universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

17-β-estradiol has no effects on fear processes within multiple threat of shock sessions in men

Verfasser

Jan Sören Seidel

Angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer. nat.)

Wien, Juli 2014

Studienkennzahl: A 298

Studienrichtung: Psychologie

Betreuer: Dr. med. univ. Igor Riečanský, Ph.D.

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Danksagung

An dieser Stelle möchte ich mich bei den Personen bedanken, ohne deren Hilfe die vorliegende Diplomarbeit in ihrer jetzigen Form sicher nicht hätte realisiert werden können.

Zunächst möchte ich mich bei meinem Betreuer Dr. Igor Riečanský sowie Dr. Christoph Eisenegger bedanken, die mir während der Planung, Durchführung und Auswertung, genau wie während dem Verfassen der Arbeit stets mit Rat und konstruktiver Kritik zu Seite standen und damit deren Qualität im positiven Sinne beeinflussten.

Ein ganz großes Dankeschön geht an meine slowakischen VersuchsassistentInnen Mgr. Veronika Rohárikova, Rastislav Rovný, Bc., Mgr. Jana Murinova, RNDr. Barbora Cimrova, PhD. und Matus Linsky, die mir nicht nur bei der Durchführung der Experimente tatkräftig unter die Arme gegriffen haben, sondern mich auch in die Kunst der slowakischen Sprache einzuführen versuchten (leider ohne großen Erfolg). Trotzdem ein letzter Versuch: Srdečná vd'aka za všetko!

Weiterhin ist hier die Universität Wien zu erwähnen, welche durch die Vergabe eines Förderstipendiums mehr Flexibilität und Handlungsmöglichkeiten bei der Durchführung der experimentellen Untersuchungen zuließ.

Ein herzlicher Dank geht ebenfalls an die *Aktion Österreich-Slowakei*, die mir durch die Bewilligung eines Stipendiums zur Deckung von Wohn- und Reisekosten den Aufenthalt in Bratislava erst möglich gemacht hat.

Dankbar bin ich vor allem meinen beiden Korrekturleserinnen Frau Svenja Seidel und Mrs. Clarissa Mosley, B.A, Grad Dip.Psych. B.Sc (Hons), die mir so manche inhaltliche und sprachliche Schwäche aufzeigen konnten.

Meinen Eltern, die mich schon immer auf so viele verschiedene Arten unterstützt und motiviert haben gilt ebenfalls besonderer Dank.

Nicht zuletzt möchte ich meiner besseren Hälfte Mag. Sandra Kogler danken, die mir so vieles verzeiht und mir mit ihrer Liebe und Zuneigung auch in schweren Zeit den Rücken stärkt und mir beim Verfassen der vorliegenden Arbeit ebenfalls zur Seite stand.

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List of abbreviations

Ag/AgCl	silver chloride
am	before midday
ANOVA	analyses of variances
BLA	basolateral amygdala
BMI	body mass index
BNST	bed nucleus of the stria terminals
CeA	central nucleus of the amygdala
cm	centimetres
CO_2	carbon dioxide
D2:D4	digit ratio
dB	decibel
dB FS	decibels relative to full scale
DC	direct current
DSM	Diagnostic and Statistical Manual of Mental Disorders
e.g.	for example
et al.	and others
EMG	electromyographic; electromyography
ER	estrogen receptor
FPS	fear-potentiated startle
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid receptor A
GHQ-12	12-item General Health Questionnaire
HPA	hypothalamic–pituitary–adrenal
Hz	Hertz
i.e.	that is
INPP	Institute of Normal and Pathological Physiology
M	mean

mA	milliampere
MDMA	3,4-methylenedioxy-N-methylamphetamine; ecstasy
ms	milliseconds
mm	millimetres
M.I.N.I.	M.I.N.I. International Neuropsychiatric Interview
NEO-FFI	NEO Five Factor Inventory
PET	positron emission tomography
PFC	prefrontal cortex
pm	after midday
PTSD	post-traumatic stress disorder
S-anxiety	state component of the STAI
SD	standard deviation
STAI	State-Trait Anxiety Inventory
T-anxiety	trait component of the STAI
VAS	visual analogue scale

Abstract

Gonadal steroid hormones play a crucial role in numerous neuronal and mental processes. Prior studies suggest that estrogens could modulate fear reactivity. We tested this assumption in an experimental study. In a double-blind crossover design we transdermally administered a placebo and 17-β-estradiol on two different test days to 32 healthy adult male volunteers. Prior to the application of the substances as well as two, four, and six hours afterwards we assessed the magnitude of the fear-potentiated acoustic startle reflex in a threat of shock paradigm. In addition, in each test session subjects rated their feelings of anxiety, sleepiness, and unpleasantness. We found statistically significant modulation of fear-potentiated startle and affective ratings over time and experimental conditions. However, we did not observe any significant effects of estradiol. Our results indicate that estradiol does not significantly modulate fear reactivity.

Keywords: fear, anxiety, amygdala, fear-potentiated startle, threat of shock, sex hormones, estradiol, estrogen, double-blind crossover.

Zusammenfassung

Sexualhormone spielen bei zahlreichen neuronalen und psychologischen Vorgängen eine wichtige Rolle. Frühere Studien lassen dabei vermuten, dass Östrogene einen Einfluss auf Furchtreaktionen haben könnten. Um diese Annahme experimentell zu untersuchen wurde 32 gesunden männlichen Freiwilligen innerhalb einer doppelblinden Crossover-Studie an zwei unterschiedlichen Tagen entweder ein Placebo oder 17-β-Östradiol transdermal appliziert. Jeweils vor Substanzapplikation sowie zwei, vier und sechs Stunden danach überprüften wir die Intensität des furchtpotenzierten akustischen Schreckreflexes innerhalb eines Threat-of-Shock-Paradigmas. Weiterhin gaben die Versuchsteilnehmer bei jeder Testung an wie ängstlich, müde und unwohl sie sich gerade fühlten. Wir fanden statistisch signifikante Veränderungen hinsichtlich des potenzierten Schreckreflexes genau wie der Gefühlswahrnehmung in Abhängigkeit sowohl von Zeit als auch Versuchsbedingung. Ein Einfluss von Östradiol war allerdings in keiner der Bedingungen erfassbar, was vermuten lässt, dass dieses Hormon keine direkte Wirkung auf die Verarbeitung von Schreckstimuli hat.

Keywords: Furcht, Angst, Amygdala, Furcht-potenzierter Schreckreflex, Threat-of-Shock, Sexualhormone, Östradiol, Östrogen, Doppelblinde Crossover-Studie.

1 Introduction

1.1 Fear and anxiety

The protection of one's own physical integrity in the presence of dangerous threats, like a confrontation with a predator, is an evolutionary challenge almost all living creatures have to face. Organisms, therefore, have developed several adaptive defensive approaches to increase their survival chances in such threatening situations (Blanchard, Yudko, Rogers, & Blanchard, 1993; Mobbs et al., 2009). In the presence of danger higher evolved species can rely on two distinguishable defense strategies (Walker & Davis, 2008): phasic fear and a more sustaining state of anxiety (Blanchard et al., 1993; Fanselow, 1994; Grillon, 2008; Mobbs et al., 2009). While spatial and temporal distance to a threatening object (Davis, Walker, Miles, & Grillon, 2010; Fanselow, 1994; Mobbs et al., 2009) as well as environmental cues, like the availability of escape routes (Blanchard et al., 1993), determine the perceived level of risk and the selection of a particular defense strategy both fear and anxiety lead to certain physiological, attentional, and behavioral alterations (Grillon, 2008; Walker, Toufexis, & Davis, 2003).

Davis et al. (2010) describe fear as a response to predictable, clear and imminent threats or painful objects. A fearful organism exhibits an attentional focusing on the menacing cue, while the perception of additional sensual stimuli is attenuated (Grillon, 2008). Fear is associated with a higher arousal in the autonomic nervous system, preparing the organism to perform immediate fight or flight reactions as an attempt to reduce the impact of the danger (Fanselow, 1994; Öhman, 2005; Walker et al., 2003). Naturally, such fear reactions are intensive events of short duration (Davis et al., 2010; Miles, Davis, & Walker, 2011; Walker et al., 2003; Walker, Miles, & Davis, 2009).

As opposed to fear, anxiety is a context-dependent, aversive state that mainly arises in unknown, ambivalent environments or in situations of possible, but unpredictable danger (Davis et al., 2010; Grillon, 2008; Grillon, Baas, Lissek, Smith, & Milstein, 2004). Grillon (2008)

describes anxiety as a state of risk assessment, which is accompanied by sustaining, yet unspecific, concerns about future harm. Thus, organisms experiencing anxiety show persistent feelings of distress, insecurity, and a negative affect as well as an increased vigilance (Blanchard et al., 1993; Davis et al., 2010; Grillon, 2008). The duration of anxiety can vary considerably from several minutes to hours (Grillon, 2008; Walker & Davis, 2002). Similar to fear reactions, anxiety evokes a higher sympathetic arousal but in contrast to fear, overall sensual sensitivity is not narrowed but enlarged (Grillon, 2008). Thus, the chance of perceiving and reacting to a real threat in a timely manner is increased. Moreover, to forestall confrontations with possibly threatening environments, anxiety motivates avoidance behavior (Davis et al., 2010; Maner & Schmidt, 2006). Despite the differences, fear and anxiety are linked to each other: anxiety serves as a risk assessment that can facilitate phasic fear reactions such as fleeing or fighting in case of detecting real dangers (Grillon & Charney, 2011; Walker et al., 2003).

1.2 Clinical implications

The adaptive value of well-functioning fear and anxiety systems has an obvious evolutionary advantage (Lang & Bradley, 2010; Nischke, Sarinopoulos, Mackiewicz, Schaefer, & Davidson, 2006). However, dysfunctions in one or both of these defense systems can lead to specific pathological manifestations. Patients suffering from anxiety disorders often exhibit excessive elevations of worries about future disasters as well as chronic states of hypervigilance (see American Psychiatric Association, 2013; Rosen & Schulkin, 1998). Most of these symptoms are not linked with a particular object or real dangers but rather with unspecific as well as contextual hazards. Thus, many anxiety disorders are considered as dysfunctions of anxiety rather than fear processes. For instance, the generalized anxiety disorder can be seen as a pathological increase in normal anxiety functions (American Psychiatric Association, 2013; Grillon, 2008). An exception to this are specific phobias which are characterized by irrational and extreme fight or flight responses in the presence of a particular aversive cue or object, resulting from an impaired fear reactivity (American Psychiatric Association, 2013; Öhman, 2005; Rosen & Schulkin, 1998). Further, both panic disorder and post-traumatic stress disorder (PTSD) show typical characteristics of extreme fear, as well as anxiety (Grillon,

2008; Grillon et al., 2004, 2008, 2009; Waddel, Morris, & Bouton, 2006).

Anxiety disorders entail not only considerable individual but also societal burdens: Wittchen et al. (2011) report a 12-month prevalence of the most common mental disorders in Europe including patients from all EU Member States as well as Switzerland, Iceland, and Norway in 2010. With a prevalence rate of 14 percent anxiety disorders constituted the most frequent mental disorders with stable morbidity rates (see Alonso et al., 2004; Wittchen & Jacobi, 2005), causing direct and indirect costs of approximately € 74.4 billion per year (Gustavsson et al., 2011). Data from the United States are even more dramatic: According to Kessler et al. (2005), the lifetime prevalence for US citizens to meet DSM-IV criteria for at least one anxiety disorder amounts to 28.8 percent. Further, specific phobias can be seen as one of the most frequent isolated mental disorders, with prevalence rates ranging from 6 to 8 percent in European countries (Alonso et al., 2004; Wittchen & Jacobi, 2005; Wittchen et al., 2011) and up to 12.5 percent in the United States (Kessler et al., 2005).

Modern treatment for anxiety disorders consists of the administration of anxiolytic drugs like benzodiazepines (Baas et al., 2002; Grillon, 2008). While this kind of treatment shows satisfying success rates in the therapy of generalized anxiety disorder, it is not effective against specific phobias (Baas et al., 2002; Hermans, Putman, Baas, Koppeschaar, & van Honk, 2006; Scaife, Langley, Bradshaw, & Szabadi, 2005). Baas et al. (2002) propose that the mode of action of benzodiazepines lies in the reduction of sensual sensitivity and distress, events occurring in states of anxiety rather than fear. Adverse side effects and frequent rates of substance dependence (Miles et al., 2011) also speak against the use of benzodiazepines for the treatment of pathological fear processes. Against this backdrop, and in consideration of the high prevalence rates, the lack of effective medical treatments to reduce or cure the negative impact of pathological fear is disappointing. This highlights the fact that a better understanding of the underlying neuronal processes of fear reactions is urgently required.

1.3 Neuroanatomical correlates of fear

Despite many years of research efforts the neuronal mechanisms of fear are not yet fully understood. Nonetheless, relationships between the activity of several brain regions and fear reactions in mammals have been identified (e.g., Davis, 1992, 2006; Fanselow, 1994; Mobbs et

al., 2009; Phillips, 1992; Walker et al., 2003). A key role in fear-learning as well as regulating short-duration fear responses is attributed to the amygdala (Davis et al., 2010; Grillon, 2008; Lebron-Milad & Milad, 2012; Rosen & Donley, 2006; van Wingen, Ossewaaarde, Bäckström, Hermans, & Fernández, 2011; Walker et al., 2003), a well-connected midbrain structure that integrates input from several brain areas (see Davis, 2006). In animal research experimental manipulations of the amygdala, but not of other brain regions, lead to significant alterations in fear-associated activity and behavior (Davis, 1992; Miles et al., 2011; Ryan & Davis, 2011; Tye et al., 2011). These findings are consistent with studies using modern neuroimaging techniques in humans (Fisler et al., 2013; LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998; Phelps et al., 2001).

In particular, two components of the amygdala are involved in fear processes: the basolateral amygdala (BLA), consisting of the lateral, basolateral, and basomedial nuclei of the amygdala, and the central nucleus of the amygdala (CeA; Rosen & Donley, 2006; Tye et al., 2011; Walker et al., 2009). When sensory input from cortical, hippocampal, or thalamic systems signalling danger reaches the amygdala a neuronal circuit is activated, sending signals from the BLA to the CeA. The resulting activation of the CeA arouses several brain areas by efferent output including the lateral hypothalamus, the brain stem, or the pontine reticular formation by efferent output (Davis, 1992, 2006; Miles et al., 2011; Walker et al., 2003). This results in a broad bandwidth of autonomic, electrophysiological, and behavioral responses, manifesting as the subjective experience of fear (Grillon, 2008; Lang & Bradley, 2010). The BLA is not only connected to the CeA but also projects to the bed nucleus of the stria terminals (BNST), a brain area associated with the regulation of stress and sustained anxiety (Grillon, 2008; Toufexis, Myers, & Davis, 2006; Waddel et al., 2006). Due to structural and neurochemical similarities, spatial proximity, and neuronal connections between CeA and BNST (Miles et al., 2011; Tye et al., 2011; Walker et al., 2009), these structures are considered to be strongly associated (Toufexis et al., 2006). Moreover, Davis et al. (2010) report that the neuronal fibers projecting from the BLA to the BNST run right through the CeA. Thus, pathological impairment of the BLA-CeA connection will deteriorate the transmission of BLA signals to the BNST (Tye et al., 2011; Walker et al., 2009), resulting in abnormal reactions of fear and anxiety. These findings provide a possible explanation of the resemblance between fear and anxiety.

1.4 The fear-potentiated startle paradigm as a suitable model of fear in animals and humans

The examination of treatment effects on fear requires an adequate experimental operationalization of fear processes. As a widely accepted method to quantify fear of a specific imminent danger both in animals and humans (e.g., Grillon et al., 2004; Klumpers et al., 2010; Paschall & Davis, 2002; Walker et al., 2003) Brown, Kalish, and Farber (1951) introduced the cue-specific fear-potentiated startle (FPS) paradigm. In their experiments with rodents Brown et al. (1951) paired an unconditioned aversive stimulus (i.e., an electric shock) with a neutral cue to induce a classically conditioned learned response. The neutral cue then became a conditioned stimulus which, when presented in the absence of the aversive stimulus, produced the same fear reaction as the initial aversive stimulus. Brown et al. (1951) demonstrated that once conditioned to react to the neutral cue by means of this form of classical conditioning trained rodents subsequently showed a higher startle reflex to brief noise bursts compared pre-conditioning response rates to brief noise bursts in the absence of the cue. Brown and colleagues concluded that the existence of the conditioned cue led to a fearful apprehension of the imminent aversive stimulus, resulting in a more intensive startle reaction (see also Davis, 2006; Walker et al., 2003).

The startle reflex can be defined as an inherent, involuntary response to a possibly threatening stimulus of short duration that serves as an immediate defense reaction (Grillon, 2008). In humans, startling stimuli lead to a contraction of facial and skeletal musculature that elicits, among other things, the eye blink reflex (Blumenthal et al., 2005; Davis, 1992, 2006; Hermans et al., 2006). This fast, instinctive closing of the eyelids in order to prevent eye damage by a threat is measurable via electromyographic methods (EMG) at the orbicularis oculi muscle (Blumenthal et al., 2005) and can be seen as a reliable method to measure human fear reactivity. Typically, startle potentiations are observed when an imminent confrontation with a threatening object is very likely (see Fanselow, 1994). It is important to note that an increase in the startle magnitude is not the result of a pairing between the aversive stimulus and the startling one. It is the administration of startle stimuli in the presence of a cue associated with the aversive stimuli that is essential for an increase in startle reaction (Davis, 2006).

Over the years the original FPS paradigm was modified in terms of design and contextual conditions. For instance, different conditioned cues (e.g., visual, auditory, tactile, or olfactory; Paschall & Davis, 2002) as well as differing aversive stimuli (disturbing pictures, CO_2 administration, or air blasts to the larynx; Grillon et al., 2004) could successfully replicate results of the original paradigm.

Prior studies emphasize that lesions of the BNST have no remarkable impact on FPS while lesions of the CeA have (Davis, 2006; Grillon, 2008; Walker et al., 2003). Furthermore, sedative drugs like benzodiazepines were found to have a reducing effect on startle only in the absence of the conditioned stimuli (see Baas et al., 2002; Miles et al., 2011; Scaife et al., 2005). Patients suffering from PTSD (Grillon et al., 2004, 2009) or panic disorder (Grillon et al., 2008) also exhibit abnormal startle responses within FPS studies, compared with healthy controls. These findings suggest that FPS is related to fear processes rather than anxiety, a further indication that FPS is a useful tool to examine fear.

In addition to the afore-mentioned mental disorders and medical drugs, the normal or the potentiated startle reflex can also be influenced by psychoactive substances like alcohol (Curtin, Lang, Patrick, & Stritzke, 1998) or cocaine (Willick & Kokkinidis, 1995). Furthermore, evidence is pointing towards effects of personality traits, in particular neuroticism and extraversion (Corr et al., 1995), on the startle reflex.

1.4.1 Threat of shock

Despite its great research value FPS experiments suffer from several limitations. The necessity of a previously conducted fear acquisition, i.e., the linking of unconditioned aversive stimuli with neutral cues requires multiple training sessions, sometimes even several training days (Toufexis, Myers, Bowser, & Davis, 2007). Therefore, FPS studies are not only time-consuming procedures; they also require test subjects to endure a great number of aversive inconvenient stimuli. In addition, test persons undergoing FPS experiments show strong habituation effects over time (Davis et al., 2010), hence, statements about medical treatment effects based on classical FPS methods must be viewed sceptically.

In order to solve these problems Grillon, Ameli, Woods, Merikangas, and Davis (1991) introduced the threat of shock paradigm, a modification of FPS techniques suitable especially for human test subjects. Threat of shock experiments consist of different test phases or

experimental conditions. While in one condition only startling stimuli occur (i.e., *baseline*, or *safe*), the other condition comprises both startling stimuli and the possible administration of aversive pain stimuli (*fear-potentiating*, or *threat*). At any time, participants are aware of the respective test phase, and, hence know whether an aversive stimulus is to be expected or not. It was found that the apprehension of aversive stimuli, rather than the actual experience, leads to an increase in the startle magnitude within the threat condition (see Figure 1; Blumenthal et al., 2005; Davis, 2006; Grillon et al., 1991).

The threat of shock paradigm offers some advantages compared to FPS methods: While FPS relies on classical conditioning techniques, examinations via threat of shock experiments are based on the fearful anticipation of aversive stimuli to enhance startle responses (Davis, 2006; Grillon et al., 1991; Grillon, 2008). This has an advantage compared to FPS sessions, as confounding effects of inter-individual variability in associative learning can be controlled in threat of shock procedures (Toufexis et al., 2007). As threat of shock studies employ only a very small number of aversive stimuli, or even completely avoid their application, inconveniences for test subjects can be reduced and the frequency of painful experiences can be minimized (Davis et al., 2010). Furthermore, the threat of shock paradigm can be viewed as a more economic approach as it does not require training for fear conditioning. Even more importantly, threat of shock experiments exhibit a satisfactory resilience to habituation effects. It has been shown that even several threat of shock test sessions within one day do not lead to extreme habituation effects (Klumpers et al., 2010). These findings advocate the use of this paradigm in order to evaluate effects of medical treatment on fear reactivity (Baas et al., 2002; Klumpers et al., 2010). In sum, the threat of shock paradigm can be seen as a reasonable extension of traditional FPS methodology as well as a useful model of normal and pathological fear in humans with various clinical applications. For this reasons the threat of shock paradigm was used to evaluate fear responses in this experiment.

1.5 The role of sex hormones in fear processing

Not only in mammals but in all vertebrates hormones, a group of biosynthesized messengers, have a pivotal impact on numerous functions throughout the organism. A notable group of

these messengers are steroid-based gonadal, or sex, hormones (McEwen, 2001; van Wingen et al., 2011). Sex hormones are involved in the control of morphological development as well as the regulation of biological, cognitive, affective, and social functions (Bos, Panksepp, Bluthé, & van Honk, 2012; Toufexis et al., 2006). Interestingly the length ratio between index and ring finger (D2:D4 digit ratio) is considered as an indicator of the in utero sex hormone concentration in humans (Fink, Manning, Neave, & Tan, 2004), determining several physiological and psychological characteristics (Wacker, Mueller, & Stemmler, 2013).
Sex hormones act via different cellular mechanisms (Bos et al., 2012; Gasbarri, Tavares, Rodrigues, Tomaz, & Pompili, 2012; McEwen, 2001): In a non-genomic mode of action, hormones bind to specific receptors within the membrane of particular cells leading to fast, but short-lived, intracellular chain reactions. Due to their steroidal structure sex hormones are also able to easily cross the cellular membrane and enter the cell nucleus in order to directly influence the expression of genes (Pfaff et al., 2000). This genomic pathway may need more time to exert its modifying effects, which are, however, longer-lasting than the non-genomic ones. Sex steroids can also affect cells in an indirect genomic way by binding to membrane receptors which are linked with G-proteins. When activated G-proteins translocate to the cell nucleus in order to influence gene transcription (McEwen, 2001; Norbury et al., 2003).

1.5.1 Testosterone and estradiol

Amongst others, two classes of sex steroids have a remarkable influence on sexual dimorphisms in vertebrates: Androgens, with their most potent representative being testosterone, and estrogens (Lebron-Milad & Milad, 2012). Among other effects, testosterone seems to influence fear reactivity in humans. Hermans et al. (2006) used a threat of shock paradigm (Grillon et al., 1991) in a double-blind crossover design to examine the effects of a single dose of testosterone on fear processes. Four hours after the administration of a placebo their female test subjects exhibited elevated startle magnitudes as well as elevated expressions of subjectively perceived fear and unpleasantness within threat (i.e., FPS) relative to safe conditions. The administration of testosterone, however, led to a noticeable reduction in the magnitude of the FPS four hours after its application, as compared to the placebo. Interestingly testosterone had no significant impact on the startle effect in the safe condition. These results indicate that the exogenous administration of testosterone has mitigating effects on states of

fear, but might not affect anxiety.

Testosterone can further be metabolized into the potent estrogen 17-β-estradiol, a process catalyzed by the enzyme aromatase (Bos et al., 2012; Ghayee & Auchus, 2007; Takahashi et al., 2006). Male vertebrates, which are not able to produce estrogens directly benefit from this conversion (Lebron-Milad & Milad, 2012; McEwen, 2001). Research highlights the influence of estrogen on an organism's attentional and perceptual systems (Pfaff et al., 2000), cognitive and memory functions (Norbury et al., 2003) as well as emotional and mood states (Gasbarri et al., 2012). Estrogens function to regulate behavioral patterns (Pfaff et al., 2000) and act as a mediator in the perception of pain intensity (Amandusson & Blomqvist, 2013). In addition, estrogens might have neuroprotective effects in psychiatric disorders like Alzheimer's disease and against neurotoxins (McEwen, 2001; Norbury et al., 2003; but see Marriot & Wenk, 2004). Besides this, results yielded by other studies suggest influencing effects of estrogen on fear.

Previous research supporting estrogens' protective effect against fear includes studies indicating that blood concentration of estrogens seems to be related to fear learning processes: Female rats within metestrous phase of their menstrual cycle (i.e., low estrogenic blood levels) exhibit deficits in fear extinction in a conditional fear-learning paradigm relative to those in their proestrous phase (i.e., high estrogen), or males (Milad, Igoe, Lebron-Milad, & Novales, 2009). According to that, healthy human women with low systemic levels of estrogen due to an early follicular phase in their menstrual cycle (Milad et al., 2010), or oral contraceptives (Merz et al., 2012), show an altered fear-extinction memory, compared to those with high estrogen levels, or men. Glover et al. (2013) revealed differences in the ability to inhibit conditioned fear in female patients suffering from PTSD in phases when their estrogenic blood level is low compared to patients with high levels and women without PTSD diagnosis. Certainly the concentration of other possibly influencing hormones, like progesterone, changes within the menstrual cycle as well as the relative hormone ratios (Lebron-Milad & Milad, 2012; Merz et al., 2012; van Wingen et al., 2011). Nevertheless, estrogen plays a germane role in these mechanisms (Milad et al., 2010). Work that highlights high estrogen sensitivity of hippocampal structures (Gasbarri et al., 2012; McEwen, 2001) supports this argumentation. The hippocampus plays a critical role in learning mechanisms (Gasbarri et al., 2012) and is

associated with states of anxiety and contextual fear conditioning (Anagnostaras, Gale, & Fanselow, 2001; Grillon, 2008). Furthermore, the amygdala and the hippocampus interact with each other in several ways. For instance, Phelps (2004) describes not only influences of the amygdala on memory consolidation of emotionally intense experiences, but also effects of the hippocampal formation on imagined as well as real fearful objects. This suggests an hippocampal mediation on direct fear processes.

Recent studies emphasize a relationship between estrogen and neurotransmitters such as choline, noradrenaline, γ-aminobutyric acid (GABA), dopamine and serotonin (Gasbarri et al., 2012; McEwen, 2001; McEwen, Akama, Spencer-Segal, Milner, & Waters, 2012; Norbury et al., 2003). For instance, Fadok, Dickerson, and Palmiter (2009) showed in a FPS paradigm with mice that dopamine is a necessary component for the exhibition of adequate fear behaviors. In turn, dopamine is able to regulate the synthesis of estradiol by controlling aromatase activity in a rapid manner (Balthazart, Baillien, & Ball, 2002). Further, dopamine indirectly regulates amygdala-dependent activity by affecting the excitability of BLA neurons (Kröner, Rosenkranz, Grace, & Barrionuevo, 2005). Therefore it is possible that estrogen may unfold its effects by modifying the dopaminergic system.

Further evidence for an influencing effect of estrogen can be seen in its synthesis. Testosterone can be converted into estradiol by aromatase (e.g., Ghayee & Auchus, 2007). While the production of aromatase is observed in various areas throughout the body and the brain a high distribution of aromatase is located in brain areas associated with fear. In order to detect aromatase concentration in the brain of rats Takahashi et al. (2006) used a PET study to track the radioactive tracer [^{11}C]vorozole that reversibly binds to aromatase receptors. Takahashi and colleagues subsequently found a high aromatase distribution in the medial amygdala and the BNST especially in male rats. In their experiments with human participants Biegon et al. (2010) also point towards elevated levels of aromatase within the amygdala. Thus, the reducing effects of testosterone on fear reported by Hermans et al. (2006) may be partly achieved by metabolized estradiol (Milad et al., 2010).

Women naturally show higher estrogen levels than men. Hence, regarding the higher prevalence rates for anxiety disorders in women compared to men (Lebron-Milad & Milad, 2012; Wittchen et al., 2011), the assumption of fear-reducing effects of estrogenic steroids seems paradoxical at first glance. To reconcile this apparent contradiction Toufexis et al. (2006,

2007) suggest opposing effects of the major estrogen receptors (ER) α and β in mechanisms underlying fear and anxiety. While the activation of ER-α might have an anxiogenic effect the activation of ER-β might be associated with a decrease in anxiety. Thus, a sex-specific distribution, or a selective inhibition or activation of ER α and β in fear-associated brain structures could lead to observed differences in normal and pathological fear in men and women (Gasbarri et al., 2012; Lebron-Milad & Milad, 2012; Toufexis et al., 2006).
In conclusion, the reported findings imply influences on fear processes by estrogen. Nevertheless, due to lack of research, statements about a direct relationship between estrogen and fear processes cannot be made. To investigate this issue, we conducted a double-blind crossover design, comparing the effects of the most potent estrogen 17-β-estradiol with a placebo using a modified threat of shock paradigm. To our knowledge, the present study is the first one addressing this question; it aims to improve our knowledge of fear in humans which could make an important contribution to the development and implementation of new treatment strategies for anxiety disorders.

1.6 Hypotheses

The primary goal of the present work was to examine the effects of 17-β-estradiol on normal and fear-potentiated startle reflexes as well as affective feelings in humans. The fact that testosterone can be converted into estradiol by aromatase, an enzyme with a high distribution in fear-related brain areas (Biegon et al., 2010), suggests that the observed decline in fear reactivity after the administration of testosterone (Hermans et al., 2006) could be contributed to metabolized estradiol. Therefore, we hypothesized that the administration of estradiol will reduce the magnitude of fear-potentiated startle and negative affective states in threat conditions and administration of placebo will produce no change. We also anticipated that estradiol will not effect baseline startle responses within safe conditions. Exogenous administration of estradiol leads to a temporal elevation in estrogenic blood levels that peaks approximately two hours after administration and declines continually afterwards (Eisenegger, von Eckardstein, Fehr, & von Eckardstein, 2013). We therefore anticipated an alteration of estradiol's impact on FPS and affective states over time, with the highest effect two hours after application and lesser effects at later times.

2 Methods

2.1 Test subjects

Thirty-two right-handed healthy men between 20 and 32 years (mean = 24.19 years, SD = 3.18), who were recruited via flyers or online announcements, participated in the study. After receiving detailed information about the experimental procedure, each test subject gave his written informed consent. All participants were of normal weight, had no impaired hearing ability as well as no exaggerated perception of anxiety. Women were not included due to the fact that the menstrual cycle leads to significant fluctuations in their estrogen blood concentration. Exclusion criteria were past or current mental or neurological disorders, such as schizophrenic psychosis, mood disorders, or epilepsy, affecting individuals or first-degree relatives. Individuals with endocrine diseases or dysfunctions of the liver as well as individuals with no startle responses (non-responders) at the initial measurement were excluded from participation in the study. Additional exclusion criteria were the abuse of psychoactive substances like coffee, alcohol, or cigarette smoking, consumption of illegal drugs, and long term pharmacological treatment. Test subjects were directed to avoid alcohol-containing beverages for 24 hours and caffeine-containing drinks for at least one hour before the measurements. A thorough pre-selection process ensured that only individuals who fit in our test sample joined the investigation. Firstly, interested persons completed the 12-item General Health Questionnaire (GHQ-12; Goldberg & Williams, 1988; Sarkova et al., 2006) the trait-component (T-anxiety) of the State-Trait Anxiety Inventory (STAI; Spielberger, Gorsuch, Lushene, Vagg, & Jacobs, 1983), as well as an anamnestic questionnaire including the M.I.N.I. International Neuropsychiatric Interview (M.I.N.I.; Sheehan et al., 1998) and a screening about the abuse of psychoactive substances online. Persons who did not demonstrate an exaggerated anxiety trait (i.e., T-anxiety score < 44) were invited to an initial testing for further selections (see 2.3.1). For their participation in this initial selection process, subjects received remuneration in the amount of € 10. Nevertheless, only individuals who exhibited satisfying results in all online tasks and the initial test session were accepted to join the

main study. As a result of our rigorous screening process only 34 out of the initial 64 examined individuals (53.1%) met our criteria. Since one test subject refused to continue the experimental investigations after the first test day and a post-examination exclusion of another subject due to insufficient startle responses, the final test sample consisted of 32 participants. All experiments were carried out between May and November 2013 in the Laboratory of Cognitive Neuroscience at the Institute of Normal and Pathological Physiology (INPP) in Bratislava, Slovakia. The study was approved by the Ethical Committee of the INPP.

2.2 Experimental design and substance administration

In order to examine the effects of estradiol on startle reactivity as well as affective states over time participants underwent four threat of shock sessions on two non-consecutive test days with a gap of on average one week between the days. The experimental manipulation consisted of the administration of two different substances: On each experimental day participants were administered either a gel containing estradiol (Divigel, Orion Pharma, Zug, Switzerland) or a placebo gel. Both substances, estradiol or placebo gel, were applied on chest, shoulders, and upper arms of the test subjects. The placebo gel contained no estradiol but could not be distinguished from the Divigel based on appearance, odor or consistence. The order of which of the gels were administered was randomized and counterbalanced throughout the sample; neither participants nor examiners had knowledge of the respective substance applied (double-blind crossover design).

2.3 Procedure

2.3.1 Initial test sessions

Individuals who met the criteria were invited to an initial test session where they not only were informed about the study design, but also underwent more examinations: Subjects whose results in the GHQ-12 or the anamnestic questionnaire did not clearly preclude the presence of a mental or neurological disorder were investigated in more detail. During this session subjects underwent a detailed structured interview focused at their consumption of psychoactive substances, including caffeine, alcohol, nicotine, cannabinoids, methamphetamine, MDMA

(ecstasy), cocaine, amphetamines, opiates, hallucinogens, and organic solvents. For each substance, test subjects were asked to report frequency and the most recent date of consumption. At the beginning of the initial testing as well as both experimental sessions, urine of subjects was tested on the presence of cotinine (i.e., nicotine consumption; Diagnostik Nord, Schwerin, Germany) cannabis, amphetamines, methamphetamine, MDMA, cocaine, barbiturates, benzodiazepines, LSD, and opiates (BIOGEMA, Košice, Slovak Republic). Afterwards, their body mass index (BMI[1]) was calculated. Subjects then underwent a single threat of shock session in order to become familiar with the experimental procedure. To identify and exclude individuals with hearing impairments, subjects were presented white noise in different sound intensities (between -58.3 and -88.3 dB FS). Individuals were judged as hearing-impaired when they could not hear noise of at least -78.3 dB FS. Furthermore, individuals' startle reactivity was tested. Subjects who exhibited no visible startle responses at EMG-frequencies of 100 μV within the initial threat of shock session were classified as 'non-responders' and not invited to participate in the main studies. At the end of the initial testing, subjects completed the Slovak version of the NEO Five Factor Inventory by Costa and McCrae (NEO-FFI; Ruisel & Halama, 2007).

2.3.2 Main test sessions

Most of the test days (90.6%) of the experimental investigation were set seven days apart. Due to illness, one subject was not able to come to our lab on his second day testing, so this subject's investigation occurred 13 days after the first one. Further, one participant was tested six and one ten days after the first examination.

On each experimental day testing started at 09:30 am and ended at approximately 05:30 pm. When the test subject arrived he was asked for a saliva sample in order to determine their current endogenous hormone levels. To detect concentration changes throughout the test days, further saliva samples were obtained every hour until the end of the experiment. An initial startle measurement session (session I; baseline session of the day) was carried out. In preparation for the experimental condition gel was applied, the experimenter was blind to whether estradiol or placebo was being used. Participants then completed the state component of the STAI (S-anxiety). On the first testing day, the hands of subjects were also scanned

[1]The body mass index as an indicator for body fat is calculated according to the formula BMI $= \frac{\text{kg}}{\text{m}^2}$

to determine the D2:D4 digit ratio. Each test subject was then asked for a urine sample for multiple screening drug tests (see 2.3.1). Further threat of shock sessions were performed two, four, and six hours after gel administration (sessions II, III, and IV). Between the sessions participants stayed in a separate room next to the laboratory. They were allowed to read, watch movies, or use the internet. However, social interaction and leaving the laboratory was not allowed. Contact between examiners and test persons was also minimized. A lunch break was introduced after the second threat of shock session.

To determine possible fluctuation in pain sensitivity, participants were asked whether they noticed any changes in intensity of shock probes on a five-point Likert scale (*significantly less – moderately less – no changes – moderately more – significantly more*) after finishing the test session II as well as IV. In the very end of the investigation (i.e., after the second test day), subjects were interviewed about their feelings and beliefs about administration order of substances. At the close of the test participants were paid a reward of € 100 for their study participation.

2.3.3 Acoustic stimulation and shock administration

During the whole experimental session participants listened to 58.3 dB(A) white noise in order to minimize the impact of environmental noises. Presented startle stimuli consisted of brief, loud bursts of white noise (105 dB(A), 50 ms). All sounds were presented by binaural ER-2 Tubephone Insert Earphones (Etymotic Research, Groove Village, USA). Unpleasant electric shocks were administered using a Digitimer DS5 isolated bipolar constant current stimulator (Digitimer, Letchworth Garden City, UK) with electrode applied at the back of the hand. On each test day, the intensity of stimulation was determined at the beginning of the first experimental test session. Participants were administered shocks (i.e., 0.5% of DC 2.5 mA, 150 ms) with gradually increasing intensities and asked to rate their pain on a nine-point scale (0 = *imperceptible*; 8 = *most agonizing pain*). Current intensity evoking *severe pain* (scale point = 5) was then used as the aversive threat stimulus in the threat of shock session. Shock delivery was controlled by a MATLAB script using COGENT toolbox (Cooper & Fox, 1998).

2.3.4 Electrophysiological data acquisition

Occurrence and magnitude of startle-induced eye blinks were measured by electromyography (EMG): Two reusable sintered Ag/AgCl ring electrodes with an external diameter of 10 mm (EASYCAP, Herrsching, Germany) were attached below each eye as suggested by Blumenthal et al. (2005); one electrode was applied below the lower eye-lid while second electrode was placed approximately two centimeters laterally to the first one. Ground electrode was placed on the forehead. In order to improve skin-electrode impedance, skin beneath the electrodes was scarified with a sterile needle. Then, electrodes were filled with EMG conductive gel (Adagel; Neuris, Pieštany, Slovak Republic). Impedances were kept below 3 kΩ. EMG data was recorded using a DC-amplifier Nexus-10 with a sampling rate of 2048 Hz (Mind Media, Herten, the Netherlands).

Threat of shock sessions

In order to measure subjects' fear reactions we conducted a threat of shock paradigm (Grillon et al., 1991). Test persons sat in a relaxed position, approximately 50 cm away from a Samsung SyncMaster 943B monitor (Samsung, Seoul, South Korea). They were instructed to look at the monitor screen and avoid movements of the body or the eyes to reduce measurement artifacts. Test sessions began with presenting background noise, without any presentation of startling stimuli, for 3 minutes. Following this twelve acoustic startle stimuli were presented. The habituation block was followed by alternating threat and safe blocks (three blocks of each condition). Both threat and safe blocks contained six acoustic startle stimuli. In safe blocks no electric impulses were administered. In threat 0, 1, or 2 electric shocks were applied. Within each block, the interstimulus interval between the startle probes varied randomly between twenty and thirty seconds with an average of 25 seconds. The sequence of blocks (safe-threat vs. threat-safe) was counterbalanced throughout the sample and kept constant for each individual over all sessions of both test days. The number of shocks administered during one test session was randomized, but no more than three shocks were presented. During the testing the current block (condition *habituation*, *safe*, or *threat*) was displayed on the monitor screen. An example of a test session with safe-threat order is presented in Figure 2. To assure the test subjects that no shock probes would occur within safe blocks the shock electrode was

plugged off the stimulator. Altogether, each threat of shock session thus consisted of 48 startle stimuli in seven discrete blocks. One sessions lasted approximately twenty-five minutes.

2.4 Assessment of subjective feelings during test blocks

At the end of each block, participants rated their current feelings of anxiety (*How anxious do you feel?"*), sleepiness (*"How sleepy do you feel?"*), and unpleasantness (*"How unpleasant do you feel?"*), using a visual analogue scale (VAS), ranging from *"not at all"* to *"very much"* (see Figure 3). Judgments of anxiousness, sleepiness, and unpleasantness, indicated on the visual analogue scales, were linearly converted to scores ranging from 0 to 100. Average ratings for both safe and threat conditions, respectively, were calculated within each session.

2.5 Measurement of D2:D4 digit ratio

In order to length of index and ring fingers, we scanned the ventral surface of both hands of test subjects. Images were then analyzed with the software AutoMetric 2.2 (DeBruine, 2004). The lengths of fingers were measured from the ventral proximal crease to the finger tip (Fink et al., 2004; see Figure 4). The ratio of the length of the index and the ring finger (D2:D4 digit ratio) was averaged.

2.6 Data processing and analysis

2.6.1 Startle response measurements

EMG data were further processed in MATLAB using the EEGLAB toolbox (Delorme & Makeig, 2004). After digitally filtering (28 – 800 Hz passband) data were epoched and rectified. EMG data were visually controlled for artifacts (e.g., eye movements, stimulus-unrelated eye blinks) and affected epochs were excluded from further analysis. In the remaining epochs, the magnitude of the startle response was determined as the peak of EMG amplitude in the time interval from 20 to 150 ms after stimulus onset. According to suggestions of Blumenthal, Elden, and Flaten (2004; see also Blumenthal et al., 2005;

Klumpers et al., 2010), peak values were z-transformed with respect to data from all single-epochs data (all blocks and sessions) of given subject and converted into T-scores using the formula: $T = z * 10 + 50$. Within each session, T-score values from the safe and threat conditions, respectively, were averaged. Since this thesis is focused on the influence of estradiol on fear-potentiated startle, we excluded the habituation stimuli from further analyses.

2.7 Statistical analyses

SPSS Version 20 (IBM, Armonk, USA) was used for all statistical analyses. In order to eliminate outlying observations startle reflex data (see 2.6.1) as well as averaged VAS ratings were winsorized within groups regarding the different substances administered, test sessions, and conditions. Observations below the 25^{th} percentile minus 1.5 times the interquartile range were set to this value. Likewise, observations exceeding the 75^{th} percentile plus 1.5 times the interquartile range were set to that value.

Our test sample was matched for numerous variables, such as subjects' age, BMI, mental and somatic health, abusive behavior, or medication. However, it was not possible to deal with some more variables in a similar manner, namely participants' D2:D4 digit ratio as well as their scores within the NEO FFI subscales Neuroticism and Extraversion. For each session and substance, Pearson product-moment correlation coefficients were calculated to assess the association between trait measures of D2:D4 digit ratio, neuroticism and extraversion on one hand and eye blink startle magnitude and subjective ratings of current affective feelings (VAS scores) on the other hand.
In order to ensure that state conditions did not differ between both test days in our test sample we conducted paired t-tests to compare the respective daily manifestations of participants' S-anxiety scores of the STAI, shock intensity ratings before each baseline session, and changes in the perceived shock intensity two and six hours after substance administration.

A mixed-design (split-plot) analysis of variances (ANOVA) was adopted to test the experimental effects on startle magnitude and subjective feelings. In the case of a violation of the assumption of sphericity determined via Mauchly's test data were adjusted using

Greenhouse-Geisser ($\varepsilon < .75$) or Huynh-Feldt correction ($\varepsilon > .75$), as recommended by Field (2009). In order to examine the influence of estradiol on the startle reflex we calculated a $2 \times 2 \times 4 \times 2$ ANOVA, with the within-subject factors *Substance* (placebo, estradiol), *Test Condition* (safe, threat), *Test Session* (I, II, III, IV), and a between-subject factor *Order of Substance Administration* (1^{st} day placebo and 2^{nd} day estradiol vs. 2^{nd} day placebo and 1^{st} day estradiol) with the *magnitude of the startle reflex* (i.e., averages of the T-score values) as the dependent variable. Analogous ANOVAs were calculated with the ratings of anxiousness, sleepiness, and unpleasantness as dependent variables.

For all statistical tests, the level of statistical significance (α) was set at .05. In the case of significant omnibus F-tests, post hoc calculated pairwise comparisons were conducted using Bonferroni-corrected t-tests (Field, 2009).

3 Results

Table 1 contains descriptive statistics of the constitutional variables age, D2:D4 digit ratio, body mass index (BMI), the scores of the GHQ-12, the subscales neuroticism and extraversion of the NEO-FFI, and T-anxiety of the STAI as assessed in our sample. An overview of the means (M) and standard deviations (SD) of startle response magnitudes regarding substance applied, session, and condition are shown in Table 2. Additionally, the intensities of the perceived affective states anxiousness (Table 3), sleepiness (Table 4), and unpleasantness (Table 5) are presented.

3.1 Analysis of potentially confounding variables

None of the correlation coefficients between the trait variables D2:D4 digit ratio, neuroticism and extraversion score and both the EMG magnitude of the FPS (see Table 6), as well as affective states (Table 7, 8, & 9) indicated more than a weak association. Furthermore, the state variables S-anxiety, perceived pain intensity of the presented shocks and changes in pain intensity two and four hours after gel administration showed no significant differences between test days, as checked by paired t-tests (all $t < 1$; see Table 10). Thus, it is unlikely that these variables could confound the experimental effects of estradiol administration.

3.2 Effects of estradiol on the eye blink response

The analysis of group differences in startle response magnitudes (see Table 11) revealed a highly significant overall effect of the factor *Test Session*, $F(3,90) = 37.93, p < .001, \eta^2 = .558$, with higher startle responses in test session I (M = 53.04, SD = 0.35) compared with all other test sessions, as examined by post hoc tests. Further, in session II (M = 49.51, SD = 0.32) subjects showed significant higher startles than in session III (M = 47.92, SD = 0.30). Test session IV (M = 48.58, SD = 0.38) did not significantly differ from test sessions II or III. Further, a highly significant main effect of *Test Condition* was found,

$F(1,30) = 69.42, p < .001, \eta^2 = .698$, with higher startle responses in threat (M = 51.93, SD = 0.35) compared to safe blocks (M = 47.60, SD = 0.19). Neither a main effect of *Substance*, $F(1,30) = 0.002, p = .964, \eta^2 < .001$, nor interaction effects between the within-subject variables were found (see Figure 5). The overall effect of the between-subject factor *Order of Substance Administration* was also not significant, $F(1,30) = 0.19, p = .666, \eta^2 = .006$. Moreover, the administration order had no significant influence on any of the within-subject effects (see Table 11).

3.3 Effects of estradiol on affective states

A significant main effect on perceived anxiousness (see Table 12) was revealed for *Test Session*, $F(1.92,57.66) = 7.22, p = .002, \eta^2 = .194$. Subsequently conducted post hoc comparisons of *Test Session* indicated that anxiety in test session III (M = 2.37, SD = 0.54) was lower compared with session I (M = 3.94, SD = 0.92), session II (M = 4.2, SD = 1.03), as well as test session IV (M = 3.55, SD = 0.83). *Test Condition* showed also a significant main effect, $F(1,30) = 11.36, p = .002, \eta^2 = .275$, indicating that participants felt overall significantly less anxious in safe (M = 2.76, SD = 0.61) than threat conditions (M = 4.26, SD = 1.02). Additionally, there was a significant *Session* $\times$ *Test Condition* interaction, $F(1.88,56.39) = 7.81, p = .001, \eta^2 = .207$ (see Figure 6). The applied *Substance* had no significant impact on anxiousness. Likewise, there was no significant effect of the *Order of Substance Administration* (see Table 12).

An overall effect on sleepiness (see Figure 7) was found for *Test Session*, $F(2.64,81.87) = 6.66, p = .015, \eta^2 = .177$, with higher ratings in session III (M = 28.01, SD = 3.73) compared with session I (M = 19.1, SD = 2.84) and session IV (M = 18.16, SD = 2.85), as well as higher ratings in session II (M = 24.87, SD = 3.66) compared with session I, as examined by post hoc tests. Further, *Test Condition* showed a significant main effect, $F(1,31) = 8.73, p = .006, \eta^2 = .22$: Study participants were less sleepy in threat (M = 21.14, SD = 2.82) than in the safe conditions (M = 23.94, SD = 3.05). A significant interaction effect of *Substance* $\times$ *Session* $\times$ *Test Condition* $\times$ *Order of Substance Administration*, $F(2.2,65.93) = 3.99, p = .02, \eta^2 = .117$, suggests that the perceived sleepiness depends on

whether the placebo or estradiol was applied first (Figure 8). No other significant effects were found (see Table 13).

Feelings of unpleasantness were significantly influenced by the factor *Test Condition*, $F(1,30) = 11.52, p = .002, \eta^2 = .278$, with higher unpleasant feelings in threat (M = 7.54, SD = 1.61), compared with safe blocks (M = 5.89, SD = 1,25). No more group differences could be found (see Table 14; Figure 9).

4 Discussion

The aim of the present work was to investigate possible anxiolytic effects of estradiol by measuring fear potentiation of acoustic startle response and affective feelings. In summary, both the startle response magnitude and current affective states were affected mainly by different test sessions and conditions. An influence of substances, in particular estradiol, could not be found. The order of substance administration had – with one exception – no impact on the startle responses or the affective states.

In contrast to our expectations, we found no effects of estradiol: Subjects exhibited no attenuation of fear-potentiated startles and affective feelings after the administration of estradiol. These results are surprising, given the number of studies indicating an impact of estrogens on fear processes (e.g., Lebron-Milad & Milad, 2012; Merz et al., 2012; Milad et al., 2009, 2010; Toufexis et al., 2006, 2007). However, these studies primarily examined estradiol's influence on fear conditioning and found a positive effect on fear extinction learning in FPS experiments. Startle potentiation elicited by the threat of shock paradigm is based on the fearful anticipation of an aversive event rather than conditioned fear learning (Grillon et al., 1991). With its important role in learning processes, the involvement of the hippocampus might differ between the two ways of startle potentiation (Grillon, 2008; Lissek et al., 2013; Phelps, 2004) and hippocampal or amygdaloid estrogen receptors could mediate contextual and cue learning rather than influencing the apprehension of threatening stimuli. From this point of view it is conceivable that estradiol affects only conditioned FPS but not anticipatory fear processes.

Participants showed the highest startle responses, perceived anxiety and unpleasantness as well as lowest feelings of sleepiness in test session I compared with later ones. These findings are largely consistent with Klumpers et al. (2010), indicating increased fear reactivity and negative affects in the first session with a moderate habituation over time. However, habituating effects did not influence the differences between fear-potentiated and baseline startle: threat of shock sessions were characterized by higher startle magnitudes and generally

more negative affective states compared to safe blocks. In this respect, our outcomes are consistent with prior threat of shock experiments.
Interestingly both overall startle responses and negative feelings tended to be lowest in test session III. In particular, subjective anxiousness was markedly reduced in threat conditions, with levels comparable with those in safe blocks (see Figure 6). A possible explanation of this could be the timing of the present study. All test subjects were allowed to have lunch only after the second threat of shock session. Digestion processes lead to several endogenous changes, resulting in feelings of comfort and relaxation. Subjects frequently reported such states especially before or after test session III. Thus, reduced fear reactivity might relate to the timing of food intake, an effect that should be considered in future research.

In contrast to prior findings (Corr et al., 1995), we cannot report any effects of the personality traits neuroticism and extraversion on startle magnitude or affective feelings. An explanation for this could be found in the measured personality variables of our test subjects. Influences of estradiol on fear may exist in individuals with high manifestations in neuroticism or low manifestations in extraversion only. On average, however, our participants scored relatively low in neuroticism and high in extraversion (see Table 1), which could be the reason why we did not find any anxiolytic effects of estradiol. Furthermore, Grillon, Dierker, and Merikangas (1998) compared fear reactions between adolescents whose parents suffered from diagnosed anxiety disorders and such with healthy parents. Their results indicate not only startle differences between these two groups, but also between male and female participants. Women are two to three times more often affected by mood and anxiety disorders than men (Kessler et al., 2005; Wittchen et al., 2011), disorders modulated by neuroticism trait (Griffith et al., 2010). Thus, according to findings of fear-reducing effects of exogenous testosterone in female individuals (Hermans et al., 2006), it is possible that testosterone's metabolite estradiol has only modulating effects on fear processes in women. Future research should investigate this issue.

Brain structures regulating anticipatory fear involve the amygdala and hippocampal formation (Phelps, 2004), as well as the prefrontal cortex (PFC; Lissek et al., 2013; van Wingen et al., 2011). Therefore, it is recommended that future research takes a closer look at substances

already known to modulate neuronal activity in these brain areas. Accordingly, Lebron-Milad and Milad (2012) report that progesterone may modulate the communication between the amygdala and the PFC. Moreover, progesterone's metabolite allopregnanolone has anxiolytic effects when infused into the amygdala in rats, probably by influencing the activity of $GABA_A$-receptors (Lebron-Milad & Milad, 2012; Reddy & Jian, 2010; Schüle, Nothdurfter, & Rupprecht, 2014). Opposed to benzodiazepines, which also act via GABA-receptors, allopregnanolone seems to have anxiolytic effects but does not influence spontaneous locomotor activity (see Schüle et al., 2014), suggesting different mechanisms of action of these two agents. Lebron-Milad and Milad (2012) further suggest that testosterone could directly influence the connectivity between the amygdala and the orbitofrontal cortex via androgen receptors. Additionally, as mentioned by Hermans et al. (2006), testosterone could act indirectly as a prohormone within the GABAergic system. For instance, testosterone may exert its anxiolytic effects through its metabolite androstanediol, which can also act by influencing the $GABA_A$ receptors (Reddy & Jian, 2010). Thus, while estrogen might regulate fear learning processes, direct fear reactivity might be modulated by testosterone or progesterone, or their metabolites androstanediol or allopregnanolone (van Wingen et al., 2011).

Nevertheless, the use of estradiol could have some benefits in clinical treatment of certain anxiety disorders. As recently reported by Grillon et al. (2008, 2009), both patients suffering from panic disorder as well as PTSD show symptoms of elevated anxiety, which are moderated by contextual fear cues. For instance, Milad et al. (2010) reported that estrogen blood levels in women who experienced traumatic events predicted the development of a PTSD. Estradiol could be effective in reducing the impact of conditioned fear in these anxiety disorders, a research area worth of further investigations.

To summarize, while regulation of fear processes is complex, our understanding of the underlying mechanisms is limited. Future research should therefore not only focus on potentially anxiolytic substances, but also on the interactions between these substances and neurotransmitter systems. Moreover, differences in concentration of substances such as testosterone, estradiol, or progesterone might have distinct anxiolytic effects. The saliva samples

we collected from our test subjects may be useful in order to investigate these issues. While the analysis of these samples was not completed to date and therefore could not be reported in the current study it is possible that distinctive relationships can be revealed between specific hormone levels and fear reactivity within the threat of shock paradigm.

When interpreting the results of the present work several limitations should be considered. Due to technical difficulties, mostly trigger failures, some startle responses were not recorded in an accurate fashion and similar problems led to an exclusion of a single test session of three participants. Overall, however, we missed less than 5% of data. Several subjects found the threat of shock sessions exhausting and monotonous. Finally, our test sample of 32 participants may be too small to observe existing substance effects.

5 Conclusion

Despite several previous reports of modulation of fear processes by estrogens in a threat of shock paradigm, we did not observe any effect of estradiol administration in healthy adult men. Moreover, estradiol did not affect subjective feelings of anxiety or unpleasantness induced by the threat of shock. Our results indicate that estradiol plays no significant role in fear reactivity.

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A Figures

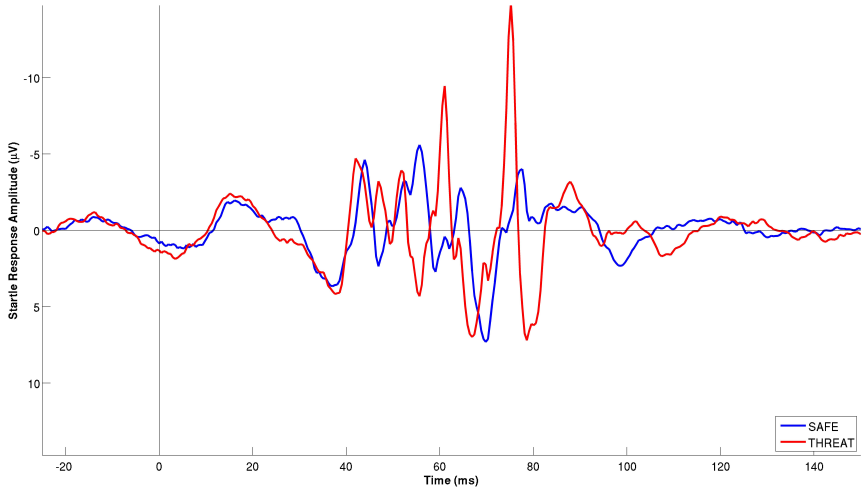

Figure 1: Differences in the eye blink startle amplitudes in respect of safe and threat conditions within a threat of shock paradigm

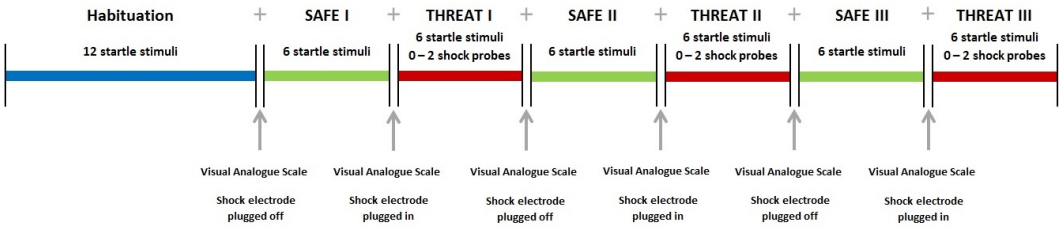

Figure 2: Exemplary threat of shock session procedure, starting – after habituation phase – with safe condition. After each block, visual analogue scales (VAS) were administered.

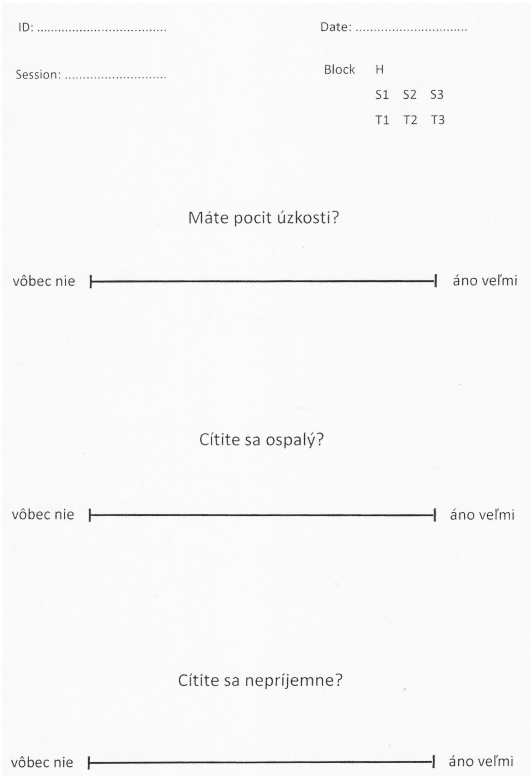

Figure 3: After each block, participants answered three questions about their actually perceived states of anxiousness (*How anxious do you feel?*), sleepiness (*How sleepy do you feel?*), and unpleasantness (*How unpleasant do you feel?*) via visual analogue scales (VAS).

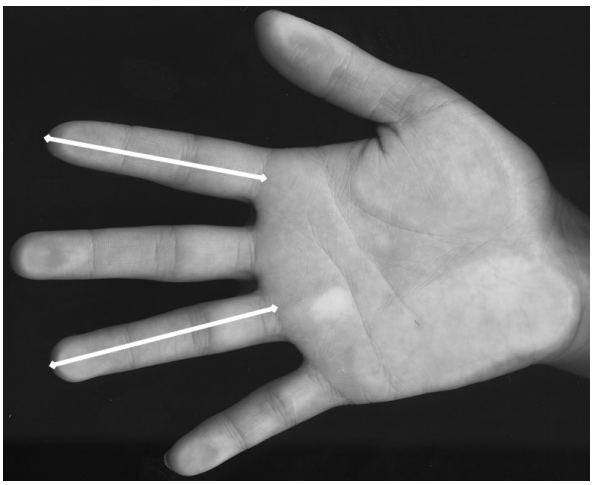

Figure 4: The D2:D4 digit ratio was examined by evaluating the lengths of the digit and the ring finger and calculating their ratio for each hand.

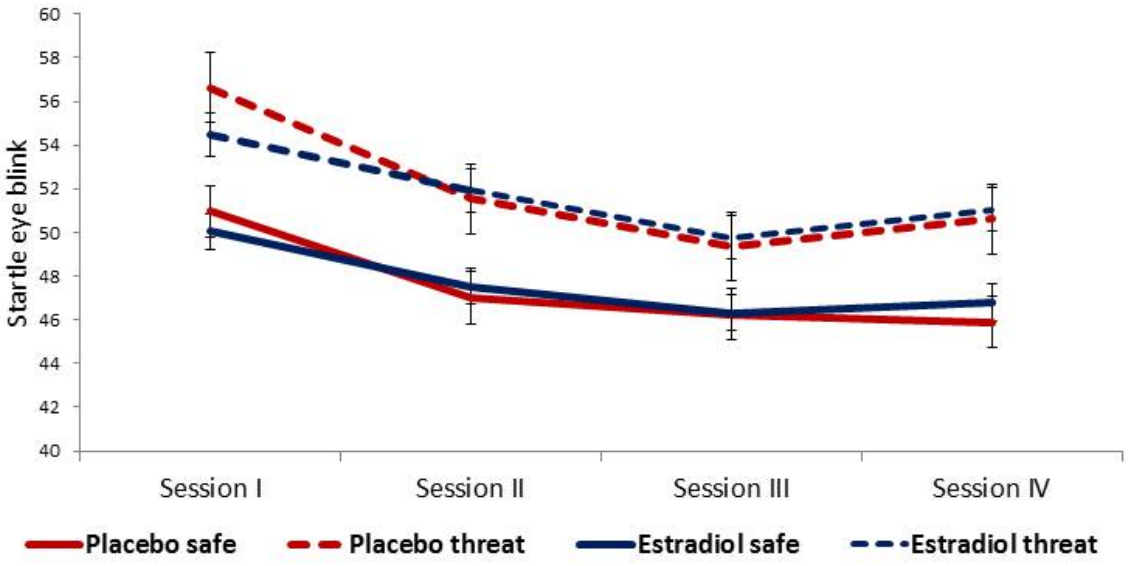

Figure 5: Intensity of startle eye blink responses with regard to different substances, sessions, and test blocks (averaged T-score values). Error bars represent standard errors of the mean

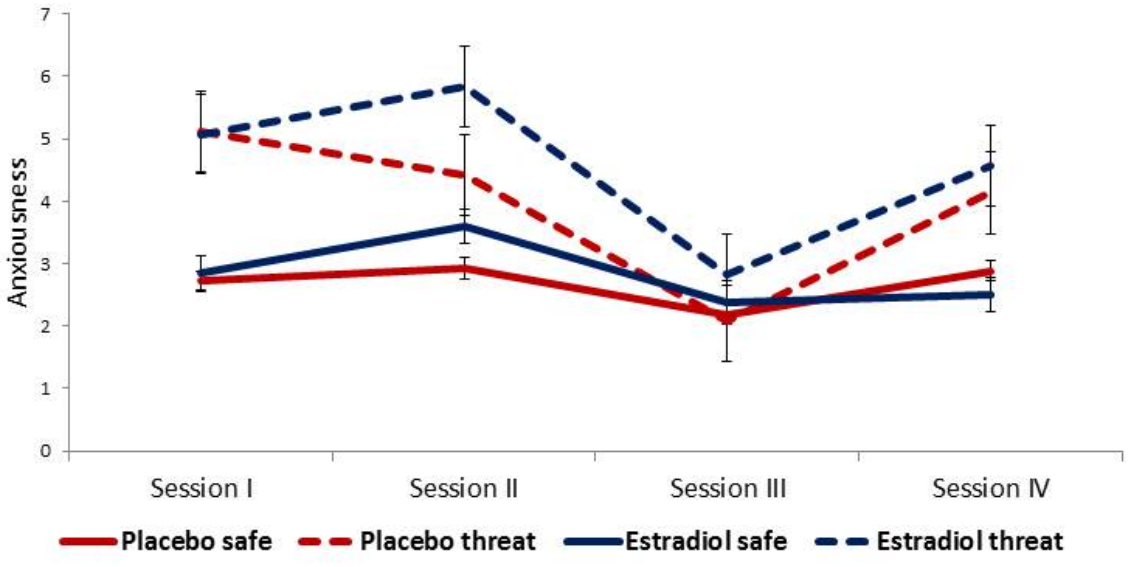

Figure 6: Changes in perception of anxiety over time in respect of different threat of shock conditions. Error bars represent standard errors of the mean

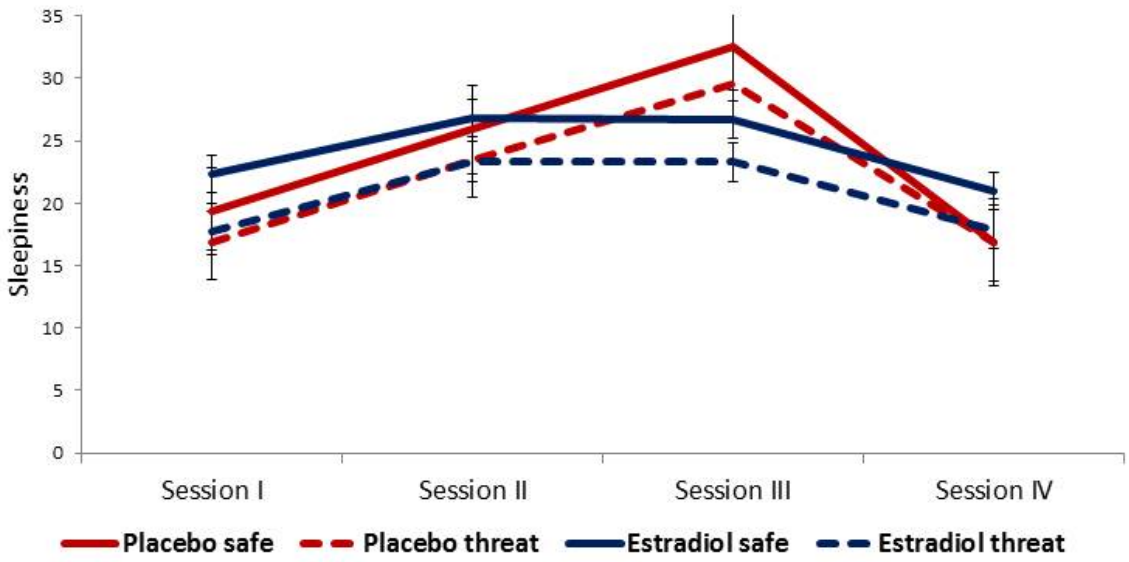

Figure 7: Changes in perception of sleepiness over time in respect of different threat of shock conditions. Error bars represent standard errors of the mean

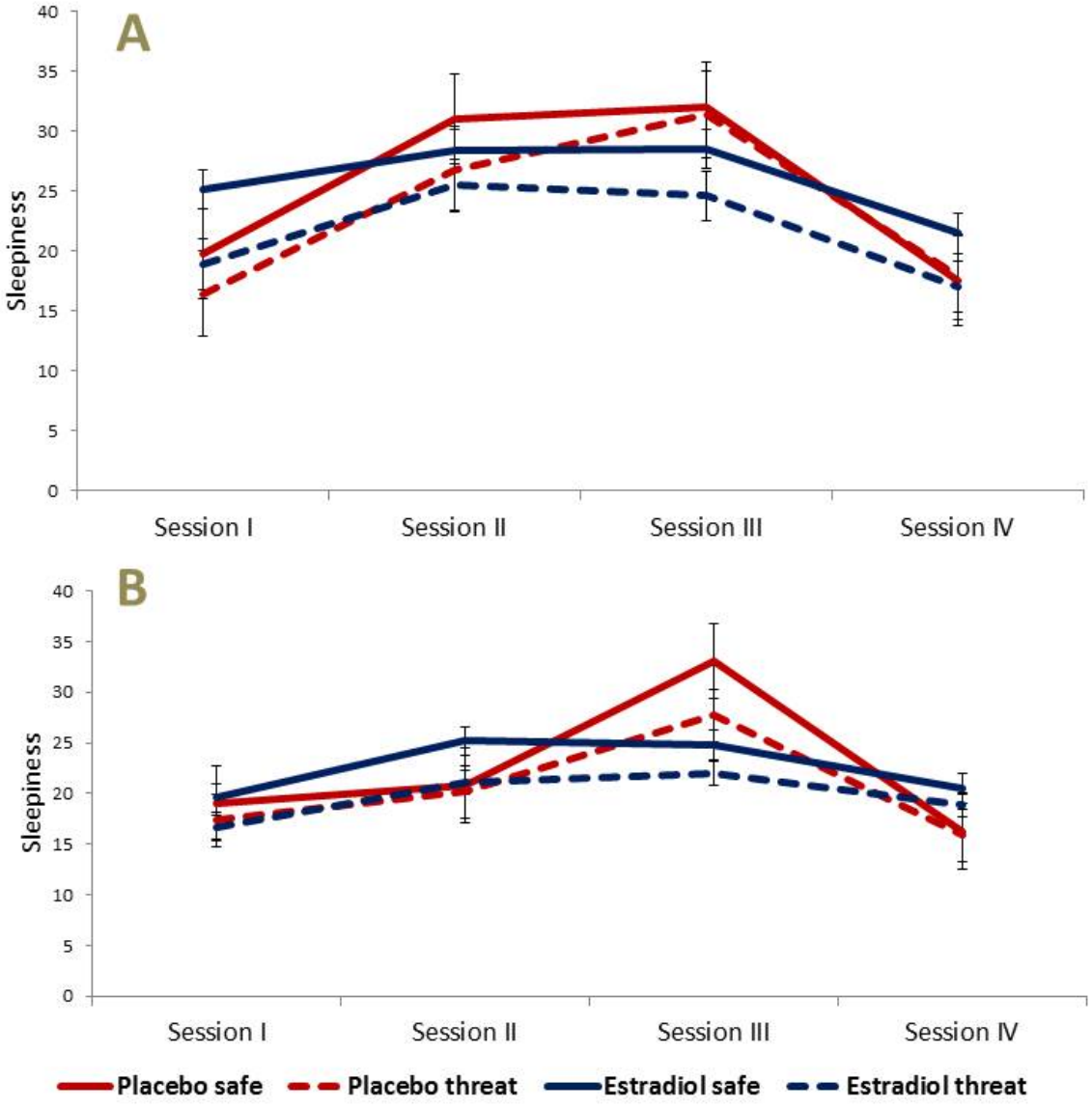

Figure 8: Differences in the perception of sleepiness with regard to substance application order.
A: Administration of placebo on the first test day, administration of estradiol on the second test day.
B: Administration of estradiol on the first test day, administration of placebo on the second test day. Error bars represent standard errors of the mean

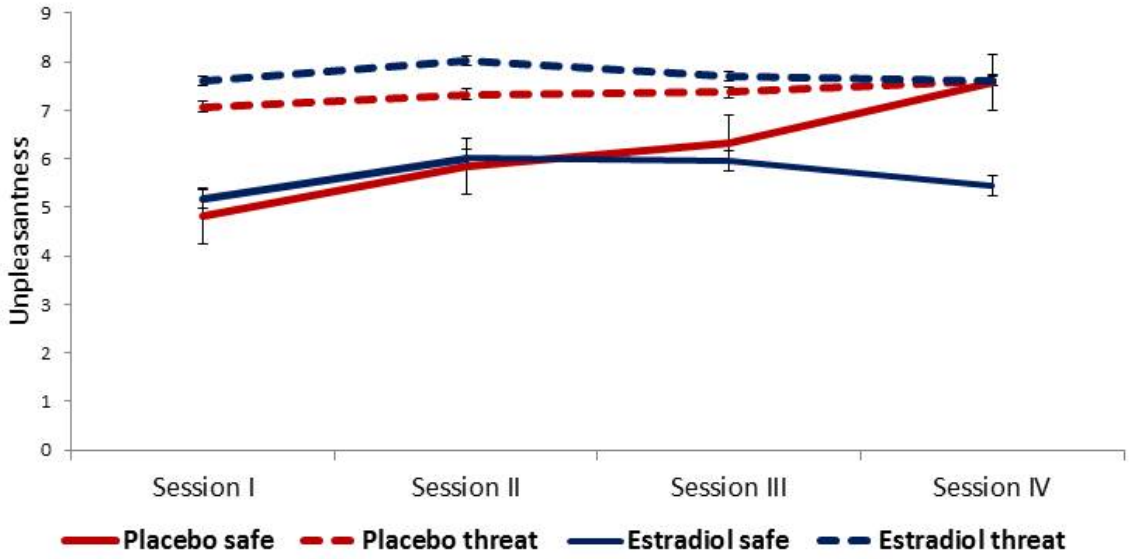

Figure 9: Changes in perception of unpleasantness over time in respect of different threat of shock conditions. Error bars represent standard errors of the mean

B Tables

Table 1: *Descriptive Statistics of Trait Variables*

	Mean	SD	Min	Max
Age	24.19	3.18	20	32
D2:D4	0.95	0.02	0.91	1
BMI	23.02	1.70	20.00	26.50
GHQ-12	1.69	2.21	0	9
Neuroticism	13.65	6.28	2	26
Extraversion	31.68	6.45	17	43
T-Anxiety	33.69	5.596	22	43

Means, standard deviations (SD), minimal and maximal values of subject's demographic data

Table 2: *Startle Eye Blink Magnitudes at Different Test Conditions*

Session	Condition	Placebo	Estradiol
I	Safe	50.98 (3.93)	50.05 (4.47)
	Threat	56.64 (7.93)	54.47 (5.14)
II	Safe	47.01 (2.93)	47.53 (3.36)
	Threat	51.56 (5.09)	51.95 (4.52)
III	Safe	46.23 (2.78)	46.32 (2.80)
	Threat	49.36 (3.50)	49.76 (4.72)
IV	Safe	45.88 (2.58)	46.79 (2.79)
	Threat	50.61 (4.71)	51.06 (5.88)

Note. Mean (standard deviation) of averaged startle responses

Table 3: *Subjective Perception of Anxiety at Different Test Conditions*

Session	Condition	Placebo	Estradiol
I	Safe	2.72 (3.70)	2.86 (3.72)
	Threat	5.12 (7.74)	5.08 (6.98)
II	Safe	2.93 (4.23)	3.60 (5.06)
	Threat	4.42 (6.65)	5.84 (8.99)
III	Safe	2.19 (2.99)	2.38 (3.03)
	Threat	2.08 (2.57)	2.82 (3.79)
IV	Safe	2.89 (3.92)	2.50 (3.21)
	Threat	4.14 (6.06)	4.57 (6.52)

Note. Mean (standard deviation) of averaged VAS ratings

Table 4: *Subjective Perception of Sleepiness at Different Test Conditions*

Session	Condition	Placebo	Estradiol
I	Safe	19.39 (18.57)	22.34 (20.09)
	Threat	16.90 (15.43)	17.77 (15.99)
II	Safe	25.90 (25.85)	26.79 (22.59)
	Threat	23.47 (23.43)	23.32 (21.58)
III	Safe	32.54 (24.44)	26.67 (21.60)
	Threat	29.54 (24.46)	23.31 (20.79)
IV	Safe	16.86 (16.02)	21.00 (19.27)
	Threat	16.85 (16.53)	17.92 (17.57)

Note. Mean (standard deviation) of averaged VAS ratings

Table 5: *Subjective Perception of Unpleasantness at Different Test Conditions*

Session	Condition	Placebo	Estradiol
I	Safe	4.81 (6.87)	5.17 (6.47)
	Threat	7.06 (10.34)	7.61 (9.32)
II	Safe	5.84 (8.42)	6.01 (7.46)
	Threat	7.32 (10.12)	8.02 (10.38)
III	Safe	6.31 (8.87)	5.95 (7.98)
	Threat	7.37 (9.90)	7.69 (10.19)
IV	Safe	7.57 (10.73)	5.45 (7.52)
	Threat	7.61 (10.59)	7.61 (10.97)

Note. Mean (standard deviation) of averaged VAS ratings

Table 6: *Pearson Correlation Coefficients Between D2:D4 Digit Ratio, Neuroticism as well as Extraversion and the Startle Responses*

Substance	Session	Condition	Digit Ratio	Neuroticism	Extraversion
Placebo	I	Safe	-.067	-.097	-.121
		Threat	-.001	.131	-.128
	II	Safe	.056	-.110	-.155
		Threat	-.165	-.057	-.181
	III	Safe	-.023	-.031	.026
		Threat	.100	.075	.243
	IV	Safe	.134	-.168	-.102
		Threat	-.020	.047	-.014
Estradiol	I	Safe	-.141	-.040	.288
		Threat	.002	.046	.277
	II	Safe	.073	.318	.074
		Threat	.135	.023	.022
	III	Safe	$< .001$	.143	-.122
		Threat	-.101	.040	.034
	IV	Safe	-.090	.019	-.059
		Threat	.031	-.094	.139

Table 7: *Pearson Correlation Coefficients Between D2:D4 Digit Ratio, Neuroticism as well as Extraversion and Subjective Anxiousness*

Substance	Session	Condition	Digit Ratio	Neuroticism	Extraversion
Placebo	I	Safe	.208	-.219	.167
		Threat	.245	-.247	.153
	II	Safe	.225	-.242	.173
		Threat	.289	-.302	.201
	III	Safe	.141	-.118	.080
		Threat	.148	-.178	.176
	IV	Safe	.240	-.158	.045
		Threat	.415	-.160	.166
Estradiol	I	Safe	.222	.077	-.035
		Threat	.267	-.077	.079
	II	Safe	.279	.020	.005
		Threat	.263	-.074	.039
	III	Safe	.201	-.006	.068
		Threat	.242	-.100	.117
	IV	Safe	.092	-.088	-.038
		Threat	.170	-.122	.026

Table 8: *Pearson Correlation Coefficients Between D2:D4 Digit Ratio, Neuroticism as well as Extraversion and Perceived Sleepiness*

Substance	Session	Condition	Digit Ratio	Neuroticism	Extraversion
Placebo	I	Safe	.090	-.130	.061
		Threat	.082	-.167	.113
	II	Safe	.196	-.176	.118
		Threat	.180	-.199	.172
	III	Safe	.072	-.266	-.025
		Threat	.008	-.290	-.035
	IV	Safe	.167	-.039	-.103
		Threat	.130	-.102	-.077
Estradiol	I	Safe	.274	-.110	.152
		Threat	.236	-.135	.207
	II	Safe	.183	-.097	.152
		Threat	.147	-.173	.168
	III	Safe	.115	-.053	-.028
		Threat	.104	-.098	-.005
	IV	Safe	.194	.285	-.267
		Threat	.177	.164	-.243

Table 9: *Pearson Correlation Coefficients Between D2:D4 Digit Ratio, Neuroticism as well as Extraversion and Perceived Unpleasantness*

Substance	Session	Condition	Digit Ratio	Neuroticism	Extraversion
Placebo	I	Safe	.123	-.085	-.007
		Threat	.212	-.131	.049
	II	Safe	.033	-.093	-.080
		Threat	.090	-.097	-.043
	III	Safe	.084	-.085	-.123
		Threat	.092	-.036	-.188
	IV	Safe	.123	-.161	-.104
		Threat	.232	-.150	-.044
Estradiol	I	Safe	.119	.155	-.111
		Threat	.115	.019	-.010
	II	Safe	.274	.252	-.064
		Threat	.261	.093	-.006
	III	Safe	.036	.068	-.150
		Threat	.082	.014	-.040
	IV	Safe	-.017	.038	-.182
		Threat	.048	.022	-.142

Table 10: *Anxiety States, Subjective Shock Intensities, and Changes in Pain Intensity on Both Test Days*

	Test Day				
	Placebo	Estradiol	df	*t*	*p*
S-Anxiety Score	32.41 (5.60)	32.45 (7.09)	28	0.04	.972
Perceived Pain Intensities	17.12 (15.15)	17.63 (12.46)	31	0.25	.807
Intensity Changes (2 Hrs. After Administration)[a]	2.50 (0.81)	2.70 (1.12)	29	0.72	.476
Intensity Changes (6 Hrs. After Administration)[a]	2.92 (0.91)	2.72 (1.31)	24	-0.59	.558

Note. Means (standard deviations), degrees of freedom, t-statistics and p-values

[a] Values are mean scores on a 5-point Likert scale (1 = *significantly less painful*, 5 = *significantly more painful*)

Table 11: *Main and Interaction Effects of Group Factors on the Eye Blink Startle Response*

	df_{model}	df_{error}	F	P
Substance	1	30	0.002	.964
Session	3	90	37.927	$<.001$[1]
Condition	1	30	69.415	$<.001$[2]
Administration Order	1	30	0.190	.666
Substance × Administration Order	1	30	1.334	.257
Session × Administration Order	3	90	0.174	.914
Condition × Administration Order	1	90	0.063	.804
Substance × Session[a]	2.69	35.424	1.985	.129
Substance × Condition	1	30	0.350	.559
Session × Condition	3	90	2.158	.098
Substance × Session × Administration Order[a]	2.69	35.42	1.890	.144
Substance × Condition × Administration Order	1	30	0.686	.414
Session × Condition × Administration Order	3	90	1.459	.231
Substance × Session × Condition[a]	2.64	79.06	0.615	.586
Substance × Session × Condition × Administration Order[a]	2.64	79.06	1.735	.173

Note. degrees of freedom (*df*), *F*-statistics and *p*-values of the different group factors

[a]Huynh-Feldt correction

[1]$\eta^2 = .558$

[2]$\eta^2 = .698$

Table 12: *Main and Interaction Effects of Group Factors on the Perception of Anxiety*

	df_{model}	df_{error}	F	P
Substance	1	30	1.414	.244
Session[a]	1.92	57.66	7.216	.002[1]
Condition	1	30	11.363	.002[2]
Administration Order	1	30	3.581	.068
Substance × Administration Order	1	30	2.547	.121
Session × Administration Order[a]	1.92	57.66	0.795	.452
Condition × Administration Order	1	30	1.294	.264
Substance × Session[a]	1.52	45.64	1.109	.350
Substance × Condition	1	30	2.197	.149
Session × Condition[a]	1.88	56.39	7.812	.001[3]
Substance × Session × Administration Order[a]	1.52	45.64	2.569	.101
Substance × Condition × Administration Order	1	30	0.592	.448
Session × Condition × Administration Order[a]	1.88	56.39	1.969	.152
Substance × Session × Condition[a]	1.96	58.81	1.066	.350
Substance × Session × Condition × Administration Order[a]	1.96	58.81	0.488	.612

Note. degrees of freedom (*df*), *F*-statistics and *p*-values of the different group factors

[a]Greenhouse-Geisser correction

[1]$\eta^2 = .194$

[2]$\eta^2 = .275$

[3]$\eta^2 = .207$

Table 13: *Main and Interaction Effects of Group Factors on the Perception of Sleepiness*

	df_{model}	df_{error}	F	P
Substance	1	30	0.027	.870
Session[a]	2.72	81.72	6.539	.001[1]
Condition	1	30	8.491	.007[2]
Administration Order	1	30	0.218	.644
Substance × Administration Order	1	30	0.003	.956
Session × Administration Order[a]	2.72	81.72	0.422	.719
Condition × Administration Order	1	30	0.144	.707
Substance × Session	3	90	2.573	.059
Substance × Condition	1	30	3.737	.063
Session × Condition	3	90	1.909	.134
Substance × Session × Administration Order	3	90	0.608	.612
Substance × Condition × Administration Order	1	30	0.937	.341
Session × Condition × Administration Order	3	90	2.068	.110
Substance × Session × Condition[b]	2.20	65.93	1.061	.357
Substance × Session × Condition × Administration Order[b]	2.20	65.93	3.994	.020[3]

Note. degrees of freedom (*df*), *F*-statistics and *p*-values of the different group factors

[a]Huynh-Feldt correction

[b]Greenhouse-Geisser correction

[1]$\eta^2 = .179$

[2]$\eta^2 = .221$

[3]$\eta^2 = .117$

Table 14: *Main and Interaction Effects of Group Factors on the Perception of Unpleasantness*

	df_{model}	df_{error}	F	P
Substance	1	30	0.002	.963
Session[a]	1.76	52.86	0.657	.504
Condition	1	30	11.523	.002[1]
Administration Order	1	30	2.224	.146
Substance × Administration Order	1	30	0.105	.748
Session × Administration Order[a]	1.76	52.86	0.396	.649
Condition × Administration Order	1	30	0.840	.367
Substance × Session[a]	2.13	63.80	0.791	.465
Substance × Condition	1	30	2.686	.112
Session × Condition[b]	2.78	83.35	2.601	.062
Substance × Session × Administration Order[a]	2.13	63.80	0.532	.601
Substance × Condition × Administration Order	1	30	0.037	.849
Session × Condition × Administration Order[b]	2.78	83.35	2.108	.110
Substance × Session × Condition[b]	2.60	78.04	1.186	.318
Substance × Session × Condition × Administration Order[b]	2.60	78.04	1.696	.181

Note. degrees of freedom (df), F-statistics and p-values of the different group factors

[a]Greenhouse-Geisser correction

[b]Huynh-Feldt correction

[1]$\eta^2 = .278$

Curriculum Vitae

Zur Person

Name:	Jan Sören Seidel
Geburtsdatum	21.11.1986
Geburtsort	Bad Kreuznach
Staatsbürgerschaft:	deutsch

Ausbildung

Oktober 2014	Voraussichtlicher Abschluss des Diplomstudiums Psychologie
02. August.2010	Erste Diplomprüfung Psychologie (mit Auszeichnung bestanden)
ab März 2008	Diplomstudium Psychologie an der Universität Wien
März 2007	Abitur am Gymnasium am Römerkastell, Bad Kreuznach

Praktika

April 2013 – Juli 2014	Praktikum an der Social, Cognitive and Affective Neuroscience (SCAN) Unit, Fakultät für Psychologie, Institut für Psychologische Grundlagenforschung und Forschungsmethoden, Universität Wien
Okt. 2012 – Feb. 2013	Praktikum am Institut für Bewusstseins - und Traumforschung, Wien
Juni – Juli 2012	Sechs-Wochen-Praktikum im Rahmen des Psychologiestudiums, Neuropsychologisches Labor des Sozialmedizinischen Zentrums Baumgartner Höhe Otto-Wagner-Spital und Pflegezentrum, Wien

Berufstätigkeit

ab März 2013	Freiberufliche Mitarbeit am Institut für Bewusstseins- und Traumforschung, Wien
April – Dez. 2007	Zivildienst bei der Ökumenischen Sozialstation Rüdesheim-Stromberg e.V., Hargesheim