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Reward-related processing of visual food cues: neuroendocrine and stress mechanisms

Autor:

Diana S. Ferreira de Sá, Mestre Psicologia

(Integriertes Masterstudium in Psychologie,
Universidade de Coimbra)

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Gutachter:

Prof. Dr. med. Hartmut Schächinger

Prof. Dr. Melly S. Oitzl

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Department of Clinical Psychophysiology

Institute of Psychobiology – University of Trier

Affiliation of the Supervisors

Prof. Dr. med. Hartmut Schächinger

Department of Clinical Psychophysiology – Institute of Psychobiology – University of Trier

Prof. Dr. Melly S. Oitzl

Department: Structural and Functional Plasticity of the Nervous System - Swammerdam Institute for Life Sciences - University of Amsterdam

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General Abstract

Every day we are exposed to a large set of appetitive food cues, mostly of high caloric, high carbohydrate content. Environmental factors like food cue exposition can impact eating behavior, by triggering anticipatory endocrinal responses and reinforcing the reward value of food. Additionally, it has been shown that eating behavior is largely influence by neuroendocrine factors. Energy homeostasis is of great importance for survival in all animal species. It is challenged under the state of food deprivation which is considered to be a metabolic stressor. Interestingly, the systems regulating stress and food intake share neural circuits. Adrenal glucocorticoids, as cortisol, and the pancreatic hormone insulin have been shown to be crucial to maintain catabolic and anabolic balance. Cortisol and insulin can cross the blood-brain barrier and interact with receptors distributed throughout the brain, influencing appetite and eating behavior. At the same time, these hormones have an important impact on the stress response. The aim of the current work is to broaden the knowledge on reward related food cue processing. With that purpose, we studied how food cue processing is influenced by food deprivation in women (in different phases of the menstrual cycle) and men. Furthermore, we investigated the impact of the stress/metabolic hormones, insulin and cortisol, at neural sites important for energy metabolism and in the processing of visual food cues.

The Chapter I of this thesis details the underlying mechanisms of the startle response and its application in the investigation of food cue processing. Moreover, it describes the effects of food deprivation and of the stress-metabolic hormones insulin and cortisol in reward related processing of food cues. It explains the rationale for the studies presented in Chapter II-IV and describes their main findings. A general discussion of the results and recommendations for future research is given.

In the study described in Chapter II, startle methodology was used to study the impact of food deprivation in the processing of reward related food cues. Women in different phases of the menstrual cycle and men were studied, in order to address potential effects of sex and menstrual cycle. All participants were studied either satiated or food deprived. Food deprivation provoked enhanced acoustic startle (ASR) response during foreground presentation of visual food cues. Sex and menstrual cycle did not influence this effect. The

startle pattern towards food cues during fasting can be explained by a frustrative nonreward effect (FNR), driven by the impossibility to consume the exposed food.

In Chapter III, a study is described, which was carried out to explore the central effects of insulin and cortisol, using continuous arterial spin labeling to map cerebral blood flow patterns. Following standardized periods of fasting, male participants received either intranasal insulin, oral cortisol, both, or placebo. Intranasal insulin increased resting regional cerebral blood flow in the putamen and insular cortex, structures that are involved in the regulation of eating behavior. Neither cortisol nor interaction effects were found. These results demonstrate that insulin exerts an action in metabolic centers during resting state, which is not affected by glucocorticoids.

The study described in Chapter IV uses a similar pharmacological manipulation as the one presented in Chapter III, while assessing processing of reward related food cues through the startle paradigm validated in Chapter II. A sample of men was studied during short-term food deprivation. Considering the importance of both cortisol and insulin in glucose metabolism, food pictures were divided by glycemic index. Cortisol administration enhanced ASR during foreground presentation of "high glycemic" food pictures. This result suggests that cortisol provokes an increase in reward value of high glycemic food cues, which is congruent with previous research on stress and food consumption.

This thesis gives support to the FNR hypothesis towards food cues during states of deprivation. Furthermore, it highlights the potential effects of stress related hormones in metabolism-connected neuronal structures, and in the reward related mechanisms of food cue processing. In a society marked by increased food exposure and availability, alongside with increased stress, it is important to better understand the impact of food exposition and its interaction with relevant hormones. This thesis contributes to the knowledge in this field. More research in this direction is needed.

Index of Figures

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Index of Publications

This doctoral thesis consists of one chapter with general introduction, and three other chapters, which are published as 'Original Research Articles' in international peer-reviewed journals. The author is the first author of all three articles (one of these, a shared first authorship, meaning that both authors contributed equally to the study). The articles are presented in their original published/submitted form, except from formatting changes (e.g., figure labeling, references).

Content	***has been published:***
Chapter II	**Ferreira de Sá, D. S.**, Plein, D. E., Schulz, A., Oitzl, M. S., Blumenthal, T. D., & Schächinger, H. (2014). Acoustic startle reactivity while processing reward-related food cues during food deprivation: Evidence from women in different menstrual cycle phases and men. *Psychophysiology*, 51 (2), 159-67. doi: 10.1111/psyp.12166 (Impact Factor 2014: 3.26)
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Chapter IV	**Ferreira de Sá, D. S.**, Schulz, A., Streit, F. E., Turner, J. D., Oitzl, M. S., Blumenthal, T. D., & Schächinger, H. (2014). Cortisol, but not intranasal insulin, affects the central processing of visual food cues. *Psychoneuroendocrinology*, 50C, 311-320. doi: 10.1016/j.psyneuen.2014.09.006. (Impact Factor 2014: 5.591)

Index of Abbreviations

ACTH	adrenocorticotrophic hormone
AgRP	agouti-related protein
ANOVA	analysis of variance
ANS	autonomic nervous system
ARC	arcuate nucleus
ASM	affective startle modulation
ASR	acoustic startle response
AUCg	area under the curve with respect to ground
BMI	body mass index
BOLD	blood oxygen level-dependent
CASL	continuous arterial spin labeling
CBF	cerebral blood flow
cm	centimeter
CRH	corticotropin-releasing hormone
CSF	cerebrospinal fluid
dB	decibels
EMG	electromyography
EPI	echo planar imaging
FD	food deprivation
FP	midfollicular phase of the menstrual cycle
FOV	Field of view
FWE	family-wise-error
g	gram
GI	glycemic index
GR	glucocorticoid receptor
HPA	hypothalamic-pituitary-adrenal
Hz	Hertz
IAPS	International affective picture system
IV	intravenous
IU	international unit
kg/m^2	kilogram per square meter
kHz	Kilohertz

LP	midluteal phase of the menstrual cycle
M	mean
min	minutes
ml	milliliter
mm	millimeter
ms	milliseconds
MNI	Montreal Neurological Institute
MR	mineralocorticoid receptor
MRI	magnetic resonance imaging
M_0	equilibrium magnetization
NC	normal food consumption
nmol/l	nanomol per liter
NPY	neuropeptide Y
PET	positron emission tomography
PVN	nucleus paraventricularis of the hypothalamus
PYY	pepetide YY
rCBF	regional cerebral blood flow
ROI	region of interest
s	seconds
SD	standard deviation
SE	standard error
SEM	standard error of the mean
SPM	Statistical parametric mapping/map
T	Tesla
T_1	longitudinal relaxation time
T_2	transversal relaxation time
TE	echo time
TR	repetition time
VAS	visual-analog-scale

Chapter I − General Rationale

Throughout human history, eating habits and food resources have changed drastically. Modern industrialized society is marked by the increasing availability of food and exposure to food cues, especially of palatable food, rich in fat and carbohydrates, which is perceived as highly rewarding (Adam and Epel, 2007; Dallman et al., 2005). Simultaneously, metabolism-related disorders, like obesity, metabolic syndrome, diabetes and eating disorders, have substantially risen in the last years (Regitz-Zagrosek et al., 2006; Seidell, 1995).

Our body follows a complex principle of homeostasis, necessary to the healthy function of the organism (Woods, 2009). Human energy consumption has evolved to adapt to the demanding metabolic needs of the brain. Human brain metabolism accounts for ~20-25% of resting metabolic rate, a much higher rate than the observed in other primates (Leonard et al., 2003). It is therefore not surprising that the brain plays the main role in maintaining energy homeostasis, a process achieved by balancing energy consumption and expenditure. This requires the exchange and integration of a vast array of peripheral and central nervous signals (Woods, 2009).

Eating behavior is not only regulated by biophysiological mechanisms, but also influenced by environmental factors. Exposition to appetitive food cues increases brain metabolism, particularly in brain areas related to drive and reward seeking, as the orbitofrontal cortex, the amygdala, and the striatum (Gottfried et al., 2003; LaBar et al., 2001; Morris and Dolan, 2001; Wang et al., 2004). Cues that signal food intake increase cravings and hunger, and also produce a set of anticipatory bodily responses with the function of optimizing food digestion, acting as conditioned stimuli (Mattes, 1997; Nederkoorn et al., 2000). It was previously shown that exposure to food advertisement provokes increased food consumption both in children and adults, independently of reported hunger (Andreyeva et al., 2011; Harris et al., 2009).

The aim of this work is to further extend the knowledge on food cue processing and specifically on how it can be affected by metabolic states (food deprivation or satiation) and important metabolic hormones (insulin and cortisol). Additionally, effects of those hormones on neural activation during resting state are reported.

1.1 Measuring reward-related processing of food cues

Various methods are used in the study of food cue processing. Some of those comprise the modified stroop task (Brody et al., 2004; Channon and Hayward, 1990), the visual-probe paradigm (Hou et al., 2011; Mogg et al., 1998), analysis of event-related potentials (Blechert et al., 2010; Leland and Pineda, 2006; Stockburger et al., 2008) and functional magnetic resonance imaging (Cornier et al., 2007; Garcia-Garcia et al., 2013; Siep et al., 2009). Following the line of research in drug cue processing, Drobes and colleagues (2001) adapted the startle modulation paradigm to the study of food cue processing. This was also the methodology chosen for two of the studies presented in this thesis. Startle methodology allows inferring motivational states, free of interference from subjective perception, task characteristics, or intentional deviations (Grillon and Baas, 2003). Additionally, it has the advantage of following long established protocols, it is relatively easy to apply, and requires comparably less resources.

A startle response occurs as a short abrupt response to an unexpected stimulus of high intensity. It is followed by a whole or partial body movement which assumes a protective function. It is therefore considered to be a defensive reflex (Lang et al., 1990). The startle eye blink reflex is one of the most stable components of the startle response and easy to quantify. In laboratory context, it is common to use unexpected auditory stimulation to study startle eye blink, as measured by electromyographic activity of the orbicularis oculi muscle (Blumenthal et al., 2005; Koch, 1999). The primary circuit of acoustic startle comprises three synapses: cochlear root neurons, nucleus reticularis pontis caudalis and facial motor nucleus (Davis, 1998). It is suggested that the modulation of the startle reflex is mediated by the central nucleus of the amygdala that projects to the nucleus reticularis pontis caudalis, especially concerning startle potentiation. Moreover, the nucleus accumbens is hypothesized to play a role in startle attenuation by appetitive cues (Grillon and Baas, 2003). Acoustic startle eye blink reflex (ASR) magnitude is highly sensitive to emotional, motivational and attentional states of the individual, constituting an excellent physiological measure of affective/cognitive processes (Grillon and Baas, 2003). According to the emotional priming model (Lang et al., 1998), emotions are organized in the brain in a two-factor motivational structure: appetitive and defensive. When one of these motivational systems is engaged, there will be a priming effect for affectively congruent stimuli. Therefore, in context of aversive emotion the defensive ASR is accentuated, while in contrast, if the appetitive system is activated by positive emotion there is attenuation of the ASR (Bradley et al., 1993).

Food cues are highly salient stimuli, most probably due to their importance for survival. The salience of food stimuli is further enhanced during fasting. Food deprivation directs attention towards food stimuli (Stockburger et al., 2009) and increases their ratings of arousal and interest (Drobes et al., 2001). Short-term food deprivation provokes a set of endocrine and neuronal changes. For instance, the circulating levels of the gut hormones insulin, leptin and peptide YY (PYY) decrease, while ghrelin levels rise (Neary et al., 2004; Woods et al., 1998). In response, neuronal activity of neuropeptide Y (NPY) and agouti-related protein (AgRP) increase, exerting an orexigenic effect through the hypothalamus. The substructures arcuate nucleus (ARC) and paraventricular nucleus (PVN) of the hypothalamus play an important role in central regulation of feeding, with the latter being responsible for integrating signals relevant for energy homeostasis with the thyroid and hypothalamic-pituitary-adrenal (HPA) axis (Neary et al., 2004). The brain stem has extensive points of connection with the hypothalamus, and likewise has high density of glucose sensitive neurons. The brain stem impacts energy regulation by receiving, integrating, and projecting gustatory, vagal and other nutritional signals to higher brain structures (Marty et al., 2007; Smith and Ferguson, 2008). Fasting can be regarded as a threat to the homeostasis of the body, which might elicit stress responses (Pacak & Palkovits, 2001). On another turn, stress also influences food intake by its action in the hypothalamic appetite-satiety centers (Tsigos, & Chrousos, 2005).

Drobes and colleagues (2001) showed that food deprived participants present higher ASR during food cue presentation, although rating food pictures as more arousing and interesting. The authors suggested that food deprived subjects might enter in an aversive motivational state of frustration nonreward (FNR). In contrast to these results, a study in food deprived participants found that high cravers showed decreased ASR during food cue presentation (Hawk et al., 2004). A major difference of this experiment was that in vivo cues were presented and these were available for consumption during the study, therefore no frustration was induced. Rejeski and colleagues (2010) showed that participants to whom consumption of food was withheld for a longer period presented higher ASR during food presentation. This connection between food availability and attenuation of the ASR strengthens the hypothesis of a FNR effect during states of hunger. The concept of FNR was first brought by Amsel (1958) and consists in the aversive response to the frustration provoked by the absence of an expected and anticipated reward. This effect has the potential to affect instrumental behavior, motivation and emotion. In a study from Amsel and Rousall (1952) rats were trained to run down an alley with two goal boxes, each with a food reward. Removal of reward in the first box enhanced running to the second box. The authors assumed that FNR facilitated this

behavioral response by inducing emotional and motivational changes, and a consequent state of drive towards a stimulus.

Men and women show different nutritional behavior and metabolic characteristics (Asarian and Geary, 2006; Mittendorfer et al., 2001), and these factors may influence the effect of food deprivation on startle during food cue presentation. For women, the different phases of the menstrual cycle have been shown to affect eating behavior (Buffenstein et al., 1995; Dye and Blundell, 1997; Farage et al., 2009). The menstrual cycle has a normal length of 21-35 days and can be divided in two phases that are separated by ovulation: the follicular (FP) and the luteal phase (LP). The FP starts with the menses' onset and continues until ovulation. The LP starts with the ovulation and lasts until the onset of a new menses (for a review see Despopoulos and Silbernagl, 2003). Throughout the menstrual cycle there is a large fluctuation of, among others, two very important hormones: estrogen and progesterone. During the early FP, levels of progesterone and estrogen are low. Estrogen levels start to rapidly increase at the late state of the FP, while progesterone starts to rise with the beginning of the LP, reaching its peak at the mid-luteal phase together with a second peak of estrogen. In the late LP, estrogen and progesterone levels start to decline and reach the baseline shortly before the onset of a new menses (Farage et al., 2009). Studies have shown that during the LP there is an increase in energy intake (Barr et al., 1995; Dalvit, 1981; Li et al., 1999) and preference for high caloric foods (Dye and Blundell, 1997; Johnson et al., 1994; Li et al., 1999).

The first experiment reported here (Chapter II) aimed to replicate and extend the knowledge on FNR effects during food deprivation. A within-subject design was applied, in contrast to the previous studies that were based on between-subject samples (Drobes et al., 2001; Friederich et al., 2006; Hawk et al., 2004; Mauler et al., 2006), allowing for correlational analysis. The experiment included women in different menstrual cycles and men in order to explicitly focus on potential sex and menstrual cycle effects. Our findings replicated the previous studies, with attenuation of ASR by pleasant foreground pictures, but enhanced ASR by presentation of food pictures, when participants where food deprived for 18-hours. As expected, food pictures were rated as more appetitive and arousing during deprivation. No effects of sex or menstrual cycle were found. Furthermore, the change in ASR observed between the two food conditions (normal food consumption and food deprivation) correlated with the change in ratings of hunger and motivation to eat, opening the question on the role of metabolic hormones in this effect.

1.2 Stress, hormones and metabolism

The hormones insulin and cortisol play an important role in maintaining energy homeostasis and in biopsychobehavioral aspects of eating, as appetite and satiety (Dallman et al., 2007a; Peters et al., 2004). Furthermore, both hormones are implicated in the stress response (Maniam and Morris, 2012).

In a stress situation, activation of the sympathetic nervous system (SNS) and of the HPA axis prepares the body to react to the demands of the stress stimuli (Tsigos and Chrousos, 2002). The SNS, part of the autonomous nervous system (ANS), provides the rapid control of functions important in the fight/flight response. It releases catecholamines, mainly epinephrine (adrenaline) and norepinephrine, and increases heart rate and peripheral vasoconstriction, among others. On its turn, HPA axis activation initiates a sequence of complex physiological events. The paraventricular nucleus of the hypothalamus releases corticotrophin releasing hormone (CRH) that stimulates the secretion of adrenocorticotropin hormone (ACTH) from the anterior pituitary. In response, the adrenal cortex promotes the synthesis and release of GCs into the bloodstream. GCs exert negative feedback on the HPA axis at the level of the hypothalamus, hippocampus, and pituitary, maintaining basal function of the axis as well as the termination of acute stress responses. Cortisol is the main GC in humans, and corticosterone is the main GC in rodents. GCs can bind to two different receptors that will elicit different effects: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Due to its higher binding affinity to cortisol, the MR is almost fully occupied under low GCs levels and has been implied in maintaining the responsiveness of the stress systems under basal conditions. GR is activated under increased GCs levels elicited by stress or circadian rhythm and it modulates the reestablishing of homeostasis (for a review see Gunnar and Quevedo, 2007; Ulrich-Lai and Herman, 2009).

Stress and consequent release of stress hormones also interfere with eating behavior. The increase in GCs induces a catabolic response in order to mobilize energy resources (Tataranni et al., 1996) and to maintain carbohydrate homeostasis (Laugero, 2001). This is essentially achieved by protein breakdown (Berneis et al., 1997), gluconeogenesis (Mutel et al., 2011) and increase in lipolysis (Peckett et al., 2011). According to the theoretical model of Reward Based Stress Eating (Adam and Epel, 2007), cortisol stimulates the intake of calorically dense food and is a potential neuroendocrine mediator between stress and eating through its effects on central reward circuitry. The influence of cortisol in reward might be mediated by other

neuropeptides/hormones like leptin, insulin and NPY, which not only interact with GCs but also have central role in energy regulation (Adam and Epel, 2007). In contrast, hypocortisolemia prevents obesity (Okada et al., 1992), and induces lower carbohydrate consumption (Laugero, 2001). Furthermore, the anorexic effects provoked by adrenalectomy and resulting hypocortisolemia are reversed by the administration of GCs (la Fleur, 2006).

It is known that the hormone insulin, produced by the pancreatic ß-cells, also influences food-associated behavior (Brody et al., 2004), even when hypoglycemia in the periphery is controlled (Dallman et al., 2007b; Hallschmid et al., 2004). Brody and colleagues (2004) showed that insulin-induced hypoglycemia provoked selective attention towards food stimuli. On the other hand, insulin affects reward-related processing of food cues, by decreasing reward or motivating properties of food (Figlewicz et al., 2007). Insulin is secreted in response to elevated concentration of glucose in the blood, as seen with the ingestion of carbohydrates. It promotes glucose uptake into fat and muscle tissues, glucose storage as glycogen in liver and muscle, and inhibits gluconeogenesis and glycogenolysis (Khan and Pessin, 2002). Insulin has the potential to cross the blood-brain barrier via a saturable transport mechanism in order to reach the central nervous system's components of the insulin signaling pathway. Receptors are widely distributed throughout the brain, with higher density in the hypothalamus, hippocampus and olfactory nerve (Plum et al., 2005). Insulin acts, together with leptin, as an "adiposity signal". It informs the brain of adipose stores' size, initiates negative feedback in structures that control eating behavior, and regulates adipose mass (Figlewicz and Sipols, 2010). Although evidence that insulin acts at a central level exists since a considerable amount of years, this topic has only recently gained a bigger status in human research. It is suggested that central insulin affects, among others, glucose homeostasis, food consumption, body weight, attention and memory (Stockhorst et al., 2004). The experimental manipulation of central effects of insulin by intravenous appliance of this hormone is limited by severe peripheral side effects, i.e. hypoglycemia (Fish et al., 1986). Insulin applied intranasally can prevent such peripheral side effects while it provides a direct route to the central nervous system (CNS). Given that insulin reaches the cerebral spinal fluid (CSF) within 30 min, it is possible that intranasal application follows a fast extraneuronal pathway: it crosses the nose mucosa, diffuses thought the olfactory epithelium into the CSF, and reaches the brain receptors (Born et al., 2002).

In the periphery and under regular physiological states, insulin and cortisol exert antagonistic effects on metabolism. GC's mobilizes energy stores (catabolic effect), while insulin

promotes glucose uptake and energy storage (anabolic effect) (Dallman et al., 1995). Both hormones have the capacity of crossing the blood-brain barrier and acting on the central nervous system (Joels, 1997; Plum et al., 2005). At a central level, cortisol will stimulate food consumption in order to increase energy resources and insulin will inhibit food intake. In a stress situation, the presence of GC-induced insulin moderates the catabolic impact of stress (Dallman et al., 1993). Excess of GCs, as seen in Cushing's syndrome, is associated with increased insulin resistance, an "impaired sensitivity to the effects of insulin on carbohydrate metabolism" (Andrews and Walker, 1999, p. 513), and certain types of obesity (Rosmond, 2005). On the other hand, glucocorticoid depletion increases insulin sensitivity, resulting in higher efficiency of central insulin to reduce body weight and food consumption (Chavez et al., 1997). There is also evidence that the dysregulation of both cortisol and insulin is involved in the etiology of the metabolic syndrome, a cluster of metabolic risk factors for type 2 diabetes and cardiovascular disease (Rosmond, 2005). In the presence of insulin, stress induced GCs enhance the consumption of "comfort foods" (highly palatable, high carbohydrates), that on its turn attenuates the HPA axis stress response (Dallman et al., 2005). This seems to be a synergic effect where GCs are responsible for the general enhancement of food drive and motivation, while insulin regulates the kind of foods chosen (la Fleur, 2006). Interestingly, participants subjected to psychosocial stress showed attenuation of the HPA axis when receiving intranasal insulin, supporting the role of central insulin as another modulator of the stress response (Bohringer et al., 2008).

Considering the importance of central insulin and cortisol in energy metabolism and their complex interaction, we carried out an experiment to study the central effects of both hormones, as well as their interaction, during resting state (Chapter III). A fMRI technique – continuous arterial spin labeling (CASL) – was used to map resting state regional cerebral blood flow (rCBF) of 48 healthy male participants, food restricted, receiving either 40 IU of intranasal insulin, 30 mg of oral cortisol, both or placebo. Intranasal insulin affected neural structures involved in energy homeostasis, as it was visible by increased resting rCBF in the putamen and insular cortex. Cortisol did not exert an effect of its own or in interaction with intranasal insulin.

A third study was conducted (Chapter IV), following a similar pharmacological manipulation as the one described in the previous experiment, this time to analyze effects in affective processing of food cues. Since the FNR effect appears during states of higher motivation towards food, all participants were studied in a state of food deprivation. Data from 57

healthy male volunteers were acquired. Additionally, food pictures were divided in two categories: foods with high glycemic index (GI), and foods with low GI. Such a division was done since both hormones play a crucial role in glucose homeostasis, and therefore a difference of reward value between categories is possible. The GI is the measure of the total rise of glucose in the blood after carbohydrate ingestion by comparison to a reference food. Foods with high GI produce higher postprandial blood glucose than the foods with low GI (Foster-Powell et al., 2002). It was hypothesized that due to the anorexigenic properties of insulin, its administration would diminish the reward value of food cues. In contrast, attending the large literature on enhancement of food consumption by stress, it was expected that cortisol administration would increase the reward value of food cues. Enhanced ASR was found during presentation of "low glycemic" pictures in the four studied groups. However, enhanced ASR during foreground presentation of "high glycemic" pictures was only observed when participants received cortisol. Neither intranasal insulin nor the interaction cortisol x intranasal insulin had any effects in startle. Given that cortisol is one of the most important stress hormones and that during a stress reaction the body mobilizes energy resources in order to prepare to "fight" or "flight", it can be adaptive that cortisol increases the reward value of high glycemic food, foods that will provide faster amounts of energy.

1.3 Summary, limitations, concluding remarks

Food is one of the basic elements of life, responsible for maintaining the energy homeostasis of the body. The research described here replicates and complements findings from previous studies, where it is shown that disruption of this homeostasis by food deprivation provokes a FNR effect. It also adds that FNR is present independent of sex and menstrual cycle, denoting the stability of this effect. According to the definition of FNR by Amsel (1958), these results indicate that in states of hunger appetitive food cues induce a bigger frustration and expectably enhanced motivation and drive towards these foods. A similar pattern can be seen in addiction and drug cue processing, when addicted participants are confronted with relevant cues when in states of high craving or manipulated unavailability (Carter and Tiffany, 2001; Elash et al., 1995). It is known that the amygdala is involved in aversive startle modulation (Davis et al., 1999; Pissiota et al., 2003) and that it has a central role in reward expectancy, affect and appetitive learning (Davis et al., 1999; Gottfried et al., 2003; Holland and Gallagher, 2004). One study has inclusively shown that greater amygdala activation during the processing of visual food cues would occur when participants were food deprived (LaBar et al., 2001). It is possible to hypothesize that the enhancement of the ASR to food cues

during food deprivation could be amygdala mediated, which should be addressed in future research.

Some questions regarding these results remain unclear and would benefit from further investigation. While FNR theory seems to explain the results here obtained, it would have been interesting to see if this increase in motivation would translate into eating behavior. Sex/menstrual cycle differences in eating behavior are more evident during the peri-ovulatory period (Marino et al., 2011). For this reason, further studies should address the effects of other phases of the menstrual cycle, especially of the ovulation.

In our second study we have shown that an acute application of intranasal insulin is sufficient to affect brain regions central in regulation of metabolism during a resting state, while single cortisol application or interaction of both hormones did not have an effect. These results are in line with the importance of central insulin for energy regulation. On the other hand, the last study found that cortisol provoked a FNR effect towards pictures depicting foods high in carbohydrates, and no effects of single application of insulin or interaction were found. Taken together, these results indicate that the effect of cortisol might be situation specific, therefore not visible in a resting state. Besides, intranasal insulin is a long term signal of body weight regulation (opposite to cortisol) (Havel, 2001) what can explain why a neuronal effect was found but no physiological or motivational one. Future studies should explore the effect of chronic administration of insulin and of higher dosages than the one used in this study. All participants were studied during a controlled state of food deprivation, given that motivation towards food is expected to be bigger during hunger. However, it would be interesting to compare the effect of cortisol and insulin also during states of satiation in future studies. It is a limitation of these two experiments that only men were studied. The decision of studying only men is based in the premise that women have higher prevalence of eating disorders (Hay, 1998; Hoek and van Hoeken, 2003) and are less sensitive to the anorexic effects of central insulin (Benedict et al., 2008). Nevertheless, it is recommendable that further research on this topic includes women. Furthermore, attending the importance of food cue processing in eating disorders and obesity, this line of research should be extended to populations with these conditions.

One might question if the FNR theory can really explain the results from cortisol enhanced ASR during foreground presentation of high glycemic food pictures (Chapter IV). Acoustic startle eye blink responses are enhanced in emotionally negative contexts, and reduced during

emotionally positive contexts, and this pattern of affective startle modulation has been clearly established during the last two decades of psychophysiological research (for a review see Koch, 1999). The rationale of our study was built on a previously, well-established model (Drobes et al., 2001) that meanwhile was extended by different laboratories (Mauler et al., 2006; Rejeski et al., 2010; Rodriguez et al., 2005) and also by one of the here reported studies (Chapter II). This model suggests "frustrating nonreward" to explain that food deprivation - although increasing the appetitive value of visual food cues - enhances startle responses to acoustic noise bursts during the presentation of visual food cues. This finding cannot be explained by food deprivation-induced distortion of affective processing "per se", because startle modulation by positively-valenced nonfood cues remains unaffected by food deprivation. Overall, the current study replicates these findings, in what can be considered a validation and successful manipulation check. Given that our participants were food deprived for approximately 14 hours, increased ASR during foreground presentation of food pictures was to be expected. The new result of this study is that this effect is more dominant for low-glycemic food pictures, but that during cortisol treatment this effect is also seen to high-glycemic food pictures. These results find explanation in previous research. The adrenal steroids seem to have different actions according to the type of receptor. Mineralocorticoids stimulate fat intake, while glucocorticoids stimulate mainly carbohydrate intake. These actions of carbohydrates take place when the body is low on carbohydrate stores, as it is expected during some level of food deprivation (Tempel and Leibowitz, 1994). It was also previously shown that when under stress, people change their food consumption from meal-like foods (ex.: fruits, vegetables, meat and fish) to sweets and highly appetitive foods (similar to the high glycemic foods here depicted) (Oliver and Wardle, 1999). Stress activates the dopaminergic system, which in turn evokes pleasure seeking behavior and stress avoidance. Consumption of palatable food, high in sugar and fat, and consequent dampening of the HPA axis, might therefore be stimulated by the action of dopamine in the reward system (Maniam and Morris, 2012). It is therefore possible that the cortisol effect in FNR to high carbohydrate food cues is mediated by the dopaminergic system.

In the developed countries, economical and sociocultural changes have modified food search and consumption. What before was essential, to find and collect all the food available so that the body could subsist until finding a new source of energy, is now easily obtainable. The energy required to search for food diminished but the raise in availability of food can induce a caloric intake that exceeds our homeostatic needs. Furthermore, we are constantly exposed to cues of foods with appetitive high glycemic content, whose consumption can have strong

impact in metabolic diseases. It is important to assess how this food cue stimulation affects the population. This work was performed to extend the knowledge about food cue processing and the role of the stress/metabolic hormones cortisol and insulin in this process. Attending that both stress and metabolic diseases have seen a rise in the last years and appear to be intrinsically connected, it is essential to research these phenomena in respect to emotion and motivation, which are important determinants of human behavior. In addition, the present work emphasizes the validity of startle methodology, specifically of the ASM paradigm, to study FNR effects in food cue processing. In conclusion, this work points to the significant role that food cue processing might have before the actual consumption of food. Increased consumption of "comfort food" seen during stress can contribute to a set of metabolic diseases and therefore should be regarded with great importance. Research in this field might contribute to a better understanding of the phenomena, as well as to the development of pharmacological or cognitive interventions that can be applied before the actual consumption behavior.

Chapter II: Acoustic startle reactivity while processing reward-related food cues during food deprivation: Evidence from women in different menstrual cycle phases and men.

First author: Diana S. Ferreira de Sá

Co-Authors: Debora E. Plein, André Schulz, Melly S. Oitzl, Terry D. Blumenthal & Hartmut Schächinger

2.0 Abstract

Previous research has shown that food deprivation enhances the acoustic startle reflex when it is elicited during presentation of visual food cues. Frustrative nonreward may explain this effect, since visual food cues are also rated to be more appetitive and arousing during food deprivation. However, the impact of menstrual cycle and sex on this effect remains unclear, and it is also not known whether this effect is influenced by hunger and motivation to eat. According to a within-study design, 20 healthy women in different menstrual cycle phases and 14 healthy men participated twice, in normal and food-deprived conditions. After 18 h of food deprivation, acoustic startle was attenuated by appetitive nonfood foreground pictures, but enhanced by presentation of food pictures. No differences between menstrual cycle phases and sexes appeared. The effect correlated with hunger changes, suggesting that motivational factors play a role.

Keywords: Startle modulation, food deprivation, sex, menstrual cycle, food pictures

2.1 Introduction

Acoustic startle responsiveness is enhanced during foreground presentation of aversive slides, and attenuated during appetitive slide presentation. These effects are stronger when slides are rated to be more arousing. This pattern of effects is known as “affective startle modulation” (Bradley, Lang, & Cuthbert, 1993).

Food is biologically preestablished to act as a primary reinforcer. This is reflected in generally high appetitive ratings of nutritional stimuli. Food deprivation increases hunger and the motivation to eat, as well as appetitive and arousal ratings of food pictures (Lüthy et al., 2003). Based on the affective startle modulation paradigm, an additional attenuation of acoustic startle responsiveness during foreground presentation of food slides would be expected in food-deprived subjects. However, the opposite has been found: food deprivation has been associated with increased acoustic startle responses during foreground presentation of food pictures, while acoustic startle responses during foreground presentation of other emotional pictures, aversive or appetitive, were not affected by food deprivation (Drobes et al., 2001; Mauler, Hamm, Weike, & Tuschen-Caffier, 2006).

Drobes and colleagues (2001) interpret these results as indicative of a frustrative nonreward effect, meaning that in a state of drive to ingest food, the visual appearance of food-related stimuli that are impossible to consume would induce frustration. If the drive to consume the food is further enhanced, such as in high cravers for food, an enhanced frustrative nonreward-effect is to be expected. Exactly this was found when acoustic startle responsiveness during foreground presentation of food slides was studied in bulimic patients (Mauler et al., 2006), who are established high food cravers (Waters, Hill, & Waller, 2001). The frustrative nonreward hypothesis is able to explain the results found by two additional human startle experiments. Presentation of consumable "in vivo" foods - instead of food pictures - decreased acoustic startle responsiveness in subjects with high craving for the food items (Hawk, Baschnagel, Ashare, & Epstein, 2004). This is in accordance with the assumption that the ability to consume the presented food prevents the progression into a frustrative nonreward state. A related study that experimentally manipulated the expected duration of unavailability of in vivo food items showed increased acoustic startle responsiveness when participants knew that the presented in vivo foods would be unavailable for an extended period (Rejeski et al., 2010), a finding also compatible with the frustrative nonreward hypothesis.

Contradictory findings have also been reported. A recent study showed that bulimic females express reduced acoustic startle responsiveness during the visual foreground presentation of non-consumable foods (Friederich et al., 2006). Unfortunately, that study did not investigate interaction with sex effects and did not control for the length of food deprivation, but both variables may be of key importance and deserve evaluation. Although some of the above-cited startle studies included women and men, none of them explicitly focused on sex effects

during different states of food consumption (Drobes et al., 2001; Rejeski et al., 2010). However, significant sex differences in food intake, nutritional behavior, and metabolic rate exist (Mittendorfer, Horowitz, & Klein, 2001), and these factors may influence the frustrative nonreward effect seen during food deprivation. Women, in comparison to men, show greater tendency to restrict food intake, are more concerned with weight control, and have a higher prevalence of eating disorders (Asarian & Geary, 2006). Moreover, a high prevalence of neuroendocrine disturbances exists in females with eating disorders, often accompanied by anovulation, which prohibits the large fluctuations of sex hormones seen during a normal menstrual cycle (Morgan, 1999). Sex hormones are known to influence food cognition and feeding behavior (Buffenstein, Poppitt, McDevitt, & Prentice, 1995; Dye & Blundell, 1997; Farage, Osborn, & MacLean, 2008). For example, several studies have shown increased energy intake during the luteal phase of the menstrual cycle (Barr, Janelle, & Prior, 1995; Dalvit, 1981; Li, Tsang, & Lui, 1999), as well as a preference for high caloric nutrients (Dye & Blundell, 1997; Johnson, Corrigan, Lemmon, Bergeron, & Crusco, 1994; Li et al., 1999). Hence, studies on food processing, food deprivation, and startle should explicitly take into account possible interactions effects with sex-related factors of gender and menstrual cycle phase.

Food deprivation induces a variety of complex cognitive, affective, and motivational responses to food cues and directs attention to food stimuli (Seibt, Häfner, & Deutsch, 2007). Interactions of these responses are poorly understood. It is unclear whether hunger and the motivation to consume food may contribute to frustrative nonreward-induced changes of acoustic startle responsiveness seen during foreground presentation of food slides. Previous studies have shown that food deprivation increases hunger and motivation to eat (Lüthy et al., 2003), but such individual changes have never been correlated with frustrative nonreward effects on startle responsiveness seen during food deprivation. This could only be done when the nutritional states are varied experimentally within subjects. However, most of the previous studies had a between-subjects design (Drobes & Tiffany, 1997; Friederich et al., 2006; Hawk et al., 2004; Mauler et al., 2006), with subjects being studied after either food restriction or normal food intake. Therefore, correlation analysis of subjective hunger changes induced by food deprivation could not be conducted.

The present study, however, involved a within-subjects design in order to correlate the startle-enhancing effects of food deprivation with changes in subjective feelings of hunger and motivation to eat. To analyze the role of sex and menstrual cycle effects and the interaction

with food deprivation, our sample consisted of women studied during the follicular menstrual cycle phase, women studied during the luteal phase, and men. Participants were studied twice with different sets of slides, under food deprived and nondeprived (normal food consumption) conditions. The impact of food pictures, and neutral and positive nonfood pictures, was studied.

2.2 Methods

2.2.1 Participants

Three groups of participants were recruited from the University of Trier: 14 males; 10 females to be studied in the follicular phase of the menstrual cycle, and 10 females to be studied in the luteal phase (mean age: 23; range: 20-29). They provided written informed consent and received a moderate monetary incentive. Study procedures were approved by the local ethics committee. Exclusion criteria were: self-reported history of hearing problems, tinnitus, intolerance to lacteal products, intake of any pharmaceutical drugs, smoking, and acute medical or psychiatric symptoms/complaints. All women reported a regular cycle of 25-35 days and no use of hormonal contraceptives for at least the past 6 months. Their body mass index (BMI) had to be within normal range (men: 20-25 kg/m^2; women: 19-24 kg/m^2; according to the German Nutrition Society). Scores on the subscales "dietary restraint", "disinhibition" and "hunger" of the Three-Factor Eating Questionnaire (TFEQ; Stunkard & Messick, 1985; German version from Pudel & Westenhöfer, 1989) were used as exclusion criteria for abnormal eating habits in women. In reference to the German normalization, all the values were in the low to middle range. Due to the lack of standardized scores for males, the subscales "drive for thinness", "bulimia" and "body dissatisfaction" of the Eating Disorder Inventory-2 (EDI-2; Garner, 1991; German version from Thiel et al., 1997) were used as exclusion criteria for abnormal eating habits in men. Cutoff points were established as 1 *SD* above or below the mean score of a non-clinical control group from the German standardization.

2.2.2 Procedure

Female participants were randomly assigned to two groups (between variable): women in the mid-follicular phase of the menstrual cycle (FP, n = 10); and women in the mid-luteal phase (LP, n=10). In accordance with other studies (Li et al., 1999), FP was considered from 6-10 days after the first day of menses, while LP was calculated from 6-10 days after ovulation, which was measured with a luteinizing hormone urine test (Clearblue). They were instructed to phone the experimenter on the first day of their menses. For women in the LP group a precise day to start using the Clearblue test was calculated by taking into account the regular length of their cycle and the first day of the menses; they were instructed to contact the experimenter again as soon as a positive test result was detected.

Participants were tested in a food-deprived condition and nondeprived condition on two different days (within variable), in a randomized and counterbalanced crossover design. The interval between sessions was 1 week for men, based on previous research (Lüthy, et al., 2003), but 1 month for women, to make sure that females could be studied in the same phase of the menstrual cycle in both sessions. The experiment was run always at the same time of the day (between 11:30 am and 2:00 pm), thus avoiding increased variance from time-of-day effects on food intake (Stroebele & De Castro, 2004). Participants entered the laboratory 60 min before the experiment, which lasted 30 min. In the condition of food deprivation (FD), participants were instructed to have the last meal at 6 pm of the previous day in order to achieve an average fasting period of 18 h. In the nondeprived condition (normal food consumption, NC), participants were asked to have a normal breakfast around 8 am and received a standard snack (dark whole wheat bread with fresh cheese and garnished with chives) 60 min before the beginning of the experiment. A cover story was developed to increase participant compliance: they were told that on the day of the experimental sessions a saliva sample would be collected to check their nutritional status, with loss of monetary compensation if the required nutritional state was not achieved. On the day before the experiment, every participant was contacted by phone to again explain the fasting procedure. On both experimental days, saliva samples were collected and, as an additional way to control for compliance, participants were asked to recall their routine from waking till arriving at the experiment (diary methods; Stone, Kessler, & Haythornthwaite, 1991).

After the 60-min waiting time, the participants were prepared for electromyographic (EMG) eye blink recording of the left orbicularis oculi muscle. Skin preparation and electrode placement followed published guidelines (Blumenthal, et al., 2005).

Participants then indicated their levels of stress, arousal, current hunger, and motivation to eat on continuous visual analogue scales (VAS), ranging from 0 to 1,280 (full horizontal screen resolution), and anchored at the left margin with label “0” and at the right margin with label “7”, as well as marks at representative full numbers “1” to “6”. For stress, arousal, and motivation to eat, 0 represented “not at all” and 7 represented “very much”. Concerning the ratings of hunger, 0 meant “not hungry at all” and 7 meant “very hungry”. Participants were instructed via computer screen that a series of pictures would be displayed and that these should be viewed for the entire period of presentation. They were also informed that brief, intense sounds would be delivered throughout the experiment. Participants were asked to neither move nor speak, to sit quietly, and to avoid long periods of eye closure during the recording period. Six startle probes were presented before the experiment to serve as habituation trials. These trials were analyzed separately. Pictures were then displayed such that pictures from the same category were not presented more than two times in a row. Each picture was shown for 6 s, and an acoustic startle stimulus was presented between 2.5 and 5.5 s after picture onset in 80% of the trials. A black screen was shown for 4 s in the interpicture interval. The acoustic startle stimulus was a white noise burst (105 dB, 50 ms, instantaneous rise time) presented binaurally via USB sound card Aureon USB 5.1 (TerraTec Electronic GmbH, Nettetal, Germany) and insert earphones (ER-3A, 10 Ohm, Compumedics Germany, GmbH). The applied sound pressure level (SPL) of a continuous noise was calibrated with an HIS-L impedance simulator connected to an MFE VI front-end and analysis software ArtemiS-H (all from Head Acoustics GmbH, Herzogenrath, Germany), filter setting "A" and time constant "fast" (125 ms). Each session consisted of 60 trials. After the startle component of the experiment, participants were asked to rate the perceived appetitive value of the food pictures on a continuous VAS, ranging from 0 to 1,280 (full horizontal screen resolution), and anchored at the left margin with label “0” and at the right margin with label “9”, as well as marks at representative full numbers “1” to “8” (0 indicates extremely negative, and 9 extremely positive). Additionally, arousal ratings for all pictures were requested using the same type of scale. After completing the experimental session the subjects were debriefed or instructed for the second session and thanked for their participation.

2.2.3 Materials

Experimental material consisted of two sets of 60 pictures (120 different pictures in total), counterbalanced for the two food conditions. Pictures were collected from an internet database and from The International Affective Picture System (IAPS; Lang, Bradley, & Cuthbert, 2005). Each set contained 15 pleasant[1], 15 neutral[2], and 30 food pictures[3]. Different erotic pictures were used according to the sex of the participant: erotic couple pictures were displayed to women, while female nude pictures were displayed to men (Bradley, Codispoti, Sabatinelli, & Lang, 2001; Lykins, Meana, & Strauss, 2008). The two sets of pictures were matched for luminosity, number of displayed items, and background composition. Order of set presentation was randomized and counterbalanced.

2.2.4 Recording and analysis of the startle response

EMG responses were recorded with a BIOPAC MP 150 system and an EMG 100C amplifier at a sampling rate of 1000 Hz, with a notch filter of 50 Hz and a software band-pass filter of 28 to 500 Hz (van Boxtel, Boelhouwer, & Bos, 1998). Data were rectified and integrated online with a time constant of 10 ms (Blumenthal, 1994).

EMG blink responses were analyzed by a customized C++ based semiautomated PC program (Clip 2.0.0), which identifies response peaks in the time interval of 20-150 ms after stimulus onset, and a baseline period 50 ms prior to stimulus onset. All data were subjected to manual confirmation. Startle response amplitude was computed as the difference between peak and baseline. Trials with electrical and physiological artifacts (i.e., coinciding blinks, other facial muscular activity, multiple peaks, excessive background noise) were set to missing data, while trials with no visible startle response were scored as zero. Startle magnitudes were calculated including zero responses.

[1] Pleasant pictures depicted puppies and babies, group activities, sports, and erotica.
[2] Neutral pictures depicted nonfood-related office and household objects, cars and parts of cars, and plain buildings. IAPS picture numbers used in this study were 7002, 7025, 7050, 7090, 7100, 7130, 7140, 7185, 7186, 7217, 7224, 7235, 7705, 7710.
[3] Food pictures depicted familiar and well-appreciated foods (see appetitive ratings): burgers, pizza, lasagna, ice cream, pastry, cake, fries, sandwiches, steak, fried eggs and bacon, sausages, cheese, ham, meatballs, grilled chicken, and barbecued ribs.

2.2.5 Statistical analysis

All statistical analysis were performed with SPSS (version 17); the level of significance was set to (α) = 0.05. To test whether groups differ in startle responsiveness, individually averaged raw startle response data during the state of normal food consumption and foreground presentation of neutral pictures were tested as a dependent variable by univariate analysis of variance (ANOVA) with group (men, women in FP, and women in LP) as the independent variable. To minimize between-subject variability, individual raw data were then *T* scored using all blinks of each participant (Blumenthal, Elden, & Flaten, 2004). All further analyses of startle data were based on standardized data.

Startle habituation was studied with a 2 × 6 × 3 mixed design ANOVA with *food condition* (normal food consumption vs. food deprivation) and *time* (startle 1 to 6) as within-participants factors, and *group* (men, women in FP, women in LP) as a between-groups factor. Differences in startle responses during neutral picture presentation were studied by 2 × 3 mixed design ANOVA with *food condition* as within-participants factor, and *group* as a between-groups factor. Startle reaction during picture presentation was tested by mixed design 3 × 2 × 3 ANOVA with *food condition* and *picture category* (neutral vs. pleasant vs. food) as within-participants factors, and *group* as a between-groups factor. A priori contrast analysis was set against neutral pictures. Interaction results were clarified with paired *t* tests. Ratings for the *appetitive value* of the food pictures were analyzed by 3 × 2 mixed-design ANOVA with *food condition* as a within-participants factor and *group* as a between-groups factor. Arousal ratings of all pictures were studied by mixed design 3 × 2 × 3 ANOVA with *food condition* and *picture category* as within-participants factors, and *group* as a between-groups factor, with contrasts set against neutral pictures. A 3 × 2 mixed design ANOVA with *food condition* as a within-participants factor and *group* as a between-groups factor was used to study all the subjective ratings (*hunger, motivation to eat, stress,* and *arousal*) taken at the beginning of the experiment.

Three new indices were calculated in order to prepare for later correlation analysis of individual response differences. First, the difference between startle responsiveness during food slide presentation and neutral non-food slide presentation was calculated for the state of normal food consumption, *ΔF(NC)*, and second for the food deprived state, *ΔF(FD)*. Third, the difference between these two indices was calculated, *ΔF(FD-NC)*. The first two indices constitute a measure of startle attenuation during foreground presentation of food pictures,

while *ΔF(FD-NC)* can be considered a measure of the frustrative nonreward effect. Spearman's correlation coefficients were calculated between these three indices and other relevant variables: BMI, and subjective ratings of hunger and motivation to eat.[4]

The balanced assignment of food condition (normal food consumption vs. food deprivation first) was initially tested for order effects. Since order did not significantly affect the results, it was not included as an additional factor, to save statistical power. The Greenhouse-Geisser adjustment was applied whenever sphericity adjustment was necessary (adjusted *p* values are reported with uncorrected degrees of freedom and epsilon-values). Due to a technical failure, subjective ratings (*hunger*, *motivation to eat, stress,* and *arousal*) from one participant were missing and therefore not included in statistical analysis involving these data.

2.3 Results

2.3.1 Baseline startle responsiveness

A main effect of the factor *time*, $F(5,155) = 29.86$, $p < .001$, indicated habituation of startle, which did not interact with *food condition* or *group* ($p > .05$). Furthermore, a main effect of *food condition* was seen, $F(1,31) = 4.43$, $p = .04$, with participants presenting higher startle magnitude in the deprived condition compared to the nondeprived state. However, acoustic startle responsiveness during neutral picture presentation did not change with *food condition*, $F(1,31) = 0.27$, $p > .05$, nor interact with the *group* factor, indicating that acoustic startle responsiveness during picture presentation was not per se affected by food deprivation.

2.3.2 Modification of startle by food deprivation or picture content

Statistical analysis of the raw startle response data assessed during foreground presentation of neutral pictures in the nondeprived state showed no group differences, $F(2,31) = 1.17$, $p = .33$ (men: $M = 23.19$, $SD = 12.47$; women in follicular phase: $M = 30.45$, $SD = 24.76$; women in luteal phase: $M = 18.76$, $SD = 13.92$). All other statistical testing of within effects and interactions was based on T-scored startle data. The three-way ANOVA (between-factor: *group*; within-factors: *food condition* and *picture category*) revealed no main effect of *food*

[4] All correlations between indices and ratings were conducted with the correspondent food intake state (NC or FD) or, in the case of the third index, the difference between the two states (ΔFD-NC).

condition, $F(1,31) = 2.37$, $p > .05$, but a main effect of *picture category*, $F(2,62) = 8.63$, $p < .001$. The two-way interaction *food condition* × *picture category* was also significant, $F(2,62) = 6.01$, $p = .007$, $\varepsilon = .85$. No other significant interactions were found. Contrast analysis revealed significantly lower eye blink magnitude when startle was elicited during the pleasant as compared to the neutral picture category, $F(1,31) = 17.22$, $p < .001$, without any significant influence of food condition (see Figure 1). Contrast analysis of startle magnitude during food pictures versus neutral pictures showed no main effect of *picture category*, $F(1,31) = .74$, $p > .05$, but an interaction of *picture category* × *food condition*, $F(1,31) = 11.05$, $p = .002$. Paired t-tests revealed that during foreground presentation of food pictures, participants had higher startle magnitude when food deprived than when nondeprived, $t(34) = -3.23$, $p = .003$ (see Figure 1).

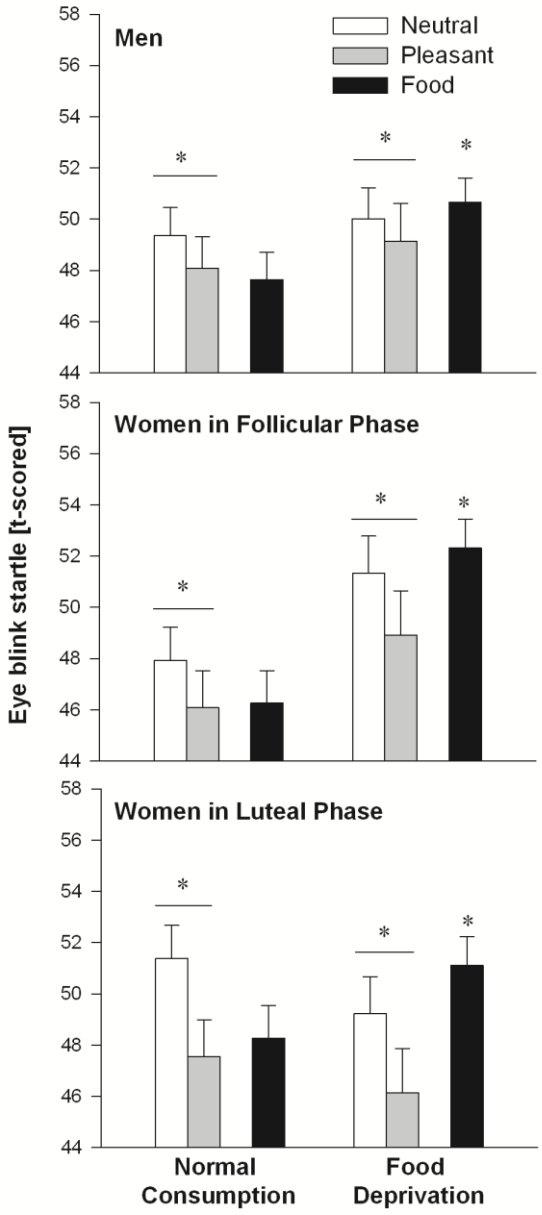

Figure 1. Mean startle eye blink for men, and women in the FP or LP of the menstrual cycle, in the condition of food deprivation (FD) or normal consumption (NC), during foreground presentation of nonfood pictures (neutral and pleasant) and food pictures. All groups show a significant decrease in the startle response from neutral to pleasant pictures in both food conditions. During presentation of food pictures, all groups show higher startle response in a state of food deprivation. Data are reported as *T* scores. Error bars indicate one standard error.

2.3.3 Affective ratings of the pictures

Mean affective ratings of the pictures by *food condition* and *group* are shown in Table 1. Analysis of appetitive ratings of the food pictures showed that they were rated with higher appetitive value during food deprivation than following normal consumption, $F(1,31) = 35.75$, $p < .001$. Arousal ratings of all pictures presented a main effect of *food condition*, $F(1,31) = 15.14$, $p < .001$, and *picture category*, $F(2,62) = 97.86$, $p < .001$, as well as a *food condition* × *picture category* interaction, $F(2,62) = 13.45$, $p < .001$, $\varepsilon = .84$. Contrast analysis revealed that both pleasant pictures and food pictures were rated more arousing than neutral pictures (respectively: $F(1,31) = 269.77$, $p < .001$; $F(1,31) = 31.43$, $p < .001$). However, compared to the neutral picture category, only the arousal ratings of food pictures were affected by food condition, $F(1,31) = 40.47$, $p < .001$. Food pictures were rated significantly more arousing during deprivation than during normal food consumption, $t(33) = -6.18$, $p < .001$. No main effect or interaction of group was found for any of the affective ratings (all *ps* > .05).

Table 1. Mean Affective Ratings of Pictures and Subjective Ratings of Hunger, Motivation to Eat, Stress, and Arousal

	Normal Consumption			Food Deprivation		
Affective ratings of pictures	Men	Women FP	Women LP	Men	Women FP	Women LP
Food						
Appetitive value	748 ± 219	808 ± 200	708 ± 194	922 ± 226	964 ± 112	863 ± 162
Arousal	404 ± 308	374 ± 252	232 ± 116	501 ± 316	503 ± 291	409 ± 269
Neutral						
Arousal	320 ± 213	164 ± 53	124 ± 49	281 ± 195	165 ± 50	139 ± 65
Pleasant						
Arousal	667 ± 280	635 ± 179	593 ± 195	698 ± 256	703 ± 178	616 ± 180
Subjective ratings						
Hunger	297 ± 60	309 ± 52	303 ± 67	750 ± 114	714 ± 108	780 ± 101
Motivation to eat	302 ± 52	355 ± 77	323 ± 67	807 ± 130	777 ± 97	829 ± 95
Stress	441 ± 150	531 ± 107	453 ± 196	387 ± 122	477 ± 131	469 ± 173
Arousal	433 ± 100	465 ± 125	427 ± 105	406 ± 131	432 ± 116	448 ± 177

Note. Results are presented by condition (normal consumption or food deprivation) and group: men, women in the follicular phase (FP), and women in the luteal phase (LP). All data represent $M \pm SD$.

2.3.4 Subjective ratings

Subjective ratings of hunger, motivation to eat, stress and arousal, measured before slide presentation, are shown in Table 1, separately by *food condition* and *group*. A significant main effect of *food condition* was found for hunger, $F(1,30) = 513.34$, $p < .001$, and motivation to eat, $F(1,30) = 515.85$, $p < .001$. Food-deprived participants reported stronger hunger and motivation to eat compared to the normal consumption condition. Subjective stress and arousal ratings were not affected by food condition ($p > .05$). There was neither a main effect of *group* nor significant interactions for any of the subjective ratings (all *ps* > .05).

2.3.5 Correlation analysis

Correlations between the indices of startle attenuation and relevant variables varied with the state of food intake. There were statistically significant correlations between *ΔF(NC)* and BMI, $r = .36$, $p = .02$, and with subjective ratings of hunger, $r_s = .44$, $p = .01$. No other significant correlation was found. On the other hand, no relationship between *ΔF(FD)* and any of the variables was found. *ΔF(FD-NC*), the index of frustrative nonreward effect, had a significant correlation with the difference (*ΔFD-NC)* in the ratings of hunger, $r_s = .37$, $p = .03$ (Figure 2a), and motivation to eat, $r_s = .41$, $p = .02$ (Figure 2b).

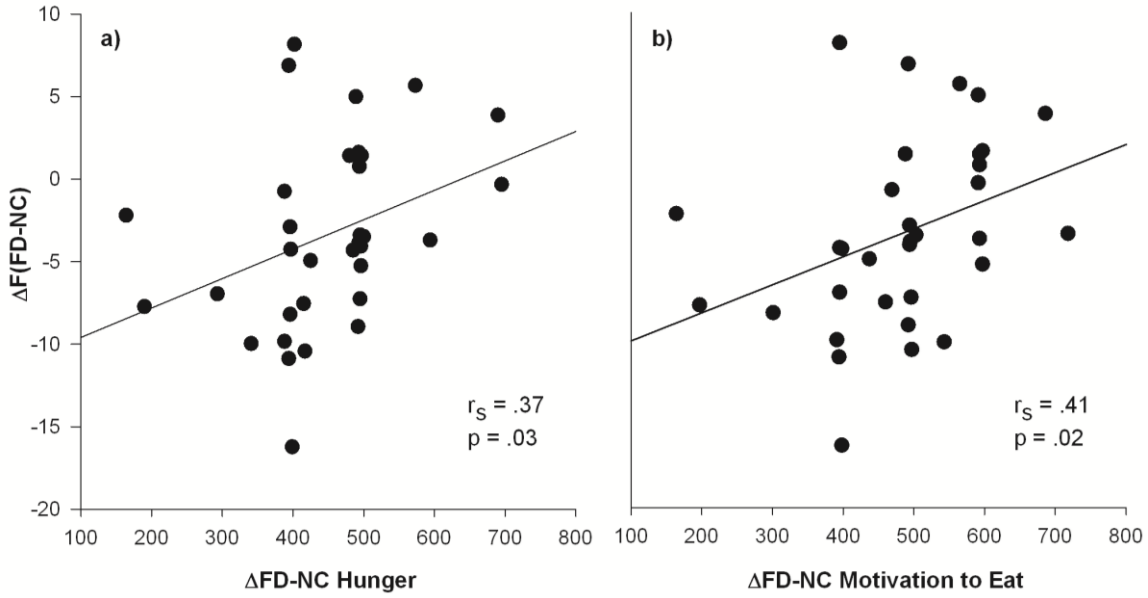

Figure 2. Scatter plot showing relation of frustrative nonreward effect index [ΔF(FD-NC)] with (a) difference of hunger ratings between the two states of food intake (ΔFD-NC Hunger); (b) difference of motivation to eat between the two states of food intake (ΔFD-NC Motivation to eat). Spearman correlation coefficients (rs) and *p* values are reported.

2.4 Discussion

This is the first within-subject design study indicating that moderate food restriction enhances acoustic startle responsiveness during the visual presentation of food slides. This effect was present in the female subjects groups studied during the follicular or during the luteal menstrual cycle phase, as well as in the male subjects group, with no differences among groups detected. Changes in hunger ratings and motivation to eat induced by food deprivation were positively correlated with the changes in startle reactivity during food picture presentation. Startle responses elicited during the presentation of nonfood (neutral and pleasant) pictures were not affected by food deprivation, either in women in both phases of the menstrual cycle, or in men. Food pictures were rated more arousing and appetitive during food deprivation, but arousal ratings of pictures from other categories remained unchanged. The higher startle response to food cues during food deprivation is in line with previous findings from Drobes and colleagues (2001), who argued that food pictures prompt an aversive motivational state (Amsel, 1958) during food deprivation, when food consumption is prevented.

These findings are based on a moderate food restriction of 18 h and noninvasive, nonstressful methods. Following Mauler and colleagues' (2006) suggestions for food deprivation studies with healthy participants, an invasive measure of compliance (e.g., serum ß-hydroxybutyric acid) was not considered to be essential. Furthermore, food-deprived participants reported significantly stronger baseline ratings of hunger and motivation to eat, thus suggesting that food condition was successfully manipulated (Lüthy et al., 2003). Importantly, no differences between food conditions in baseline stress and arousal ratings were seen. These factors have been shown to increase startle in other studies (Cuthbert, Bradley, & Lang, 1996; Grillon & Baas, 2003), but cannot explain the results of the present study.

Habituation rate of startle responsiveness with repetitive stimulation occurred as expected (Koch, 1999), and was not affected by food deprivation or group. This finding is consistent with previous research showing no sex effects on startle-alone trials (Ludewig et al., 2003; Swerdlow et al., 1993). An increase of acoustic startle reflex in the absence of other stimuli during food deprivation has been reported in animal research (Anderson, Crowell, & Brown, 1985; Meryman, 1952, as cited in Brown & Farber, 1968; Szabo, 1967), though contradictory results have been found (Armus & Isham, 1984; Fechter & Ison, 1972). In the present study, deprived subjects showed a general enhanced startle response during the habituation trials, but

this difference did not appear during the later phase of the experiment, when startle was elicited during neutral foreground slide presentation. This indicates that startle alone may be affected by food deprivation, but that this difference disappears when control slides are presented. This may reflect a task effect or additional habituation following the six habituation trials.

Participants rated pleasant pictures with higher arousal than the neutral pictures and consistently showed attenuation of the startle response to positive as compared to neutral pictures, as was expected for the affective modulation of the startle to standard emotional pictures (Bradley, et al., 1993). Food deprivation had no impact on affective responses (ratings and startle) to standard emotional nonfood pictures, similar to previous studies (Drobes, et al., 2001; Mauler, et al., 2006). Neither sex nor menstrual cycle phase interacted with the affective ratings or startle response to neutral and pleasant pictures. Although differences between men and women in picture processing have previously been found, these were primarily associated with unpleasant stimuli (Bradley, Codispoti, Sabatinelli, & Lang, 2001), which we did not include in our study. Epperson and colleagues (2007) also found no effects of menstrual cycle on startle modulation to standard emotional pictures in healthy participants.

Enhanced acoustic startle responsiveness during the visual presentation of food slides in the food-deprived state was present in all groups, independent of sex and menstrual cycle phase. Menstrual cycle classification was done on the basis of self-reports and testing for ovulation with a luteinizing hormone detection test (Clearblue). The phases of the menstrual cycle are characterized by different concentrations of the hormones progesterone and estradiol, which can modulate the startle response (Toufexis, Davis, Hammond, & Davis, 2004; Vaillancourt, Cyr, Rochford, Boksa, & Di Paolo, 2002) and affect eating behavior/cognition (Buffenstein et al., 1995; Dye & Blundell, 1997; Farage et al., 2008). The presence of anovulatory cycles is common, especially in women with eating disorders (Morgan, 1999), affecting the normal fluctuation of sex hormones. To our knowledge, this is the first food and startle study in females excluding anovulation (Drobes et al., 2001; Edler, Lipson, & Keel, 2007; Friederich et al., 2006; Rejeski et al., 2010). Confirmation by other researchers of the lack of differences among groups may have important practical implications, since such confirmation may make the recruitment of control cohorts unnecessary. Recruiting control cohorts for menstrual cycle effects is time consuming and expensive, and may increase subject burden.

Food depletion represents a challenge to energy homeostasis. It provokes an increase in food consumption (Drobes et al., 2001; Hetherington, Stoner, Andersen & Rolls, 2000; Spiegel, Shrager, & Stellar, 1989), induces strong cognitive adaptations, such as an attentional bias towards food cues (Channon & Hayward, 1990; Seibt et al., 2007), increases event-related brain potentials to food stimuli (Stockburger, Weike, Hamm, & Schupp, 2008), reinforces the value of food (Raynor & Epstein, 2003), and increases hunger and the motivation to search for and consume food (Lüthy et al., 2003). As in other studies (Drobes et al., 2001; Lüthy et al., 2003), food pictures were rated higher in arousal and appetitive value during deprivation than following normal food consumption. Arousing pictures are known to direct attention to the visual modality (Bradley, Codispoti, Cuthbert, & Lang, 2001), which may decrease acoustic startle responsiveness (Filion, Dawson, & Schell, 1998). If arousal ratings provide information about the extent of attention directed towards the visual modality, we would expect the increased arousal ratings of food pictures during deprivation in this study to result in lower acoustic startle responsiveness. However, the opposite was found, suggesting that crossmodal attentional mechanisms do not explain our results.

Hunger and motivation to eat may contribute to the changes of acoustic startle responsiveness seen during foreground presentation of food slides (Drobes et al., 2001; Mauler et al., 2006). The within-study design used in this experiment allowed for correlation analysis between hunger, motivation to eat, and startle effects. Attenuation of the startle response during presentation of food pictures was positively associated with BMI and hunger ratings in the non-deprived state, a finding which is in accordance with the affective modulation of the startle response paradigm. BMI was previously shown to be linked to a tendency to overeat in the presence of satiation (Hays et al., 2002; Hays & Roberts, 2008), and this behavior may be reflected in appetitive-like startle effects. Importantly, the food picture-induced startle effects (frustrative nonreward effect index) correlated with changes in hunger and motivation to eat. A greater effect was seen with increased difference in subjective hunger and motivation to eat, between the food deprived and the satiated condition. These findings support a frustrative nonreward effect during deprivation, where higher frustration should result from impeding high motivation. Although frustrative nonreward might explain these data, a true test of this hypothesis would require a second measure of negative affect during deprivation, which was not possible in this study.

Interestingly, addiction and drug cue reactivity research also suggest a role of stimulus availability (Carter & Tiffany, 2001; Elash, Tiffany, & Vrana, 1995). Elash and colleagues

(1995) showed that smokers showed significant startle potentiation when processing imagery scripts with an explicit smoking urge. These data from addiction research support the hypothesis of startle potentiation to appetitive cues associated with high craving and low availability. Although we have no direct measure of food craving, food-deprived subjects indicated higher ratings of hunger and motivation to eat, and rated the food cues as more arousing and appetitive, indicating a state of wanting to consume food during food deprivation.

The results of this study suggest that food pictures prompt a frustrative nonreward effect during food deprivation. Menstrual cycle phase and sex do not play a role in this effect, but the effect is influenced by changes in hunger and motivation to eat.

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2.ii AUTHOR NOTES

Address reprint requests to: Diana Ferreira de Sá, Institute of Psychobiology, University of Trier, Johanniterufer 15, D-54290 Trier, email: s1diferr@uni-trier.de.

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Chapter III: Intranasal insulin increases regional cerebral blood flow in the insular cortex in men independently of cortisol manipulation.

First authors: Thomas M. Schilling & Diana S. Ferreira de Sá

Co-Authors: René Westerhausen, Florian Strelzyk, Mauro F. Larra, Manfred Hallschmid, Egemen Savaskan, Melly S. Oitzl, Hans-Peter Busch, Ewald Naumann, & Hartmut Schächinger

3.0 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 this 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

3.1 Introduction

Adrenal glucocorticoids and the pancreatic peptide insulin jointly play key roles in energy homeostasis, co-dependably regulating food intake and metabolism (Dallman, et al. 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, et al. 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, et al. 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, et al. 2004a). Peripheral insulin accessing the brain communicates the size of adipose stores to the central nervous system (Figlewicz and 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, et al. 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, et al. 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" (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, et al. 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, et al. 2000; Schulingkamp, et al. 2000; Werther, et al. 1987). Recently, it has been demonstrated that insulin facilitates repetitive spike firing in the rat insular cortex (Takei, et al. 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 and Rolls 2004) and visual appearance (Ohla, et al. 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 (CSF) without relevant blood absorption, and consequently without peripheral side-effects such as hypoglycemia (Born, et al. 2002; Kern, et al. 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.

3.2 Methods

3.2.1 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.

3.2.2 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).

3.2.3 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 10pm 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 min) 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 min) was acquired. This was followed by five independent but subsequent functional measurement-blocks (*Block 1-5*; duration of 6:50 min 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 min after administration (Born, et al. 2002). The functional measurement-blocks therefore covered a time span of approximately 35 min, lasting 30 to 65 min 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 min 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 3 for an overview on the experimental procedure.

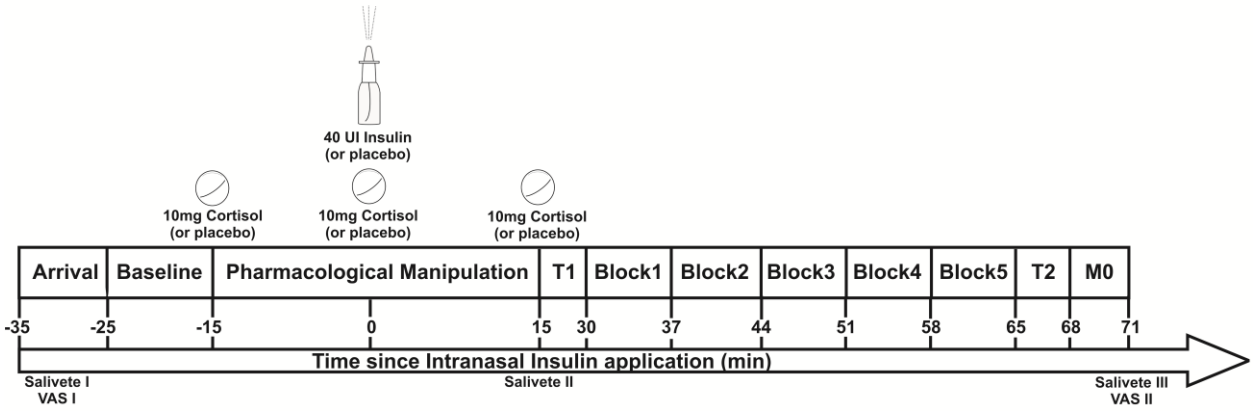

Figure 3. Flowchart of the experiment. After arrival participants entered the scanner for scan 1 (baseline). The harmacological treatment was given out of the scanner. The second scan started by recording an anatomical T1-scan before five functional-blocks, an anatomical T2-scan and a M0-scan were recorded. 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.

3.2.4 Measurements

3.2.4.1 Assessment of salivary cortisol

Salivary morning cortisol on the experimental day was assessed for all participants 0, 30, 45 and 60 min 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, et al. 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.

3.2.4.2 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*.

3.2.5 MRI-Measurements

3.2.5.1 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/100 g 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 and Alsop 1999; Wang, et al. 2008).

3.2.5.2 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, 64 × 64; 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 128 × 128 matrix. Labeling was achieved with a flow driven adiabatic inversion technique (Alsop and Detre 1996). In order to control for magnetization transfer effects an amplitude modulated version of the CASL technique was used (Alsop and 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 s 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 (T1-weighted gradient recalled echo (fast field echo); 160 slices; FOV, 256 × 192 mm; matrix, 256 × 256; 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.

3.2.5.3 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 T1-Image and then normalized to the MNI 152 average brain (Montreal Neurological Institute; Mazziotta, et al. 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 λ = 0.98 for gray matter quantification (Herscovitch and Raichle 1985), the T1 for arterial blood to 1.4 s and the labeling efficiency to α = 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 s). 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.

3.2.5.4 Selection of region of interest

Receptors for insulin and cortisol have been reported in the entire cortex as well as in subcortical structures (Sanchez, et al. 2000; Sara, et al. 1982; Schulingkamp, et al. 2000; Unger, et al. 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, et al. 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 and 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.

3.2.6 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).

3.2.6.1 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.

3.2.6.2 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.

3.2.6.3 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*.

3.2.6.4 Continuous arterial spin labeling analysis

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.

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".

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, et al. 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 min 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/).

3.3 Results

3.3.1 Salivary morning cortisol

Participants of the four groups had comparable salivary morning cortisol levels, calculated as AUCg ($F(3\ 42) = 0.51$, $p = .68$). Mean and standard error of means for each group were as follows: "insulin & cortisol" $M = 665 \pm 89$; "insulin & oral placebo" $M = 543 \pm 87$, "cortisol & intranasal placebo", $M = 562 \pm 51$, "oral & intranasal placebo" $M = 633 \pm 89$.

3.3.2 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.

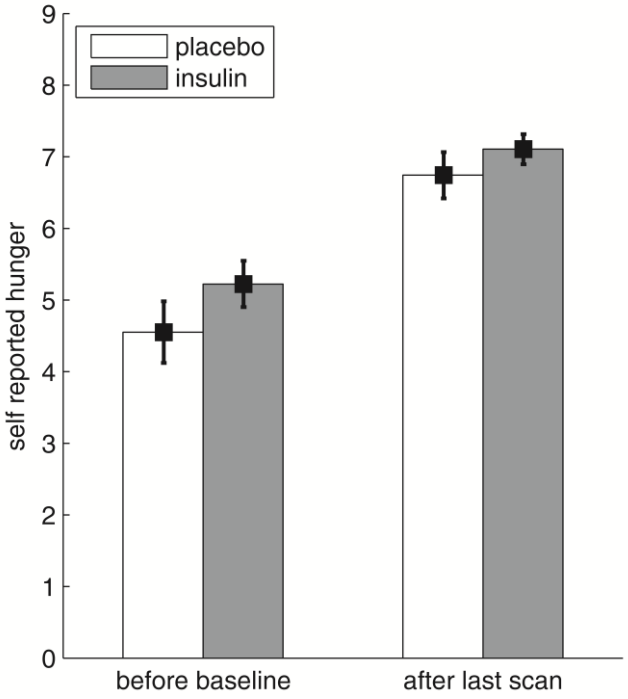

Figure 4. Values of self-reported hunger of participants receiving insulin ($N = 22$) compared with participants receiving placebo ($N = 24$) before baseline and after the last scan. Data present mean and standard error of the mean.

3.3.3 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 $p > 0.05$).

3.3.4 ROI-analysis

3.3.4.1 Primary versus Control ROI

We observed a three-way interaction "insulin" × "ROI" × "measurement-block", F (5, 210) = 2.804, p = .018, η_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 min 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 5). 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).

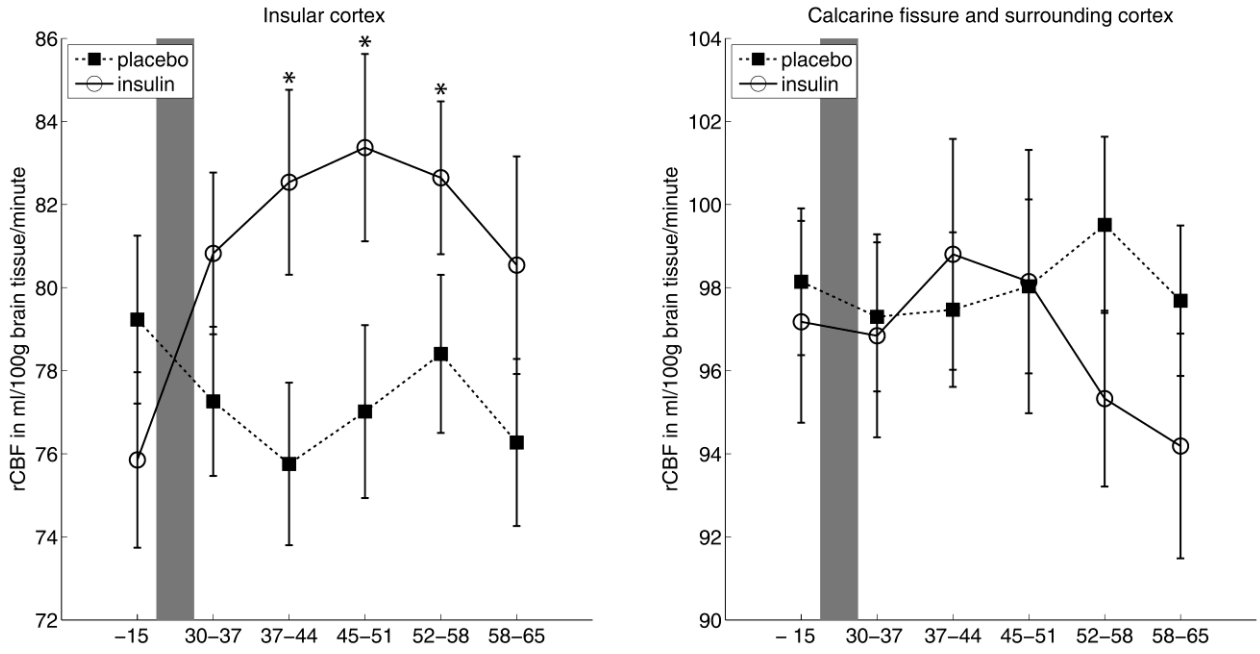

Figure 5. Regional cerebral blood flow (rCBF) over time for participants receiving insulin (N = 22) versus participants receiving placebo (N = 24); left panel: in the insular cortex; right panel in the calcarine fissure and surrounding cortex. X-axis values are in minutes relative to the application of the intranasal insulin versus 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/100 g brain tissue/minute, C = 20).

3.3.4.2 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).

3.3.4.3 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 6 and Table 2). 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.

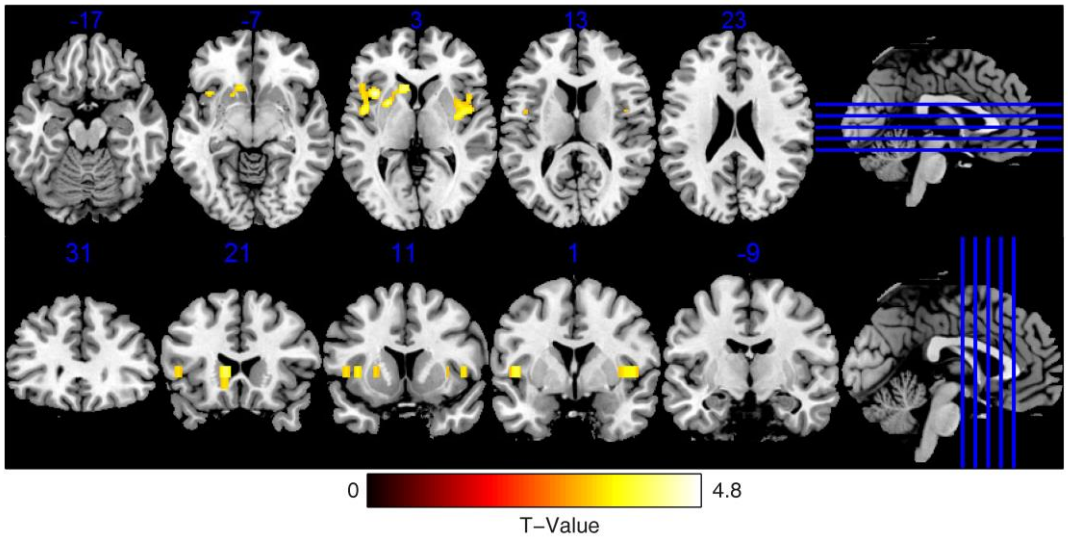

Figure 6. Baseline corrected CBF-increases in participants receiving intranasal insulin compared to participants receiving placebo in all five post-intervention measurement-blocks, corresponding to a time interval of 30-65 minutes after the pharmacological manipulation. No CBF-decreases were observed. 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 2 for more information on cluster significance values and peak *Z*-scores.

3.4 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 min 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.

Table 2. Clusters of significant activation in the voxel-based exploration in participants receiving intranasal insulin: 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 6 (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).

	Cluster-Level		**Peak-Level**			
				MNI[d] Coordinates (mm)		
Anatomical Region[a]	$P_{FWE-corr}$[b]	k_E[c]	Z-Value	X	Y	Z
Insula & 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 & 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

[a]based on the AAL template implemented in MRIcron, [b]familywise error corrected value for cluster, [c]number of active voxels per cluster, [d]Montreal Neurological Institute

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 and 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 and 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 (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 non-genomic and slow genomic cortisol effects, it has, compared to an intravenous (IV) application, the major advantage of not being invasive and stressful by itself.

This 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 and Petrides 1999; Small 2010; Veldhuizen, et al. 2011). Activity in the insular cortex is known to be modulated by subjective appetite ratings (Porubska, et al. 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 bloodflow 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 bloodflow 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 bloodflow 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 min 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 min after insulin admission and last until 65 min after. The time span of insulin effects observed in the ROI analysis of 37-58 min 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, et al. 2008; Hallschmid, et al. 2004b; 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, et al. 2012). Nevertheless, since we administered oral cortisol in order to reach steady-state cortisol plasma levels, we cannot exclude the possibility that fast non-genomic cortisol effects would not affect insulin dependent signal pathways. Direct IV-administration of cortisol into the human blood exerts non-genomic 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-adrenal (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, et al. 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, et al. 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 min 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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3.ii AUTHOR NOTES

Thomas M. Schilling and Diana S. Ferreira de Sá contributed equally to this work and share the first authorship.

Correspondence to: T. M. Schilling, Institute of Psychobiology, Department of Clinical Psychophysiology, Johanniterufer 15, D-54290 Trier, Germany, E-mail: s1thschi@uni-trier.de or D. S. Ferreira de Sá, Institute of Psychobiology, Department of Clinical Psychophysiology, Johanniterufer 15, D-54290 Trier, Germany, E-mail: s1diferr@uni-trier.de

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Chapter IV: Cortisol, but not intranasal insulin, affects the central processing of visual food cues.

First author: Diana S. Ferreira de Sá

Co-Authors: André Schulz, Fabian E. Streit, Jonathan D. Turner, Melly S. Oitzl, Terry D. Blumenthal and Hartmut Schächinger

4.0 Abstract

Stress glucocorticoids and insulin are important endocrine regulators of energy homeostasis, but little is known about their central interaction on the reward-related processing of food cues. According to a balanced group design, healthy food deprived men received either 40 IU intranasal insulin (n=13), 30 mg oral cortisol (n=12), both (n=15), or placebo (n=14). Acoustic startle responsiveness was assessed during presentation of food and non-food pictures. Cortisol enhanced startle responsiveness during visual presentation of "high glycemic" food pictures, but not during presentation of neutral and pleasant non-food pictures. Insulin had no effect. Based on the "frustrative nonreward" model these results suggest that the reward value of high glycemic food items is specifically increased by cortisol.

Keywords: Cortisol; intranasal insulin; affective startle modulation; food cues; glycemic index

4.1 Introduction

Food deprivation represents a challenge to energy homeostasis, which is of fundamental importance to all animal species. Energy homeostasis is achieved when metabolism (catabolic and anabolic forces) is in long term balance (Woods, 2009). Adrenal glucocorticoids and the pancreatic peptide insulin play a crucial role in maintaining this balance: they act and interact in complex fashion, exerting both synergic and antagonistic effects (Dallman et al., 1995). Dysfunction and a disturbed interaction of these hormones in the periphery have been linked to somatic diseases such as diabetes, metabolic syndrome, and abdominal obesity (Dallman et al., 2005). However, there is convincing evidence that both hormones act on the brain as well

(Dallman et al., 1995; Joels, 1997; Plum et al., 2005). Glucocorticoids influence nutritional behavior and food intake (Tataranni et al., 1996). Insulin acts as a satiety hormone, thereby decreasing the reward value of food cues (Schwartz, 2001). Still, little is known about interactions of insulin and glucocorticoids on the cognitive and affective processing of food cues.

Insulin is a peptide produced in pancreatic ß-cells. It is released in response to blood glucose increases, e.g. after carbohydrate ingestion. The main function of insulin is to promote glucose uptake of muscle, fat, and liver cells; the latter stores glucose in the form of glycogen (Lawrence and Roach, 1997). Brain neurons, however, also take up glucose by insulin-independent carriers. Insulin receptors are widely distributed throughout the mammalian brain, especially in areas implicated in regulation of energy homeostasis, such as the hypothalamus (Schwartz, 2001; Plum et al., 2005). Insulin originating from the pancreas reaches those receptors via the systemic circulation and transfer across the blood brain barrier, and may represent a feedback signal contributing to the neuronal control of energy homeostasis, hunger, and satiety (Schwartz, 2001). In a previous study we have shown that insulin administered intranasally - thus, entering the brain while avoiding a systemic distribution and side-effects (Born et al., 2002) - specifically increased cerebral blood flow in structures involved in the regulation of eating behavior, such as the insular cortex (Schilling et al., 2014). This finding fits well with behavioral studies indicating that intranasal insulin increases satiety, reduces food intake, and promotes weight loss (Hallschmid et al., 2004; Hallschmid et al., 2012; Jauch-Chara et al., 2012).

In humans the glucocorticoid cortisol is the end-product hormone of the hypothalamic–pituitary–adrenal (HPA) axis. The HPA axis plays an important role during stress, but also in energy homeostasis. Cortisol represents a catabolic substance that supports the mobilization of energy compounds, such as glucose (Tsigos and Chrousos, 2002). Cortisol crosses the blood-brain barrier and reaches receptors inside the central nervous system (Joels, 1997), and there is evidence that cortisol acts on central regulation of energy homeostasis and influences eating behavior. Higher cortisol reactivity to a psychosocial stressor (Epel et al., 2001), as well as glucocorticoid administration (Tataranni et al., 1996), have been associated with increased food consumption. Adam and Epel (2007) theorize in their "Reward Based Stress Eating model" that stress induced cortisol activates a reward system, motivating the intake of food. Interestingly, high caloric food may then have a dampening effect on HPA axis activity (Dallman et al., 2005), thus constituting a behaviorally-relevant negative feedback control

mechanism. Insulin may play a role in such a negative feedback mechanism, since intranasal administration of insulin dampens HPA axis responsiveness during social stress (Bohringer et al., 2008).

In the periphery and under regular circumstances insulin and cortisol interact mainly in an antagonistic fashion. Cortisol induces insulin resistance, possibly by suppression of insulin-dependent vasodilatation, inhibition of GLUT-4 translocation to the cell surface, and lipolysis activity (Andrews and Walker, 1999). Furthermore, in healthy individuals glucocorticoids stimulate compensatory insulin secretion (Nicod et al., 2003). The combined elevation of glucocorticoids and insulin increases the storage of abdominal fat and contributes to the development of obesity, type II diabetes, and metabolic syndrome (Dallman et al., 2005). However, little is known about central interactions of cortisol and insulin. A synergistic interaction has been suggested, whereby glucocorticoids may be responsible for a general rise in food intake, while insulin determines the kind of foods chosen (la Fleur, 2006).

Food consumption is influenced by several environmental factors. Exposure to food-related cues produces a multitude of anticipatory digestive and endocrinal responses that prepare the body for consumption (Mattes, 1997), and increases cravings and hunger (Nederkoorn et al., 2000). Cues that signal food intake (ex.: smell, visual presentation of food) can act as conditioned stimuli to trigger physiological activity (Nederkoorn et al., 2000). Furthermore, food cues activate reward-related brain circuits, and act as primary reinforcers of "wanting" (Berridge, 2009). Hunger additionally increases the incentive value of food cues, which are rated to be more arousing and appetitive (Ferreira de Sá et al., 2014), and receive enhanced attention (Stockburger et al., 2009), when a person is hungry.

The aim of the current study was to assess whether cortisol and insulin (intranasal administration) act on the cognitive processing of food cues, independent of each other, or when combined. Cultural factors and social expectations strongly impact eating behavior (Wildes et al., 2001), and bias the verbal report of food preferences. Therefore, a non-verbal method, free of interference from subjective perception, task characteristics, or intentional bias (Grillon and Baas, 2003), based on startle reflex modification, seems to be appropriate to address this question. Startle reflex research is long established in the study of emotional valence and approach motivation. Startle eye blink responses are interpreted on the basis of a motivational priming model: aversive context primes defensive (avoidance) responses, while appetitive context inhibits such responses (Lang et al., 1998). During affective startle

modulation (ASM), startle eye blink responses (evoked by acoustic noise bursts) are enhanced during presentation of aversive cues, and attenuated during presentation of pleasant cues (Bradley et al., 2001). Increased arousal ratings of the cues enhance these effects in both directions (Bradley et al., 1993). Our study follows a previously well-established model in startle research with food cues and different deprivation states (Drobes et al., 2001; Rodriguez et al., 2005; Mauler et al., 2006; Rejeski et al., 2010; Ferreira de Sá et al., 2014). Despite the fact that food pictures are rated as more arousing and appetitive during food deprivation, the startle response is increased during their presentation (Drobes et al., 2001; Mauler et al., 2006; Ferreira de Sá et al., 2014). This finding is best explained by a frustrative nonreward effect (Drobes et al., 2001): since deprivation increases the drive to consume food (Seibt et al., 2007), while the impossibility of achieving drive satisfaction in the face of visual food cues that cannot be consumed induces frustration and, thereby, startle enhancement. A previous study from our group replicated this pattern of startle response in a healthy sample of men and women, and showed that it correlated with changes in hunger (Ferreira de Sá et al., 2014). Two other studies showed that the increase in startle responsiveness during food presentation disappeared when the foods presented were available for later consumption (Hawk et al., 2004; Rejeski et al., 2010).

In the current study, participants were first subjected to a pharmacological manipulation where they received either sole or combined application of oral cortisol and intranasal insulin, or placebo. Then, food pictures and valenced non-food pictures were presented to the participants, while acoustic startle eye blink responses were recorded. Because cortisol and insulin play an important role in carbohydrate metabolism (Strack et al., 1995), the effects of these hormones might be specific to food category. Therefore, we presented food pictures of different carbohydrate content (high glycemic and low glycemic), but matched for total calories. In previous research on the frustrative nonreward model, startle responses were inversely correlated with the drive to consume the displayed food (Drobes et al., 2001; Ferreira de Sá et al., 2014). Therefore, we hypothesized that intranasal insulin, acting as a satiety signal, would decrease the startle response during foreground presentation of food cues and that cortisol, given its orexigenic properties, would do the opposite, especially with respect to pictures of high glycemic food.

4.2 Methods

4.2.1 Participants

Data were acquired from 57 healthy male students at the University of Trier and Trier University of Applied Sciences. All participants gave written informed consent. A moderate monetary incentive was given to every participant on completion of the study. Study procedures were approved by the local ethical committee (Landesärztekammer Rheinland-Pfalz) and are in accordance with the Declaration of Helsinki. Exclusion criteria were: tinnitus or other hearing problems; aversion for any food product or specific dietary restrictions; body mass index (BMI) outside of the normal range for men (20-25 kg/m2, according to the German Nutrition Society); drug or medication intake within the last 6 months (including nicotine), except of occasional use of pain killers and moderate caffeine consumption; acute medical or psychiatric symptoms/complaints; excessive practice of sports. Every participant was weighed and measured to validate the self-reported values of BMI. The subscales "drive for thinness" (M=9.35, SD=2.49), bulimia (M=9.53, SD=2.11), and body dissatisfaction (M=16.69, SD=5.17) of the Eating Disorder Inventory-2 (EDI-2; German version from Thiel et al., 1997) were used as exclusion criteria for abnormal eating habits. Cut-off points were established as 1SD above or below the mean score of a non-clinical control group from the German standardization. Three participants were excluded due to complete absence of startle eye-blinks (non-responders). The final sample consisted of 54 men with a median age of 24 y (range 19-36).

4.2.2 Pharmacological Manipulation

According to a double-blind design participants were randomly assigned to receive oral cortisol or oral placebo, and intranasal insulin or intranasal placebo. Thus, all participants received an oral substance and an intranasal substance, resulting in the following four substance groups: cortisol only (n = 12), insulin only (n = 13), cortisol and insulin (n = 15), placebo only (n = 14). A total of 30 mg of cortisol (Hydroson® Tabl.; Dermapharm, Gruenwald, Germany) or placebo (P-Tabletten; Winthrop, Germany) was administered in five doses (10mg + 4 x 5 mg) over a period of 60 min (15 min between doses) to assure a "steady-state like" elevation of plasma cortisol levels throughout the experimental period (see "kinetic pre-study" below). Additionally, participants received 40 units of intranasal insulin (Insulin Human Actrapid Penfill® 100 I.E./ml; Novo Nordisk, Mainz, Germany) or placebo (dilution

buffer, kindly provided by Dr. Manfred Hallschmid, University Tübingen). Two 0.1 ml puffs of substance were applied into each nostril via a high precision medical nose pump (kindly provided by Aero Pump, Hochheim, Germany). Intranasal insulin was given 45 min before the start of the experiment to ensure central effects during the time of assessment (Born et al., 2002; Schilling et al., 2014). An intranasal dose of 40 IU of insulin was chosen, as it is known that this amount reaches and affects the central nervous system within one application, without producing hypoglycemia (Born et al., 2002; Bohringer et al., 2008; Schilling et al., 2014).

4.2.3 Kinetic Pre-Study: Plasma Cortisol Concentrations

Because subjects may differ in pharmacokinetic determinants (e.g. gastric transit time, distribution volume, excretion, and metabolism) experiments following oral cortisol administration may suffer from timing problems. Uncertainties are aggravated when cortisol is taken as a single dose. This kinetic pre-study was done to assess plasma cortisol changes following a multiple dose application schedule, attempting to assure that elevated plasma cortisol levels were maintained throughout the planned experiment. Four healthy male participants (same exclusion criteria described above) received 10 mg + 4 x 5mg oral cortisol (see above), and plasma cortisol was measured every 15 min, starting 60 min before cortisol administration until 180 min after (see Figure 7). Data indicate a “steady-state-like” elevation of plasma cortisol from about 60 to 135 min after starting the cortisol administration.

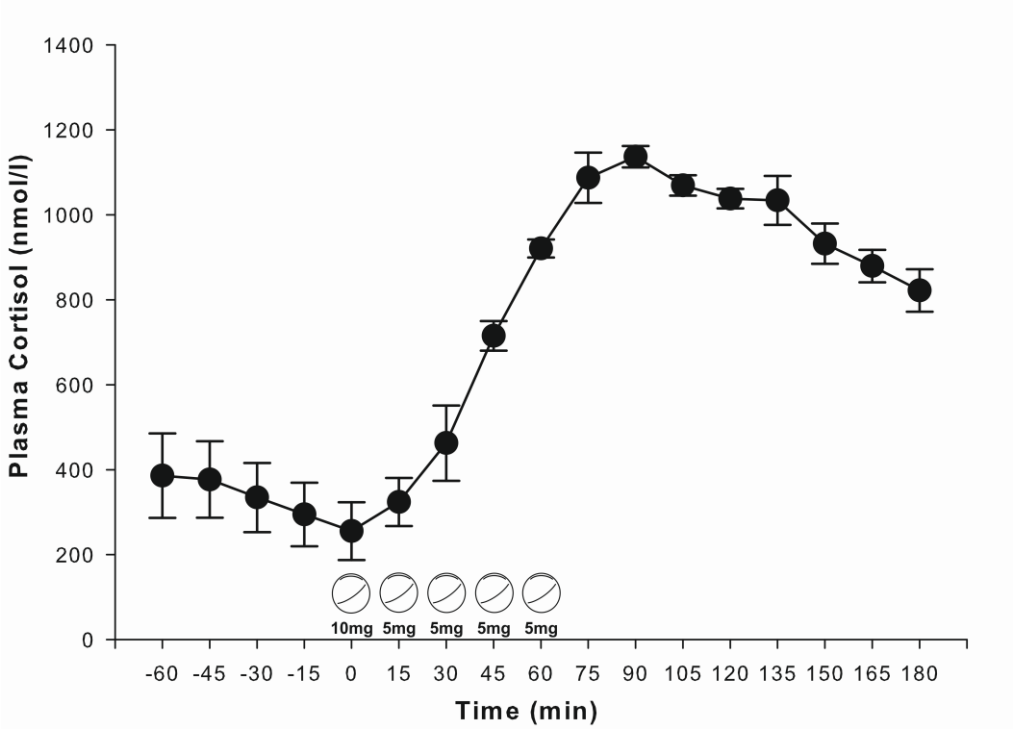

Figure 7. Kinetic pre-study: Plasma cortisol levels, in a sample of four participants, before and after administration of oral cortisol (total = 30 mg), phased in five applications (at 0, 15, 30, 45, and 60 min). Cortisol levels in blood remained high from 45 to 180 minutes after the first application of oral cortisol (all ps > .05).

4.2.4 Procedure

The experiment was always run between 1130 h and 1400 h to control for time of day effects on food intake (Stroebele and De Castro, 2004). In order to study the participants in a standardized condition, they were instructed to have their last meal at 2200 h of the previous day (ca. 14h of fasting). They were additionally asked to refrain from alcoholic beverages, sports, and coffee prior to the experiment. To increase compliance a cover story was used: they were informed that on the day of the experiment, saliva samples would be taken to verify if the fasting period had been respected, with loss of monetary compensation in case of negative results. A saliva sample was acquired at arrival and a routine recall was performed from waking till arriving at the experiment as an additional method of compliance control (diary methods; Stone et al., 1991). At arrival, participants were seated in a quiet room and subjected to a health check. After a 30 min period, the pharmacological manipulation started, according to one of the four groups described above. Before the end of the manipulation, participants were prepared for electromyographic (EMG) eye blink recording of the left orbicularis oculi muscle. Skin preparation and electrode placement followed published guidelines (Blumenthal et al., 2005). Participants were comfortably seated and received instructions via computer screen. After ingestion of the last pill (min. 60), the experimenter

left the room and the computer protocol started. They were asked to indicate their current feeling of hunger, motivation to eat, and stress on continuous visual analogue scales (VAS), ranging from 0 to 1280 (full horizontal screen resolution), and anchored at the left margin with label "0" (not at all) and at the right margin with label "7" (very much), as well as marks at representative full numbers "1" to "6". Next, participants were instructed that a series of pictures would be displayed and that they should look at them for the entire presentation time. They were further informed that throughout the experiment brief, intense sounds would be delivered. They were requested to neither move nor speak, to sit quietly, and to avoid long periods of eye closure during the recording time. Six startle probes were first presented to serve as habituation trials. Pictures were presented in pseudo-randomized fashion so that the same category was not presented more than 2 times on a row. Each picture was shown once for a 6 s period, followed by a 4 s black screen during the Inter-Picture-Interval. An acoustic startle stimulus was presented between 2.5 and 5.5 s after picture onset in 80% of the trials. The acoustic startle stimulus was a white noise burst (105 dB, 50 ms, instantaneous rise time) presented binaurally via USB sound card Aureon USB 5.1 (TerraTec Electronic GmbH, Nettetal, Germany) and audiometric headphones (Holmco PD-81, Holmberg GmbH & Co. KG, Germany). Affective ratings of the pictures were also collected during the period of steady-state-like cortisol elevation. For that purpose, after the startle procedure, each picture was shown again and participants were asked to rate it. Perceived liking (appetitive value), wanting (willingness to consume), and arousal of the food pictures were asked 85 min after the onset of the pharmacological manipulation. Arousal ratings of the neutral and pleasant pictures were collected at 110 min. Ratings were made on a continuous VAS, ranging from 0 to 9, with marks at full numbers (liking scale anchors: 0 = "extremely dislike" to 9 = "extremely like"; wanting scale anchors: 0 = "extremely unwanted" to 9 = "extremely wanted"; arousal scale anchors: 0 = "not arousing at all" to 9 = "extremely arousing"). Other experiments not related to the current publication were performed (e.g. memory experiments). In the end, participants received a standardized meal that they could eat ad libitum. Blood glucose was assessed via an ambulatory self-assessment blood glucose meter (Accu-Chek Aviva, Roche Diagnostics Deutschland, Mannheim, Germany), to reassure normal levels of sugar in the blood. Participants were debriefed and thanked for their participation.

4.2.5 Materials

Experimental material consisted of 32 pictures collected from an internet data base, based on ratings obtained in a previous study (Ferreira de Sá et al., 2014). Picture sets included 8 pleasant, 8 neutral, 8 high glycemic food, and 8 low glycemic food pictures (all food pictures depicting high caloric food).

4.2.6 Salivary cortisol assessments

Participants were asked to collect saliva samples in the morning of the experimental day: at awakening, + 15 min, + 30 min, + 45 min, and + 60 min. Saliva samples were also collected throughout the study to serve as a manipulation check for cortisol administration (at arrival, 60, 75, 115, and 135 min). Salivary cortisol was analyzed by an immunoassay with fluorescence detection (Dressendorfer et al., 1992) with a concentration detection limit of 100 nmol/L.

4.2.7 Recording and analysis of the startle response

EMG responses were recorded with a BIOPAC MP 150 system and an EMG 100C amplifier at a sampling rate of 1000 Hz, with a notch filter of 50 Hz and a software band pass filter of 28 to 500 Hz (van Boxtel et al., 1998). Data were rectified and integrated online with a time constant of 10 ms (Blumenthal et al., 2005). EMG blink responses were analyzed by a customized C++ based semi-automated PC program (Clip 2.0.0, 2010), which identifies response peaks in the time interval of 20-150 ms after stimulus onset, and a baseline period 50 ms prior to stimulus onset. All data were subjected to manual confirmation. Startle response amplitude was computed as the voltage difference between peak and baseline. Trials with electrical and physiological artifacts (i.e., coinciding blinks, other facial muscular activity, multiple peaks, excessive background noise) were set to missing data, while trials with no visible startle response were scored as zero. Startle magnitudes were calculated including zero responses.

4.2.8 Statistical analysis

All statistical analyses were conducted with IBM SPSS (version 21), the level of significance set to (α) = 0.05. Salivary cortisol measured in the morning of the experiment was averaged and studied by a 2 (cortisol) × 2 (insulin) independent factorial ANOVA. For the manipulation check of cortisol administration, saliva cortisol pre- (60 min) and post- (75 min) affective startle modulation were averaged and studied by independent t-test. Subjective ratings (hunger, motivation to eat, and stress) were analyzed by 2 (cortisol) × 2 (insulin) independent factorial ANOVAs. Startle reaction during picture presentation was tested by a mixed design 2 × 2 × 4 ANOVA with cortisol (verum vs. placebo) and insulin (verum vs. placebo) as between-groups factors, and picture category (neutral vs. pleasant vs. high glycemic food vs. low glycemic food) as a within-participants factor. A priori contrast analysis was set against neutral pictures. Interaction results were clarified with paired t-tests. Liking and wanting ratings of the food pictures were analyzed by a mixed design 2 × 2 × 2 ANOVA with cortisol (verum vs. placebo) and insulin (verum vs. placebo) as between-groups factors, and food picture category (high glycemic food vs. low glycemic food) as a within-participants factor. Due to a technical failure, wanting ratings from two participants were missing. Arousal ratings of all pictures were studied by a mixed design 2 (cortisol) × 2 (insulin) × 4 (picture category) ANOVA, with contrasts set against neutral pictures. The Greenhouse-Geisser correction was applied whenever sphericity adjustment was required (adjusted *p* values are reported with uncorrected degrees of freedom and epsilon values). Means and standard deviations are reported.

4.3 Results

4.3.1 Salivary morning cortisol

Neither a significant main effect of cortisol, $F(1, 50) = .03$, $p = .87$, nor insulin administration, $F(1, 50) = .02$, $p = .89$, was found, nor was the interaction significant, $F(1, 50) = .27$, $p = .61$. This assures that baseline cortisol levels of participants in the cortisol only (M = 10.92 ± 4.63 nmol/L), insulin only (M = 11.27 ± 4.72 nmol/L), cortisol and insulin (M = 11.65 ± 3.13 nmol/L), and placebo only group (M = 11.68 ± 3.67 nmol/L) did not differ between groups.

4.3.2 Manipulation-check

Cortisol administration increased salivary cortisol, $t(52) = 60.44$, $p < .001$, (cortisol: M = 97.92 ± 8.01 nmol/L; placebo: M = 3.45 ± 1.33 nmol/L, see Figure 8). Pharmacological manipulation of cortisol was, therefore, successful.

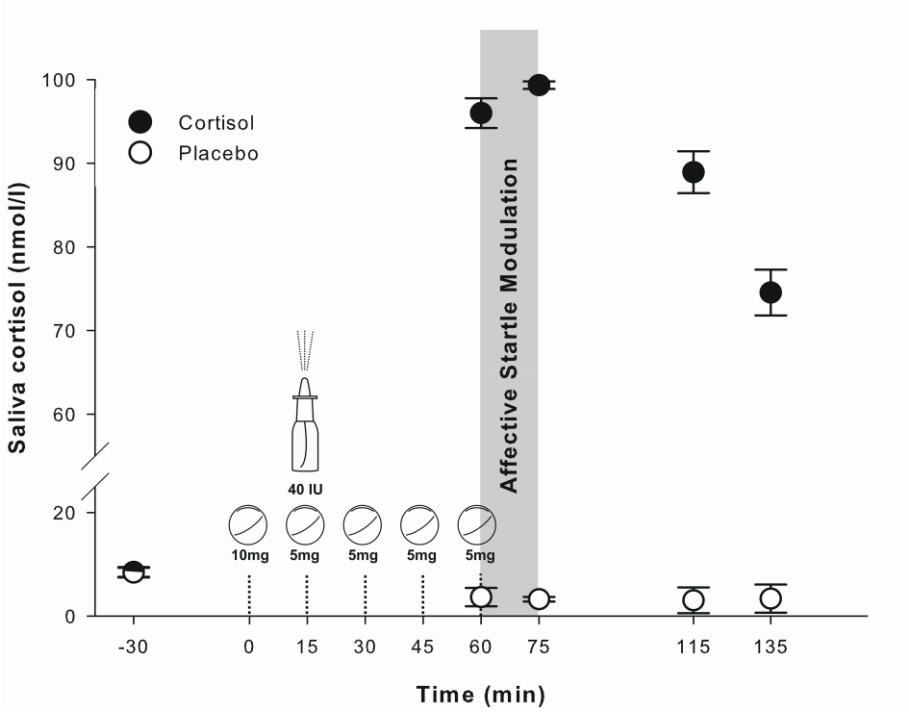

Figure 8. Salivary cortisol levels during the experiment. Salivary cortisol was assessed five times during the experiment (at -30, 60, 75, 115, and 135 min). Pharmacological manipulation consisted of: phased administration of five cortisol pills for a total of 30 mg or oral placebo (white pill), and one application of 40 IU of intranasal insulin or intranasal placebo (nasal spray). After the last procedure of the manipulation, the affective startle modulation (ASM) testing started. Cortisol administration provoked higher levels of cortisol during ASM by comparison with placebo administration (averaged pre- and post-ASM values, $p < .001$). Error bars represent ± 1 standard error.

4.3.3. Subjective ratings

Subjective ratings of motivation to eat, hunger, and stress measured at the start of the experiment are shown in Table 3 (lower panel). There was no effect of insulin nor an interaction effect of cortisol × insulin in the ratings of motivation to eat (respectively: $F(1, 50) = .16$, $p = .69$; $F(1, 50) = .35$, $p = .56$) or hunger (respectively: $F(1, 50) = .18$, $p = .68$; $F(1, 50) = .56$, $p = .46$). Cortisol reduced ratings of motivation to eat, $F(1, 50) = 5.49$, $p = .02$ (cortisol: M = 716 ± 153; M = placebo: 806 ± 124), but did not significantly affect the ratings of hunger , $F(1, 50) = 3.53$, $p = .089$, although descriptively, the mean group values point to the same tendency as the ratings of motivation to eat (cortisol: M = 675 ± 140; placebo: M =

734 ± 96.7). Subjective stress was not affected by cortisol, F(1, 50) = .04, p = .84, or insulin, F(1, 50) = 2.11, p = .15, nor cortisol × insulin, F(1, 50) = .37, p = .55.

Table 3. Mean affective ratings of pictures and subjective ratings of hunger, motivation to eat, and stress.

	Group			
	Cortisol	**Insulin**	**Cortisol and Insulin**	**Placebo**
Affective Ratings Of Pictures				
Neutral				
Arousal	2.10 ± 1.25	1.97 ± 0.97	2.32 ± 1.27	2.16 ± 1.04
Pleasant				
Arousal	6.05 ± 1.65	6.31 ± 1.02	6.48 ± 1.46	6.42 ± 0.95
Low Glycemic Food				
Liking	6.71 ± 0.70	7.29 ± 1.15	6.68 ± 1.22	7.32 ± 1.60
Wanting	6.64 ± 1.79†	7.70 ± 1.15	6.45 ± 1.78†	7.63 ± 1.03
Arousal	5.18 ± 1.35	5.93 ± 1.57	6.10 ± 1.32	5.90 ± 2.33
High Glycemic Food				
Liking	6.50 ± 1.02	7.10 ± 1.25	6.84 ± 1.04	7.02 ± 1.05
Wanting	6.70 ± 0.96†	7.37 ± 0.94	6.40 ± 1.34†	7.13 ± 1.24
Arousal	4.75 ± 1.08	5.97 ± 1.83	5.98 ± 1.30	5.57 ± 1.96
Subjective Ratings				
Hunger	696 ± 147	740 ± 103	658 ± 136	729 ± 94
Motivation To Eat	720 ± 148‡	826 ± 117	712 ± 161‡	788 ± 132
Stress	439 ± 93	393 ± 101	381 ± 74	416 ± 133

Note: Results are presented by pharmacological group: cortisol only, insulin only, cortisol and placebo, and placebo only. All data represent M ± SD.
†Main effect of cortisol (verum vs. placebo) in wanting ratings of all food pictures.
‡Main effect of cortisol (verum vs. placebo) in motivation to eat.

4.3.4 Modification of startle during foreground presentation of pictures

Startle magnitude during foreground presentation of the different picture categories is depicted on Table 4, divided by pharmacological group. There were no main effects of cortisol, F(1, 50) = .05, p = .83, or insulin, F(1, 50) = .00, p = .99, nor an interaction of cortisol × insulin, F(1, 50) = 2.59, p = .11. A main effect of picture category was found, F(3, 150) = 7.19, p < .001. The two-way interaction of cortisol × picture category was also significant, F(3, 150) = 3.08, p = .03. However, the two-way interaction of insulin × picture category, F(3, 150) = .56, p = .64, and the three-way interaction of cortisol × insulin × picture category, F(3, 150) = .28, p = .84 were not significant. Since there was no main effect nor interaction effects of insulin, graphical illustration of the startle data (see Figure 9) collapse all participants across insulin administration. A-priori planned contrast analysis revealed that startle eye blink magnitude was lower during foreground presentation of pleasant pictures

(versus neutral), F(1,50) = 5.71, p = .02. Furthermore, startle eye blink magnitude was enhanced during foreground presentation of low glycemic pictures (versus neutral), F(1,50) = 4.89, p = .03. Neither cortisol nor insulin had an effect on startle during foreground presentation of pleasant or low glycemic pictures (all ps > .05). The effect of high glycemic pictures was modulated by cortisol, F(1,50) = 5.90, p = .02, but not by insulin, F(1,50) = .01, p = .93, or cortisol × insulin, F(1,50) = .01, p = .94. Subsequent paired t-tests showed that cortisol administration enhanced startle eye blink magnitude during foreground presentation of high glycemic pictures (versus neutral), t(26) = 2.50, p = .02. This was not the case after placebo administration, t(27) = -0.64, p = .53.

Table 4. Startle magnitude during foreground presentation of neutral pictures, pleasant pictures, low glycemic food pictures, and high glycemic food pictures.

	Group			
Picture Category	**Cortisol**	**Insulin**	**Cortisol and Insulin**	**Placebo**
Neutral	30.96 ± 27.73	33.38 ± 22.99	22.99 ± 19.31	23.01 ± 10.54
Pleasant	31.6 ± 30.63	28.03 ± 21.77	19.96 ± 18.9	20.08 ± 11.52
Low Glycemic Food	35.49 ± 33.97	36.1 ± 27.27	24 ± 22.42	25.30 ± 11.03
High Glycemic Food	36.95 ± 31.98	32.08 ± 22.81	28.97 ± 29.23	22.2 ± 11.04

Note: Results are presented by pharmacological group: cortisol only, insulin only, cortisol and placebo, and placebo only. All data represent M ± SD (µV).

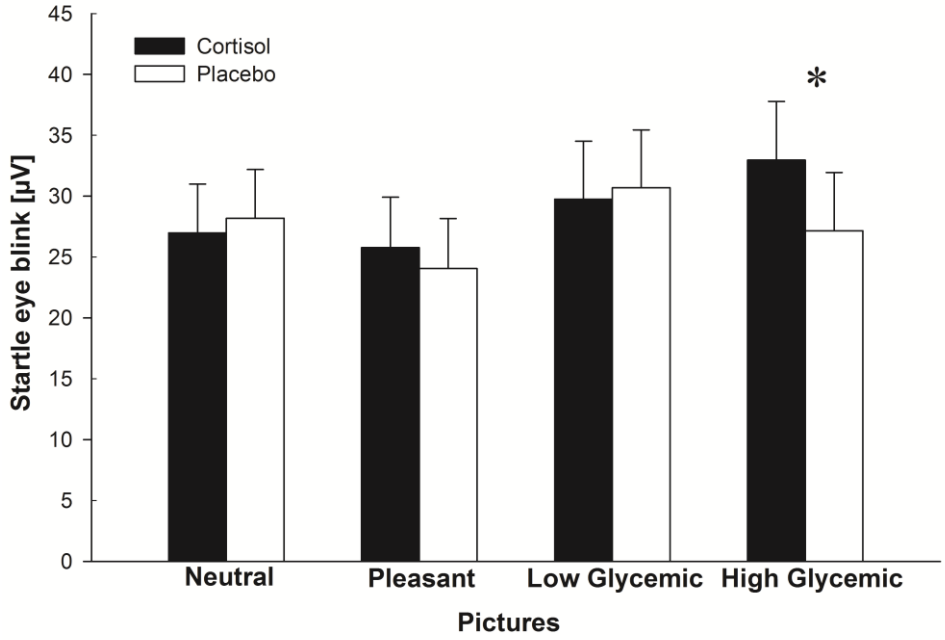

Figure 9. Mean startle eye blink with respect to drug administration (cortisol versus placebo) and picture category (neutral, pleasant, low glycemic food, and high glycemic food). Foreground presentation of pleasant pictures attenuated the startle response (p = .02) both in cortisol and placebo groups. Low glycemic pictures enhanced the startle response (p = .03) for both groups. Cortisol, but not placebo, enhanced startle response during foreground presentation of high glycemic pictures (p = .02, represented by *). Error bars indicate one standard error.

4.3.5 Affective ratings of the pictures

Affective ratings of the pictures are shown by group in table 3 (upper panel). Analysis of liking ratings revealed no significant effects (all ps > .05), although there was a tendency for less liking after cortisol intake (versus placebo), $F(1, 50) = 3.57$, $p = .065$. Cortisol administration (versus placebo) significantly reduced the ratings of wanting, $F(1,48) = 8.48$, $p = .005$. Although significant, it is important to note that ratings were still well above the midpoint of the scale (see table 3). No other main effects or interactions were found (all ps > .05). Arousal ratings were modulated by picture category, $F(3, 150) = 134.82$, $p < .001$, but no interaction with cortisol, insulin, or cortisol × insulin (all ps > .05) were detectable. Contrast analysis revealed that pleasant, low glycemic, and high glycemic pictures were rated with higher arousal than neutral pictures, respectively: $F(1, 50) = 356.2$, $p < .001$; $F(1, 50) = 199.7$, $p < .001$; $F(1, 50) = 183.2$, $p < .001$. There were no effects of cortisol, insulin, and cortisol × insulin on arousal ratings (all ps > .05).

4.4 Discussion

The present study aimed to assess the impact of two energy-regulating hormones, cortisol and insulin, on the central processing of visual food cues after moderate food deprivation. Previous studies have shown that food deprivation increases startle eye blink responsiveness during presentation of food pictures, an effect attributed to "frustrative nonreward" (Drobes et al., 2001; Ferreira de Sá et al., 2014). The current study replicates those results, but suggests that enhancement of the startle response during deprivation is more pronounced for low-glycemic food pictures. Cortisol treatment generalizes enhancement of the startle response to high-glycemic food pictures, too, suggesting that the reward value of high glycemic food items is specifically increased by cortisol. Startle responsiveness was not affected by insulin nor by an interaction of cortisol and insulin.

Food deprivation increases hunger and motivation to eat, as well as appetite and arousal ratings of food pictures (Lüthy et al., 2003; Ferreira de Sá et al., 2014). According to the startle modulation paradigm, food deprived subjects would be expected to show attenuated acoustic startle responsiveness during foreground presentation of food pictures, if those pictures are affectively positive. However, the opposite effect has been reported (Drobes et al., 2001; Mauler et al., 2006; Ferreira de Sá et al., 2014). This has been explained by a "frustrative nonreward" effect, induced by enhanced motivation to consume food (Drobes et

al., 2001) and, at the same time, the physical impossibility to immediately consume the displayed food. These effects are specific to food cues, and food deprivation does not affect startle modulation by non-food positively valenced and/or neutral pictures (Drobes et al., 2001; Mauler et al., 2006; Ferreira de Sá et al., 2014). Also in the current study with food deprived participants, normal startle magnitude attenuation was found during foreground presentation of pleasant pictures in comparison to neutral pictures. This typical affective pattern of startle modulation is not influenced by exogenous cortisol, replicating earlier results (Buchanan et al., 2001). The current study can add that intranasal insulin does not influence startle responsiveness during presentation of appetitive non-food pictures. As expected, we found enhanced startle responses during food picture presentation in every group of our participants, as a main effect significant for low glycemic food pictures. Most importantly, exogenous cortisol selectively enhanced startle responsiveness during high glycemic food picture presentation. Based on the model of "frustrative nonreward" this suggests that cortisol enhances the reward value and motivational drive towards high glycemic food cues.

Glucocorticoids such as cortisol play an important role in energy homeostasis. Previous studies have shown that glucocorticoids have orexigenic effects (Tataranni et al., 1996; Epel et al., 2001), although the stimulation of food intake might be nutrient specific. Tataranni and colleagues (1996) found that glucocorticoid administration increased the consumption of mainly carbohydrates and proteins. After stressful periods there is a tendency for humans to increase fat and sugar consumption (Wardle et al., 2000; Epel et al., 2001). Adrenalectomized rats consume lower amounts of carbohydrates (Laugero, 2001), and glucocorticoid administration reverses the anorectic effects of adrenalectomy (la Fleur, 2006). The startle enhancement during foreground presentation of high glycemic pictures provoked by cortisol observed in this study might therefore reflect a gain in reward-value of carbohydrates during high glucocorticoid levels, respectively.

It is interesting that the startle effects found in this study are not reflected in subjective reports. Rather, cortisol reduced reported motivation to eat and slightly reduced ratings of hunger as well. Food picture ratings followed the same pattern, with cortisol administration producing significantly lower ratings of wanting and a tendency for lower ratings of liking, but with no interaction with glycemic index. Startle potentiation is often seen when sufficiently arousing unpleasant pictures are paired with startle stimuli, but this was clearly not the case in the present study. Although statistically significantly reduced by cortisol, food picture ratings (liking and wanting) remain clearly in the appetitive range, suggesting no

"dislike" or "unwanting" of the presented food items. Thus, we rule out that ratings are able to explain the "above-neutral" startle reactivity enhancement provoked by cortisol during food picture presentation. This effect may be explained by two putative processes. Cortisol may specifically affect food picture processing to enhance the activation of an aversive cascade (e.g. produce greater frustration), or cortisol may rather nonspecifically translate any given aversive state into higher startle reactivity (e.g. by unselective reflex amplification processes). A previous study by Buchanan et al. (2001) did not find enhanced affective startle potentiation by cortisol treatment. Therefore, and in line with the "frustrative nonreward" model accounting for enhanced startle during food picture presentation in food deprived subjects, we conclude that the increase in startle responsiveness during foreground high glycemic food pictures provoked by cortisol is best be explained by the capability of the pictures to induce greater frustration. Furthermore, exposure to stress glucocorticoids may affect activity of the frontal cortex, thereby interacting with cognitive control of food consumption (Alonso-Alonso and Pascual-Leone, 2007), and differentially affecting physiological and subjective reactions to food cues. Startle represents a rather implicit method and cannot be voluntarily changed, while explicit ratings are subjected to intentions, cognitive modification, cultural biases, and expectations (Grillon and Baas, 2003). Dissociations between explicit and implicit measures are common and have been reported previously (Grusser et al., 2002). In the present study it may be speculated that the physical unavailability of the visually presented food items initiated a devaluation process resulting in decreased subjective ratings of liking and wanting ratings to reduce cognitive dissonance (Turner and Wright, 1965; Elliot and Devine, 1994), and that this effect may have been fostered by cortisol.

The intranasal application of insulin allows the study of central effects without insulin affecting peripheral glucose levels, i.e. inducing hypoglycemia (Born et al., 2002). Insulin acts on the brain as an adiposity and satiety signal (Schwartz, 2001), and it is known to enhance postprandial satiation and reduce food consumption (Hallschmid et al., 2004; Benedict et al., 2008; Hallschmid et al., 2012; Jauch-Chara et al., 2012). However, no published studies show a direct effect of intranasal insulin on reward-related processing of food cues. The current study did not find any main or interaction effects of intranasal insulin on the startle response during foreground presentation of food pictures, suggesting that the behavioral effects of insulin on food consumption are not mediated by those affective mechanisms that are revealed by measuring startle. In a similar vein, intranasal insulin had no impact in ratings of hunger, motivation to eat, and stress, a finding that replicates previous

results of a pharmacological study (Schilling et al., 2014). However, it has to be acknowledged that insulin is considered to be rather a long term signal for regulation of body weight and food intake (Havel, 2001). Therefore, effects of one-time insulin application might be more difficult to observe. In previous studies where changes in eating behavior were found, intranasal insulin was administered chronically (Hallschmid et al., 2004) or in higher dosage than in the present study (Benedict et al., 2008; Hallschmid et al., 2012), suggesting that 40 IU, the intranasal dosage used in the current study, may be too low to induce effects. Furthermore, it has been proposed that intranasal insulin would act only after food intake, thus in the postprandial, and not in a deprived, state (Hallschmid et al., 2012). This might also explain the lack of effect of insulin in the present study, since all participants were food deprived for approximately 14 hours, unlike a related study (Jauch-Chara et al., 2012) that used a shorter period of fasting (ca. 8h) to show that intranasal insulin increases brain energy levels, inversely correlated with food consumption.

Based on the findings that men are more sensitive than women to the effects of intranasal insulin in energy homeostasis (Benedict et al., 2008), we only included men in the current study. Furthermore, menstrual cycle effects and the higher prevalence of eating-related problems, dieting history, and food selectivity – specially related to high caloric/glycemic foods – in women would have included additional factors difficult to control. Nonetheless, it would be interesting to extend this research to women. The aim of the current study was to present data on healthy, lean individuals. Given the importance that food cue processing might have in obesity and disordered eating, future research should also focus on these groups. It is important to remark that despite the different glycemic index, the presented food pictures were balanced for caloric content. Although both types of food are expected to be perceived as palatable, the hedonic value attributed to food seems to be the main component of palatability (Yeomans, 1998). Therefore, collection of palatability ratings would have been valuable. It is a limitation that no biologically-based measure of food restriction was taken, such as blood samples for the measurement of ketone substances. Nevertheless, it has previously been shown that when working with healthy participants such a measure is not essential (Mauler et al., 2006) and that compliance is to be expected (Lüthy et al., 2003). A measure of energy balance status taken on the day of or days prior to the period of food restriction would clearly be of interest and is recommended in future studies, since it might have an impact on neuronal responses to food cues (Cornier et al., 2007). It is known that circulating glucose levels modulate reward-related brain activation by high caloric food (Page et al., 2011). Since all participants were subjected to the same state of food deprivation and

randomly distributed into groups, we expect that differences in circulating glucose at baseline are minimal and equally dispersed across the groups. Previous research has also shown that intranasal administration of 40 IU of insulin has no significant impact on blood glucose level in the periphery (Bohringer et al., 2008), so the insulin dose used here should also have no impact on free fatty acids. Nevertheless, future studies should include measures of circulating glucose to clarify potential confounds.

In conclusion, the present study shows that the stress hormone cortisol enhances acoustic startle reflex during foreground presentation of high glycemic visual food cues, while an effect of insulin was not found. Results suggest an important role of cortisol in favoring the consumption of high glycemic food during stressful periods.

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4.ii AUTHOR NOTES

Corresponding authors at: University of Trier, Institute of Psychobiology, Department of Clinical Psychophysiology, Johanniterufer 15, 54290 Trier, Germany. Tel.: +49 0651 201 3662; fax: +49 0651 201 3737. E-mail address: s1diferr@uni-trier.de (D.S. Ferreira de Sá).

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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.

Diana S. Ferreira de Sá Trier, im November 2014