error of the mean of OOc-response magnitude to startle noise (t-scored), separated by bilateral acoustic startle and visual target at either side (squares), unilateral acoustic startle and visual target at same side (circles) and unilateral acoustic startle and visual target at opposite sides (diamonds). Open symbols reflect placebo session, closed symbols cortisol session. Post-hoc simple pairwise comparisons were restricted to factor levels where a *drug*time* interaction was observed (i.e., factor level 'same'). OOc-response magnitude to startle noise was enhanced following cortisol compared to placebo infusion, when visual target and unilateral acoustic startle noise were presented in the same sensory hemi-field (circles). Asterisk indicates significant difference between post-infusion measurement time and corresponding baseline value (BL), hash difference between placebo- and cortisol session.

Testing the 2(*drug)**3(*time)**2*(startle)**2(*congruency)* factorial model revealed, as expected, that responses were higher at the ipsi-OOc than the contra-OOc ($F$ [1, 22] = 27.73, $p < .001$, $\eta_p^2 = .55$). Beside this main effect, the factor *startle* was not involved in any interaction (*drug*startle* ($F$ [1, 22] = .30), *drug*time*startle* ($F$ [2, 44] = .28),

*drug*time*startle*congruency* ($F$ [2, 44] = .63), all $p$`s > .05), indicating that all influences were similar at both OOc, independent whether it was ipsilateral or contralateral to the stimulated ear. The remaining three factors significantly interacted: *drug*time*congruency* ($F$ [2, 44] = 10.23, $p < .001$, $\eta_p^2 = .32$). Next, the three-way interaction was split into separate *drug*time* simple interactions, one for each level of the factor *congruency* ('same', 'opposite'). The *drug*time* interaction was significant within the factor level 'same' ($F$ [2, 44] = 4.68, $p = .014$, $\eta_p^2 = .18$), and approached significance within the factor level 'opposite' ($F$ [2, 44] = 2.93, $p = .064$, $\eta_p^2 = 12$). However, the interaction patterns were different. When unilateral startle stimuli and visual targets appeared at the same side, cortisol enhanced startle responsiveness. When they appeared at opposite sides, cortisol reduced startle responsiveness (see Figure 10). Simple comparisons ($C = 7$) within the level 'same' of the *congruency* factor confirmed this: a typical startle habituation was found after 'placebo' infusion at both, '1-10 minutes' ($F$ [1, 22] = 17.9, $p_{corr} = .002$), as well as '11-10 minutes' ($F$ [1, 22] = 20.3, $p_{corr} = .001$) compared to 'baseline' values. After 'cortisol' infusion habituation disappeared ('baseline' vs. '1-10 minutes' = ($F$ [1, 22] = 2.33, *ns.*; 'baseline' vs. '11-20 minutes' = ($F$ [1, 22] = 3.71, *ns.*). Direct comparisons between *drugs* at the different levels of the factor *time*, revealed stronger startle eye blink responses at '11-20 minutes' after 'cortisol' infusion as compared to 'placebo' ($F$ [1, 22] = 8.13, $p_{corr} = .046$). There was neither a significant difference between 'cortisol' and 'placebo' at '1-10 minutes' ($F$ [1, 22] = 3.60, *ns.*) nor at 'baseline' ($F$ [1, 22] = 1.15, *ns.*). Hence, compared to placebo, cortisol relatively enhanced OOc-responses when startle noises and visual targets appeared in the same sensory hemi-field (see Figure 10).

## 4.4. Discussion

Previous research suggested that the stress hormone cortisol may play a role in multisensory processing of danger cues. The current study was designed to test for rapid, presumably nongenomic effects of physiologic, low-dose cortisol on lateralized, cross-modal integration of simultaneously presented visual targets and acoustic noise stimuli. Cortisol enhanced task-induced manual choice reactions and startle eye blinks when visual targets and unilateral startle noise stimuli were presented in the same sensory hemi-field.

The current work is based on a previously established 'focused reaction time paradigm' (see Arndt & Colonius, 2003). However, some elements were changed: (i) acoustic stimuli were startling (i.e., representing a danger cue); (ii) a visual discrimination task was added, thereby inducing a context of challenge; and (iii) instead of being instructed to intentionally move their eyes to the visual target (voluntary saccades), participants performed horizontal saccades in order to solve the visual discrimination task (reflexive, stimulus-induced saccades (Leigh & Kennard, 2004)). Responses (manual choice reaction time, saccade latency, and eye blinks) reflect different levels of sensorimotor processing. Manual choice reactions involve the highest level of cognitive control, followed by task-induced, reflexive saccades. Startle eye blink responses, however, represent automatic defensive responses operated by giant cells located in the nucleus reticularis pontis caudalis (Koch, 1999), and not susceptible to direct intentional control.

One of the core findings of multisensory integration research is the "spatial principle" (Stein & Stanford, 2008): two stimuli of different modalities presented in spatial congruency are preferentially processed. This is reflected in decreased saccadic (Arndt & Colonius, 2003) and manual response time (Hughes, Reuter-Lorenz, Nozawa, & Fendrich, 1994) as well as in increased firing rates in multisensory neurons in the superior colliculus (Wallace, et al., 1996) and the thalamus (Komura, et al., 2005). In our experiment, manual reaction time to visual targets accompanied by a same sided unilateral startle noise was accelerated compared to visual targets accompanied by opposite sided unilateral startle noise, or presented alone, the latter reflecting the commonly observed 'StartReact effect' (Valls-Sole, Rothwell, Goulart, Cossu, & Munoz, 1999). Importantly, the reaction time advantage is sensitive to the spatial configuration between startle noise and visual target, thus, indicating for the first time that a "spatial principle" also contributes to the 'StartReact effect' (cf. Oude Nijhuis et al., 2007). Cortisol infusion specifically enhanced manual responses when imperative visual targets and unilateral acoustic startle noises were presented in the same sensory hemi-field. No such

cortisol effect was found when startle was presented in the opposite hemi-field, or bilaterally, or when no startle was presented. Thus, cortisol did not enhance the StartReact effect and cross-modal summation processes per se, but rather specifically strengthened the "spatial principle".

A "spatial principle" in saccadic reaction time was not observed. This appears to be in contrast to previous studies using acoustic stimuli of non-startling magnitude (Arndt & Colonius, 2003; Hughes, et al., 1994). It is possible that startle-induced blinking interferes with saccade initiation (Gandhi & Bonadonna, 2005), thereby counteracting any acceleration in saccade onset latencies. This would be supported by slower saccades in the bilateral compared to the unilateral startle condition, since bilateral startle noises evoked strongest eye blink responses. Previous studies on startle and saccadic reaction time have yielded inconclusive results. Very intense (130 dB(A)) startle noise was found to speed up saccade onset latencies in a simple reaction time paradigm (Castellote, Kumru, Queralt, & Valls-Sole, 2007), but this effect was missing when weaker startle noise (105 dB(A)) stimuli were used in a choice reaction time paradigm (Deuter, et al., 2013).

Protective, automated startle reactions are fast and appear in all vertebrates (Koch, 1999; Yeomans & Frankland, 1995). More intense startle stimuli induce stronger reactions, up to a point of asymptote. In the current study bilateral startle noise stimulation resulted in stronger OOc-responses than unilateral startle noise stimulation, an effect explained by subject's exposure to a doubling of the peripheral physical force when bilaterally stimulated. When stimulated with unilateral startle noise probes, the ipsilateral OOc reacted stronger than the contralateral OOc. This is a common finding in startle research (e.g., Schulz et al., 2009) and represents a mechanism of early spatial specificity: the protective response is stronger at the expected side of impact. This uncontrolled effect is driven by a concordant lateralized organization of sensory and motor fibers. Stronger OOc-responses were also observed when acoustic startle noise probes were accompanied by visual targets, an effect expected since startle is sensitive to both temporal summation (Yeomans, Li, Scott, & Frankland, 2002) and inhibition (Braff, Geyer, & Swerdlow, 2001). When startling stimuli are preceded by non-startling stimuli with stimulus-onset-asynchrony (SOA) between 50 and 500 ms (in humans), startle reactions are usually inhibited. Presenting them at the same time (SOA = 0 ms) will result in enhanced startle responses, an effect explained by temporal summation (Blumenthal & Berg, 1986). Temporal summation occurs both within and across modalities, but strongest summation has been observed in cross-modal stimulus settings (Li & Yeomans, 1999; Plant &

Hammond, 1989). Previously, we showed that cortisol rapidly affected the integration of consecutive (SOA = 120 ms) bilateral acoustic stimuli in a prepulse inhibition (PPI) of startle paradigm to the benefit of startle responding (Richter, et al., 2011). In the current study cortisol augmented OOc-responses to unilateral startle noise, but only when the startle stimulus was accompanied by a visual target in the same sensory hemi-field. No cortisol effects were found in other conditions (bilateral startle, unilateral startle and visual target at opposite sides, no target). Therefore, the current data indicate that the impact of cortisol is restricted to a specific spatial stimulus configuration. Interestingly, this influence is similar for both ipsilateral and contralateral OOc, suggesting that the effect is not driven by "lower cognitive" lateralized neuronal structures, but rather by reinforcement of structures integrating spatially congruent multi-modal stimuli. Thus, cortisol increases cross-modal summation of startle in a manner comparable to the enhancement of the "spatial principle" observed in manual reaction time. It has to be mentioned that the startle effect described here interacts with habituation of startle magnitude over time, which is a usual finding in startle studies and appeared after both cortisol and placebo administration.

Multiple signals appearing simultaneously in different sensory channels but the same hemi-field are more likely to be caused by one object. Same sided stimuli are, therefore, preferentially processed as stated in the "spatial principle" (Stein & Stanford, 2008). Cortisol seems to potentiate this mechanism by exclusively enhancing the integration of same side multisensory stimuli. In a dangerous environment this would allow for an enhancement of controlled behavior and selective escape reactions, as well as fostering automated defensive reactions to threatening lateralized danger stimuli. The fact that this effect is mediated by a rapid, presumably nongenomic cortisol mechanism guarantees this process to come into play as quickly as possible during perilous or stressful encounters.

Nongenomic cortisol effects appear much faster (Groeneweg, et al., 2011) than genomic effects that need at least 20 minutes to develop (Makara & Haller, 2001). Consequently, in human in vivo studies the involvement of a nongenomic cortisol mechanism is usually inferred from the time passing between drug application and effect onset (e.g., Schilling, et al., 2013; Vila et al., 2010) and an intravenous application of the hormone is mandatory.

We can only speculate on the neurobiological mechanisms underlying the observed effects. Nongenomic glucocorticoid pathways are known to target the release probability of both glutamate (Karst, Berger, Erdmann, Schutz, & Joels, 2010; Karst et al., 2005) and GABA (Di,

Malcher-Lopes, Marcheselli, Bazan, & Tasker, 2005; Di, Maxson, Franco, & Tasker, 2009), and can either facilitate or inhibit neuronal transmission. Specifically, it is known that glucocorticoids can rapidly decrease glutamate release and increase GABA release into the synaptic cleft in hypothalamic neuronal circuits (Di, et al., 2009), resulting in a decreased likelihood for postsynaptic action potentials and functional inhibition of the affected neurons. This may have great consequences for thalamic relay and filter functions (Guillery & Sherman, 2002; Sherman, 2007), an assumption supported by a recent study showing that low-dose IV cortisol infusion rapidly affected the thalamus, reduced thalamic blood flow, and decreased global EEG-power across a wide range of oscillatory frequencies (Strelzyk, et al., 2012). Indeed, the thalamus is an important, early integrator (Komura, et al., 2005; Noesselt, et al., 2010) and distributer (Cappe, Rouiller, et al., 2009; van den Brink, et al., 2013) of multisensory information as well as a modulator of inter-cortical traffic (Theyel, Llano, & Sherman, 2010). Enhanced GABAergic- and reduced glutamatergic transmission in the thalamus will impair the forwarding of afferent sensory traffic, leading to increased sensory thresholds. As a consequence, sensory signals that produce only weak neuronal responses may be attenuated, while stronger signals which easily surpass the threshold are less affected. Thus, random "noise" will be reduced and the "signal-to-noise" ratio enhanced. Given that spatially congruent cross-modal stimuli seem to produce a most robust "signal" (Komura, et al., 2005; Wallace, et al., 1996), it is likely that their processing will benefit from this "noise" reduction. Accordingly, it seems plausible that cortisol should specifically enhance the processing of spatially congruent multisensory stimuli and not of those appearing spatially apart.

## Conclusion

Our results suggest that cortisol accelerates controlled motor responses and increases automated reflexes to side-congruent multisensory stimuli via a nongenomic mechanism. This highlights the potential role of nongenomic cortisol in fast adaptation and protection against danger stimuli in stressful contexts.

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## 4.ii Author Notes – Chapter IV

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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.

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