Investigating the role of the central and the peripheral benzodiazepine receptor on stress and anxiety related parameters

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ABSTRACT

Anxiety disorders belong to the most prevalent mental disorders worldwide implying a high burden of illness. Due to their complexity and variety of underlying dysfunctions, it remains a challenging task to find optimum pharmacological treatment. Benzodiazepines, which modulate the $GABA_A$ or central benzodiazepine receptor, are among the most frequently used substances in this field. However, they hold several side effects, especially at longer application. In the search for alternatives, substances that target the translocator protein (TSPO), also known as peripheral benzodiazepine receptor, yield promising candidates. Within the present work, we aimed to compare the effects of a benzodiazepine and a TSPO ligand, which further binds to $GABA_A$ receptors, on stress and anxiety related parameters.

Within a randomized clinical trial, 60 healthy male subjects received either a daily dose of 1.5 mg alprazolam, 150 mg etifoxine or placebo for a period of five days. We applied the Trier Social Stress Test in Virtual Reality, the NPU Threat Test, resting-state functional imaging, and the Continuous Performance Test and assessed self-reports, physiological parameters, salivary and blood markers, objective performance parameters as well as changes of functional brain connectivity.

While alprazolam blunted the response of the HPA axis to acute psychosocial stress, etifoxine increased expression of TSPO independent of additional external stimulation. There were no effects of the medication on subjective or physiological measures of the stress response. Neither alprazolam nor etifoxine had an impact on the anxiety-related startle reflex, while etifoxine attenuated the fear-potentiated startle on day 1 of treatment. Alprazolam increased functional neuronal connectivity within and between several resting-state networks. In neither group, there was strong impairment of alertness or any serious adverse event or study dropout. When focusing on symptoms related to sedation, there were more reports from subjects that had received alprazolam.

The present work revealed different effects of the applied substances on molecular, physiological and neuronal markers of stress and anxiety. Thereby, especially the strength with which the GABAergic system is affected seems to play an important role, while the involvement of TSPO might be rather specific and dependent on the respective pathological state.

ZUSAMMENFASSUNG

Angststörungen gehören zu den häufigsten psychischen Erkrankungen weltweit und gehen mit einer hohen Belastung einher. Auf Grund ihrer Komplexität und Vielzahl an zugrunde liegenden Dysfunktionen bleibt es eine Herausforderung, optimale pharmakologische Behandlung zu erhalten. Benzodiazepine, welche den $GABA_A$ bzw. zentralen Benzodiazepinrezeptor modulieren, gehören zu den am häufigsten eingesetzten Substanzen in diesem Bereich. Sie verursachen jedoch, vor allem bei längerer Anwendung, zahlreiche Nebenwirkungen. Auf der Suche nach Alternativen stellen Substanzen, die das Translokator Protein (TSPO), auch bekannt als peripherer Benzodiazepinrezeptor, forcieren, vielversprechende Kandidaten dar. Ziel der vorliegenden Arbeit war es, die Effekte eines Benzodiazepins und eines TSPO Liganden, der ferner an $GABA_A$ Rezeptoren bindet, auf Stress- und angstassoziierte Parameter zu vergleichen.

Im Rahmen einer randomisierten klinische Studie erhielten 60 gesunde männliche Probanden entweder täglich 1.5 mg Alprazolam, 150 mg Etifoxin oder Placebo über einen Zeitraum von fünf Tagen. Wir setzten den Trier Sozialen Stress Tests in Virtueller Realität, den NPU Threat Test, funktionelle Bildgebung während Ruhe und den Continuous Performance Test ein und erfassten Selbstberichte, physiologische Parameter, Speichel- und Blutmarker, objektive Leistungsparameter sowie Veränderungen der funktionellen Konnektivität im Gehirn.

Während Alprazolam die Reaktion der HPA Achse auf akuten psychosozialen Stress reduziere, erhöhte Etifoxin die Expression von TSPO unabhängig von weiterer externer Stimulation. Es zeigten sich keine Effekte der Medikation auf subjektive oder physiologische Parameter der Stressreaktion. Weder Alprazolam noch Etifoxin beeinflussten den Angst-bezogenen Schreckreflex, während Etifoxin den Furcht-potenzierten Startle an Tag 1 der Behandlung reduzierte. Alprazolam erhöhte die funktionelle neuronale Konnektivität innerhalb und zwischen verschiedenen Ruhenetzwerken. In keiner der Gruppen zeigte sich eine merkliche Beeinträchtigung der Aufmerksamkeit und es waren keine schwerwiegenden unerwünschten Ereignisse oder Studienabbrüche zu verzeichnen. Mit Fokus auf unerwünschte Ereignisse in Zusammenhang mit Sedierung gab es mehr Berichte von Probanden, die Alprazolam erhalten hatten.

Die vorliegende Arbeit ergab unterschiedliche Effekte der eingesetzten Substanzen auf molekulare, physiologische und neuronale Marker von Stress und Angst. Dabei scheint vor allem die Stärke, mit der das GABAerge System beeinflusst wird, eine entscheidende Rolle zu spielen, während die Beteiligung von TSPO eher spezifisch und abhängig vom jeweiligen pathologischen Zustand zu sein scheint.

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ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
AMG	Arzneimittelgesetz (engl.: Medicines Act)
ANOVA	Analysis of variance
ANT	Adenine nucleotide transporter
AROMA	Automatic Removal of Motion Artifacts
ASI	Anxiety Sensitivity Index
ATC	Acute Toxic Class
AX-CPT	Continuous Performance Test (AX-version)
Bpm	Beats per minute
BMBF	Bundesministerium für Bildung und Forschung (engl.: German Federal Ministry for Education and Research)
BMI	Body Mass Index
BfArM	Bundesoberbehörde für Arzneimittel und Medizinprodukte (engl.: Federal Institute for Drugs and Medical Products)
BOLD	Blood oxygenation level-dependent
CCK-4	Cholecystokinin-tetrapeptide
CNS	Central nervous system
CPS.	Capsules
CRH	Corticotropin-releasing hormone
DFG	Deutsche Forschungsgesellschaft (engl.: German Research Foundation)
DICOM	Digital Imaging and Communications in Medicine
EDTA	Ethylenediamine tetraacetic acid
EPI	Echoplanar imaging
FOV	Field of view
FSL	FMRIB Software Library
GABA	Gamma-amino-butyric-acid
GCP	Good Clinical Practice
GG	Greenhouse-Geisser
GOT	Glutamic oxaloacetate transaminase
GPT	Glutamic pyruvate transaminase
GRK	Graduiertenkolleg (engl.: graduate college)
HMD	Head mounted display
HPA	Hypothalamic-pituitary-adrenal
HR	Heart rate

IC(A)	Independent Component (Analysis)
ICH	International Conference on Harmonization
IIT	Investigator Initiated Trial
IMP	Investigational Medicinal Product
ITT	Intention to treat
IU(S)	Intolerance of Uncertainty (Scale)
kDa	Kilodalton
MELODIC	Multivariate Exploratory Linear Optimized Decomposition into Independent Components
MmHG	Millimeter Quecksilbersäule (engl.: millimeter mercury)
Mg	Milligram
Min.	Minutes
MNI	Montreal Neurological Institute
MP-RAGE	Magnetization Prepared Rapid Gradient Echo
MRI	Magnetic resonance imaging
NIfTI	Neuroimaging Informatics Technology Initiative
PANAS	Positive and Negative Affect Schedule
PBS-T	Phosphate-buffered saline with Tween
PBR	Peripheral benzodiazepine receptor
PP	Per protocol
PVN	Paraventricular nucleus
Rs-fMRI	Resting state functional magnetic resonance imaging
RSN	Resting state network
RT	Room temperature
SAM	Sympathetic-adrenal-medullary
SCL	Skin conductance level
SNRI	Serotonin-noradrenaline reuptake inhibitors
SSRI	Selective serotonin reuptake inhibitors
STAI	State-trait-anxiety inventory
STAR	Steroidogenic acute regulatory protein
SVF	Stressverarbeitungsfragebogen (engl.: Stress Coping Questionnaire)
TE	Echo time
TR	Repetition time
TSPO	Translocator Protein
VAS	Visual Analogue Scales
VDAC	Voltage-dependent anion channel
VR-TSST	Trier Social Stress Test in Virtual Reality

CHAPTER 1: General introduction

"The stress of life" (Selye, 1956) – published almost 70 years ago by one of the pioneers in stress research – the title of his book outlines an issue that has lost none of its meaning. Although this term first of all describes a vital reaction that enables the organism to adapt to challenging situations, mainly referred to as the "good" so-called "eustress", we mostly think of the "bad" so-called "distress", which is related to prolonged or excessive exposure to stressful stimuli (Selye, 1976). The latter yields one of the main health risks of the 21st century promoting the development and maintenance of various somatic and mental disorders like cardiovascular diseases (Everson-Rose & Lewis, 2005), metabolic disorders (Tamashiro, Sakai, Shively, Karatsoreos, & Reagan, 2011) as well as depressive and anxiety disorders (Heim & Nemeroff, 2001), to name only a few. Besides an immense cost factor for the health system resulting from those disorders, they imply high individual burden. Within the present work, we will focus on anxiety disorders, which are globally ranked amongst the top ten under the leading causes of years lived with disability (Vos et al., 2015).

Besides psychotherapy as first choice, pharmacological treatment or a combination of both is recommended by guidelines for the treatment of anxiety disorders (Bandelow, Lichte, Rudolf, Wiltink, & Beutel, 2015). However, given the complexity and variety of dysfunctions underlying the manifestation of anxiety as well as the chronicity of the emerging disorders, it has been and will continue to be a challenging task to find optimum evidence-based treatment. Thereby, especially the promotion of "precision medicine" that emphasizes the individual with its specific conditions and needs is desirable. This requires deeper understanding of the diverse mechanisms that are involved in the action of anxiolytic compounds to obtain the most favorable ones which combine good efficacy and tolerability with only little side effects. In addition, it is necessary to further investigate biomarkers that are specific for different forms of disorders and allow reliable determination of treatment response for predicting pharmacological outcome (Bandelow, Baldwin et al., 2017). Such advance relies on preclinical and clinical research, which combines knowledge of the complex biological nature underlying pathological states with clinical aspects derived from studies in humans. Therein, it has more and more been recognized that multimodal approaches, which integrate information of molecular, physiological, neuronal, and psychological aspects of the organism, are needed.

In that sense, the present work presents a clinical trial that aimed to compare the effects of two anxiolytics, which involve different molecular mechanisms on stress and anxiety, thereby taking the aspect of multimodal measurement into account. Healthy subjects received either the

benzodiazepine alprazolam that acts via the central $GABA_A$ receptor or the less investigated benzoxazine derivate etifoxine, which likewise involves the GABAergic system but additionally acts upon the translocator protein, also known as peripheral benzodiazepine receptor. Efficacy, secondary effects as well as the involvement of underlying mechanisms were compared between those two compounds and to placebo. Thereby, we aimed to gain further insights into the role of those two protein-based systems in stress and anxiety and contribute to the improvement of pharmacological prevention and treatment options of anxiety disorders.

1.1 Overview

To encourage a comprehensive and scientifically based understanding of stress, the first chapter starts with an overview of stress concepts and introduces different kinds of stressors. This is followed by a description of the two major systems that are involved in the regulation of bodily processes related to stress – the sympathetic-adrenal-medullary system and the hypothalamic-pituitary-adrenal axis. The subsection concludes with insights on certain dysfunctions of those systems, which are strongly related to pathological states, thereby focusing on anxiety disorders.

The next main part of the introduction commences with an overview of established anxiolytic treatment. We will further go into detail for the GABAergic system as well as the translocator protein by introducing their structure and main functions as well as research underlining their role in stress and anxiety. Lastly, preclinical and clinical research on the two compounds that were investigated in the present work is summarized.

1.2 Stress

When asking people about their associations with stress, one will likely obtain several varying answers. The most stated definitions would probably be of psychological nature and related to reactions or situations that are characterized by negative experiences, high demands and burden. Interestingly, less than 100 years ago, the term stress was rather used for the description of physiological processes than psychological phenomena.

1.2.1 Overview of stress concepts

Although the description of stress related phenomena might date back earlier, it seems legit to start with one of the pioneering concepts in this field: homeostasis. First introduced by

Walter Cannon (1929), this term describes physiological efforts to keep certain parameters like body temperature or glucose levels within an adequate range to guarantee internal stability of the organism. Conclusions were based on studies which showed that external impulses like cold or heat, but also emotional distress or traumatic pain cause physical activation to restore this homeostasis. The preparation of different body systems to enable rapid reaction and adaption to threat was first summarized within the concept of "fight or flight", which granted a key role to the adrenal gland and its secretion of adrenaline also termed epinephrine (Cannon, 1929; Cannon & Lissak, 1939). Despite the later replacement of adrenaline by noradrenaline (also known as norepinephrine) as major sympathetic neurotransmitter (Euler, 1946), this constituted the first hint to one of the key stress systems, the so-called sympathetic-adrenal-medullary system (SAM) (see 1.2.2.1).

The shaping of the actual stress concept was due to Hans Selye who characterized stress as any physical reaction of an individual to external demands delineated as the general adaption syndrome (Selye, 1936). One of his numerous contributions was the identification and isolation of specific hormones, particularly glucocorticoids, which are the most crucial elements for the coordination of the bodily stress response via the so-called hypothalamic-pituitary-adrenal (HPA) axis (see 1.2.2.2) (Selye, Hall, & Rowley, 1943). Based on findings that parameters like stress-related hormones need to relocate their set-points in order to keep the internal stability throughout different states of the organism, the idea of homeostasis was complemented by the concept of allostasis, which means stability by change (McEwen, 1998; McEwen & Wingfield, 2003; Sterling, 2004). While stress actually yields an adaptive and necessary response of the body to demands, it was shown that chronic exposure results in exhaustion causing systematic sometimes irreversible changes of organs and systems (Selye, 1936). This led to the differentiation between eustress and distress with the former being acceptable even healthy (e.g. sports activities), while the latter is pathogenic and disagreeable (Selye, 1976). The wear and tear of the body resulting from overstimulation of elicited regulation processes is called allostatic load and has been related to mental and physical disorders (see 1.2.3). The underlying mechanisms can be diverse ranging from the appearance of several stressors in very short intervals to the physiological stress reaction outlasting the stressor, too small stress reactions or a lack of habituation in case of repeating stressors (McEwen, 1998; McEwen & Wingfield, 2003).

Contrary to the first theories, it was shown that different internal or external stimuli which challenge the state of homeostasis and require an adaption reaction, so-called stressors, differ with respect to the elicited response (Pacak et al., 1998). The specificity of the reaction

is dependent on the type, magnitude and duration (acute vs. chronic) of the stressor. The most common differentiation concerns threat of the physical self or intimidation of the social self (G. E. Miller, Chen, & Zhou, 2007). Physical stressors have a direct influence on physiological processes of the organism without requiring evaluation and higher cognitive processes. They comprise adverse unpleasant sensory and subjective experience associated with potential damage of body tissue and bodily threat like, for example, extreme cold, oxidative stress or pain (Pacak & Palkovits, 2001). Social stressors entail much more complex mechanisms and depend on emotional and cognitive evaluation processes (Chrousos, 2009; Goldstein & Kopin, 2007). They are more strongly determined by experiences and behavioral options of an individual (Mason, 1968). Cognitive evaluation processes as mediators between a stimulus and the respective stress reaction were first refined in the transactional model of stress by Lazarus (1966). According to that, the evaluation of a situation to be threatening is influenced by the extent to which a stressor harms the own self (primary appraisal) and meets the individuals strategies to cope with the situation (secondary appraisal). A further determinant is the occurrence of reappraisal of the situation. Especially situations that are characterized by social evaluation or exclusion as well as performance situations, which require goal-directed behavior, pose a threat to the own self and are therefore handled as powerful social stressors (Dickerson & Kemeny, 2004).

In summary, stress can be emphasized as a state of threatened homeostasis, either induced physically or perceived subjectively that is defined by the stimulus (= stressor), the perceptual processing of this input (= stress) as well as the behavioral and physiological output (= stress response) (Levine, 2005; Pacak & Palkovits, 2001). The two major physiological systems that are related to the stress response by enabling adaption and sustainment of homeostasis of an organism will be described in the following.

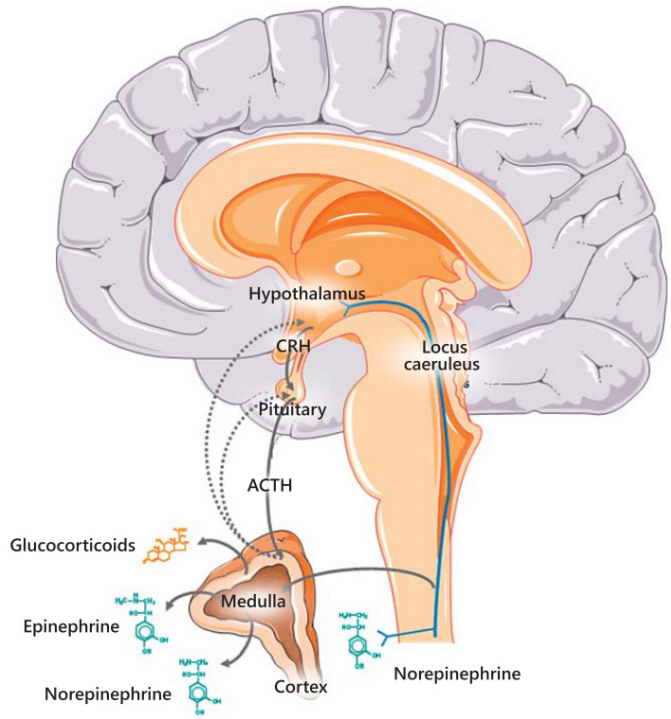
1.2.2 Major physiological stress systems

The two major physiological stress systems are needed for the supply and redistribution of energy, thereby slightly differing with respect to excitability and timing aspects. Direct confrontation with physical stressors requiring a fight-or-flight decision is mainly connected to the fast activation of the sympathetic-adrenal-medullary (SAM) system and the release of catecholamines (Valentino & van Bockstaele, 2008). Characteristics like novelty, unpredictability, uncontrollability, or intimidation of the self particularly trigger the slightly delayed hypothalamic-pituitary-adrenal (HPA) axis with subsequent release of glucocorticoids (Dickerson & Kemeny, 2004; Ursin, 1998). Importantly, those two systems are not completely

separable but rather act in parallel controlling and interacting with each other (Chrousos, 2009) (for an overview see figure 1). As the present clinical trial aimed on identifying the effects of two anxiolytic substances on parameters related to both systems, their structure and function will be described more in detail.

Figure 1

Overview of the Two Major Stress Systems



Note. Overview of the two key systems related to the stress reaction. The sympathetic-adrenal-medullary (SAM) system targets the medulla of the adrenal gland system, which is innervated by autonomic sympathetic nerves, and elicits the production of the catecholamines noradrenaline and adrenaline after stimulation by the locus coeruleus. Activity of the hypothalamic-pituitary-adrenal (HPA) axis involves the release of the corticotropin-releasing hormone (CRH) of the hypothalamus to the anterior pituitary. From there the adrenocorticotropic hormone (ACTH) is released into the blood circuitry. This vice versa stimulates the generation of glucocorticoids in the cortex of the adrenal gland. From ”Stress, the Stress System and the Role of Glucocorticoids,” by N. C. Nicolaides, E. Kyratzi, A. Lamprokostopoulou, G. P. Chrousos, and E. Charmandari, 2015, *Neuroimmunomodulation*, *22*, p. 9. Copyright 2014 by Karger AG, Basel.

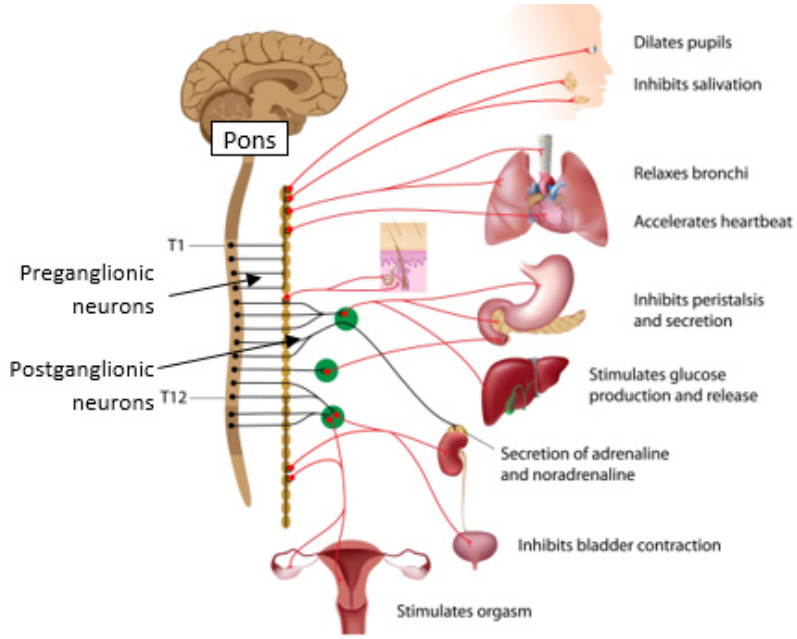
1.2.2.1 Sympathetic-adrenal-medullary (SAM) system

The sympathetic-adrenal-medullary (SAM) axis belongs to the autonomous nervous system, which together with the somatic nervous system forms the peripheral nervous system. While the somatic nervous system controls motor activity and thereby arbitrary as well as reflexive physical actions, the autonomous nervous system is responsible for the control of all vegetative organs that do not underlie conscious control (e.g. heart, sweat / salivary glands). The

sympathetic and the parasympathetic part innervate almost the same organs, thereby exerting antagonistic effects (see also figure 2). While the parasympathetic nervous system is more related to relaxation processes, the sympathetic system mainly regulates activated states of the organism. A third component is constituted by the enteric nervous system, which regulates the functions of the gastrointestinal tract. (Silbernagl & Despopoulos, 2012)

Figure 2

Overview of the Sympathetic Nervous System



Note. Schematic overview of the sympathetic part of the autonomous nervous system. Preganglionic neurons of the spinal cord are controlled by the locus coeruleus, which is located at the pons. Via release of acetylcholine (ACHT) those communicate with postganglionic neurons which run from the ganglia to the target organs. In response to stressors they regulate their activity by release of noradrenaline. Adapted from http://www.simplybehaviour.com/module-unassigned/sns-pns-ans/sympathetic-nervous-system/. In the public domain.

Activity of the sympathetic-adrenal-medullary (SAM) axis is controlled by the locus coeruleus, the main noradrenergic nucleus, which is located at the pons, a major part of the brainstem. This area holds widespread projections to the entire neuronal system including the hypothalamus, amygdala, hippocampus, septum, and the prefrontal cortex. It directly projects to sympathetic preganglionic neurons at the spinal cord, which communicate with postganglionic neurons via release of acetylcholine (ACTH). Those vice versa release noradrenaline and thereby control peripheral organs like the eyes, heart or lungs. (Benarroch, 2009; Valentino & van Bockstaele, 2008) (for an overview see figure 2)

In addition, direct projections from the locus coeruleus are sent to the chromaffin cells of the adrenal medulla, the inmost layer of the adrenal gland (see figure 1). In response to a stressor noradrenaline (20 %) and to a higher extent adrenaline (80 %) are released by the adrenal medulla into the blood circuitry. Both catecholamines bind to α- and β-adrenoreceptors that are differentially expressed depending on the respective tissue leading to organ specific effects. Binding to β-adrenoreceptors, for example, leads to stronger and faster contraction of the heart resulting in increased heart rate. Further effects comprise heightened breathing rate as well as attention and mobilization of further energy resources while digestion is decreased, altogether to enable the vital fight-or-flight response. (Silbernagl & Despopoulos, 2012)

In conclusion, circulating catecholamines like adrenaline and noradrenaline modulate behavioral function although they cannot directly cross the blood-brain-barrier. One hypothesis is that they indirectly act as neurotransmitters in the central nervous system through stimulation of the vagus nerve (Mravec, 2011). Thereby, they might act on adrenergic neurons in the nucleus tractus solitarii, which projects to the locus coeruleus, and regulate its activity by a negative feedback mechanism. However, the precise physiological mechanisms that are responsible for the central effects of peripherally active catecholamines are still under investigation (Tank & Wong, 2015).

1.2.2.2 Hypothalamic–pituitary–adrenal (HPA) axis

The main function of the hypothalamic-pituitary-adrenal (HPA) axis is the release of species-specific glucocorticoids (corticosterone in rodents, cortisol in humans) of the adrenal cortex, which play an important role for the regulation of an individual's response to stressors. As already indicated by its name this axis comprises several components of the central nervous system and the endocrine system and acts upon multiple steps that will be described in the following (for an overview see figure 1).

After registration of perceived or internally generated information related to threat, limbic and other (sub-)cortical structures of the brain like the prefrontal cortex stimulate neurons of the paraventricular nucleus (PVN), a core region in the hypothalamus (Herman et al., 2003). In response, parvocellular neurons of the PVN release the peptide corticotropin-releasing hormone (CRH) and the nonapeptide arginine vasopressin to the pituitary portal system (Tsigos & Chrousos, 2002). In a synergistic manner, both cause an activation of corticotropic cells in the anterior pituitary and trigger the release of the adrenocorticotropic hormone (ACTH) to the blood vessel system. At the zona fasciculata of the adrenal gland this results in the synthesis and release of glucocorticoids (D. B. Miller & Paul, 2002). In the

periphery, effects of glucocorticoids are mediated via binding to intracellular glucocorticoid receptors. In the central nervous system, they further bind to intracellular mineralocorticoid receptors, which are crucial for the termination of the stress response via negative feedback loops that include the pituitary, the hypothalamus and the hippocampus (Kloet, Vreugdenhil, Oitzl, & Joëls, 1998). Glucocorticoids inhibit the immune system, activate glycogen degradation in muscles and further the synthetization of glucose in the liver. Simultaneously, they increase the supply of energy of the musculature, increase heart rate, constriction of vessels, concentrations of free fatty acids and gastric acid, and lead to reductions of appetite, fatigue and sexual desire (Sapolsky, Romero, & Munck, 2000). Increased cortisol levels in response to stress where shown to be accompanied by heightened subjective stress and negative affect (Dickerson & Kemeny, 2004). In addition, glucocorticoids are the precursors for the synthesis of neurosteroids like progesterone and allopregnanolone which are known to have an important role in anxiolysis (Bali & Jaggi, 2014).

About 95 % of the glucocorticoids that are released by the adrenal glands are bound to transporter proteins, which means that 5 – 10 % are unbound. Those unbound glucocorticoids, however, are the ones that are biologically effective when cortisol molecules bind to mineral and glucocorticoid receptors (Kloet et al., 1998). Unbound cortisol is possible to get into almost all kinds of body cells and is therefore also measurable in blood or saliva, where the concentration is not influenced by corticosteroid binding globulin (Kirschbaum & Hellhammer, 1989). Depending on the nature and intensity of the stressor levels of glucocorticoids in bodily fluids were shown to rise and peak 15 to 30 minutes after exposure to acute stress with declining to baseline levels in the subsequent hours (Kloet, Joëls, & Holsboer, 2005).

In absence of any stressor, the release of cortisol is strongly regulated by the circadian rhythm, thereby supporting natural activation of the body after awakening. This is marked by an increase of cortisol release about one hour before awakening, which results in the peak of the daily cortisol level half an hour after the awakening (Fries, Dettenborn, & Kirschbaum, 2009). The lowest point of cortisol secretion is reached at about midnight with only minimal levels detectable (Weitzman et al., 1971). Therefore, studies that rely on hormone measurements should attend several suggestions to avoid undesired influence of factors like the cortisol awakening response (Adam & Kumari, 2009).

To avoid overstimulation of this system, negative feedback loops comprising the hippocampus as well as the medial prefrontal cortex react to the heightened cortisol level in the blood system and inhibit the release of CRH and ACTH in the hypothalamus and the pituitary (Tsigos & Chrousos, 2002). However, in case of overstimulation this process can be disrupted

and entail pathological states. This relation will further be examined in the following subsection.

1.2.3 Chronic stress and disease: focus on anxiety disorders

While acute stress lasting for minutes to hours implies allostatic physiological changes that are necessary to restore homeostasis, chronic or repeated exposure persisting for days to months implies excessive or prolonged activation of the related systems (Chrousos, 2009). In individuals that show high vulnerability this might almost inevitably result in disease (Ingram & Luxton, 2005) with depressive and anxiety disorders being the most common outcomes of repetitive and chronic stress (Möhler, 2012). Within the scope of this work, we will focus on anxiety disorders, which belong to the most prevalent mental disorders worldwide implying a high burden of illness and increased risk for comorbid mood and substance use disorders. Anxiety disorders comprise specific phobias with a 12-month prevalence of 10.3 %, panic disorder with or without agoraphobia (6.0 %), social anxiety disorder (2.7 %), and generalized anxiety disorder (2.2 %) (Jacobi et al., 2014). Two categories that meanwhile stand for their own but are strongly connected to stress and anxiety are obsessive-compulsive disorders (3.6 %) and posttraumatic stress disorder (2.3 %). The different forms of anxiety disorders share mental and physical signs that are marked by excessive worries and hyperarousal. Those are related to the experience of anxiety and imply risk assessment and uncertainty often triggered by generalized cues. The somatic symptoms are manifold ranging from states of fatigue to muscle tension or concentration difficulties (Vanin, 2008). They often constitute a substantial source of subjective diffuse distress in patients.

The regulation of negative emotion and generation of responses of the organism to aversive stimuli has been shown to involve specific cortical and subcortical structures in the brain. Those comprise areas of the limbic system, especially the amygdala, hippocampus, thalamus, and brainstem nuclei as well as prefrontal areas, especially the medial prefrontal cortex and the anterior cingulate cortex (Nuss, 2015). Those structures are further involved in the regulation of the stress response via control of the HPA axis and the subsequent release of cortisol (Dedovic, Duchesne, Andrews, Engert, & Pruessner, 2009; Herman et al., 2003). Prolonged dysfunctions and disrupted communication between those areas (e. g. disrupted inhibitory control of the amygdala by prefrontal structures) are crucially related to the development and maintenance of anxiety disorders (Bandelow et al., 2016; Nuss, 2015). Hyperactivity of the amygdala and further the insula has quite consistently been reported by neuroimaging studies in different forms of anxiety disorders (Etkin & Wager, 2007).

Disrupted communication between different brain regions is often traceable to dysfunctions of neurotransmitter and steroid systems, most of all those related to the release of serotonin, dopamine, noradrenaline, gamma-aminobutyric acid, glutamate, and glucocorticoids (Bandelow, Baldwin et al., 2017; Martin, Ressler, Binder, & Nemeroff, 2009). Research on the HPA axis has so far yielded heterogeneous results with blunted activation, for example, in patients suffering from posttraumatic stress disorder, while release of cortisol was seen to be heightened in panic disorder or obsessive-compulsive disorder (Martin et al., 2009; G. E. Miller et al., 2007). Thereby, hypoactivation of the HPA axis can serve as a compensatory mechanism that results from severe or chronic stress exposure, respectively, and evolves in order to protect from further overstimulation (Fries, Hesse, Hellhammer, & Hellhammer, 2005). Besides characteristics of the person including age, gender, genetic background, and previous experiences, the direction of changes related to the HPA axis depends on the severity, nature, duration, and predictability of the stressor as well as on the scheduling of measurements (G. E. Miller et al., 2007; Zorn et al., 2017). Dysregulation of HPA axis activity might even serve as a link between childhood adversity implying traumatic experiences in early life that result in epigenetic changes and later development of a mental disorder (Buchmann et al., 2014; Mello, Mello, Carpenter, & Price, 2003).

Besides the more prominent HPA axis also continuous activation of the sympathetic nervous system with diminished parasympathetic counteractivity was found due to prolonged stress. As a result, adrenaline and noradrenaline levels are found increased, whereas levels of ACTH are decreased. Subsequently, there is an increase of proinflammatory cytokines which are released from immune cells and vice versa trigger the activation of the sympathetic nervous system. (Bandelow, Baldwin et al., 2017)

However, the interdependence between pathological changes and dysregulations of the body's stress response systems needs to be further clarified and the heterogeneous data situation underlines the complexity of those disorders (Sapolsky, 2000). Based on the knowledge of the mechanisms underlying anxiety disorders, in the following, we want to shed light on treatment options with a focus on pharmacological options and involved receptor systems.

1.3 Anxiolytic therapy

Within this subchapter, we will first provide a brief overview of established pharmacological options for the treatment of anxiety disorders. As the present work aimed to investigate the effects of two compounds that differently modulate the central and the peripheral

benzodiazepine receptor, those two receptors will be described more in detail. This includes information on their structure and function as well as their role in systems related to stress and anxiety. Subsequently, insights on the therapeutic effects of the two investigational substances alprazolam and etifoxine gathered from animal and human studies are provided.

1.3.1 Overview of established anxiolytics and related target systems

The German guidelines on the treatment of anxiety disorders recommend psychotherapy with the highest evidence for cognitive behavioral therapy, especially exposure techniques, followed by pharmacological treatment or a combination of both (Bandelow et al., 2015). Anxiolytics are amongst the most prescribed psychoactive substances worldwide, particularly in the Western world (Altamura et al., 2013). Drugs of first choice, especially for generalized anxiety disorder, panic disorder and social anxiety disorder comprise serotonin reuptake inhibitors (SSRIs) like escitalopram and serotonin-noradrenaline reuptake inhibitors (SNRIs) like venlafaxine. They address the fact of disrupted central serotonergic and noradrenergic functions that are present in anxiety disorders. By selectively inhibiting the reuptake of those neurotransmitters from the synaptic gaps, those substances increase their availability within the central nervous system, thereby promoting anxiolytic effects. (Dell'Osso, Buoli, Baldwin, & Altamura, 2010)

Further pharmacological interventions include the administration of tricyclic antidepressants like clomipramine, monoamine oxidase inhibitors like moclobemide, anticonvulsants like pregabalin or azapirones like buspirone, however, the two latter ones being used less frequently (Bandelow, Michaelis, & Wedekind, 2017). Newer approaches emphasize beta-blockers like propranolol, especially for symptomatic relief, or augmentation with atypical antipsychotics like risperidone, which show promising effects but poor tolerability (Farach et al., 2012). In general, while any of the stated compounds was shown effective in their way, each of them also entails cons like, for example, tolerance development, abuse liability or delayed onset of therapeutic action, to name only a few. Thus, the pharmacological treatment of anxiety disorders remains a challenge.

It could be due to the complexity of those disorders and poor tolerability to some substances that the class of benzodiazepines are still amongst the leading substances prescribed for anxiety disorders, although not recommended by guidelines (Stahl, 2002; Starcevic, 2014). Benzodiazepines exert rapid anxiolytic effects by modulation of the inhibitory GABAergic system. However, due to their concomitant side effects ranging from sedation to tolerance and abuse liability, especially if taken over longer periods, their application should be considered

thoroughly (Lader, 2011). Promising alternatives for the anxiolytic therapy are yielded by compounds that target the translocator protein, which was shown to be involved in the regulation of neurosteroids like allopregnanolone (Nothdurfter, Baghai, Schüle, & Rupprecht, 2012; Rupprecht et al., 2010). As the GABAergic system plays an important role not only for the effects of benzodiazepines, but also for substances that target the translocator protein, this protein complex will be described more in detail in the following.

1.3.2 Central benzodiazepine receptor: $GABA_A$ receptor

Gamma-aminobutyric acid (GABA), which is synthesized from glutamate, is one of the major inhibitory transmitters in the central nervous system and responds very fast, while being present in up to 40 % of all neurons in the central nervous system (Bloom & Iversen, 1971; Roberts & Frankel, 1950). The respective receptors are constituted by proteins which are located at the membranes of those neurons. They can be separated into very fast acting ones that form ion channel pores and are controlled by ligands ($GABA_A$ / $GABA_C$) and metabotropic ones that are coupled to G-proteins ($GABA_B$) and mediate long-term actions of the neurotransmitter (Watanabe, Maemura, Kanbara, Tamayama, & Hayasaki, 2002).

Since benzodiazepines and other anxiolytic ligands unfold their effects by binding to specific sites of the ligand gated $GABA_A$ receptor, the structure and function of this type will be further specified (Sieghart & Sperk, 2002).

1.3.2.1 Structure and function of the $GABA_A$ receptor

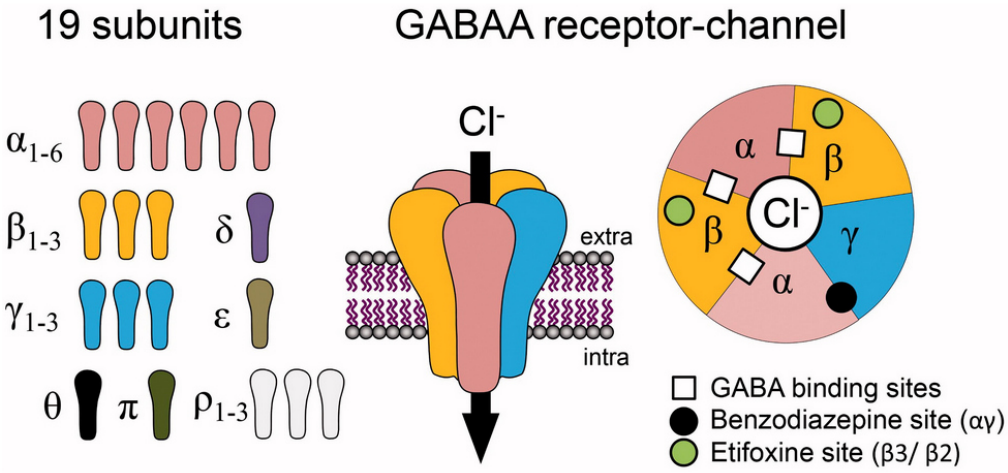
The $GABA_A$ receptor consists of five binding sites, which are organized around a central channel that allows the permeability of chloride ions into the cell after direct binding of two molecules of the neurotransmitter GABA or modulation by any other agonist. Increased influx of chloride ions leads to hyperpolarization of the membrane causing a reduced reactivity to excitatory neurotransmitters and thereby inhibition of the respective neurons. (Sieghart, 2006)

To date, at least 19 different subunits of this complex were identified (Poisbeau, Gazzo, & Calvel, 2018) (for an overview see figure 3). In vitro studies revealed that at least one α-, β- and γ-subunit are necessary to guarantee proper functioning of the receptor (Levitan et al., 1988; Verdoorn, Draguhn, Ymer, Seeburg, & Sakmann, 1990). The combination of various subunits allows the formation of many subtypes and thereby binding of different ligands. GABA itself binds to an interface between α and β subunits, which occurs twice on each receptor. Besides that, the most prominent substances binding to this complex are benzodiazepines with the

highest affinity to the specific benzodiazepine binding site, which is located between the α_1 and γ_2 subunits. Also other substances like neurosteroids (mainly α subunit), barbiturates (α / β and γ / β subunits) or the TSPO ligand etifoxine ($\beta_{2,3}$ subunits) can bind to the receptor and thereby modulate transmission of the inhibitory transmitter GABA. (Poisbeau et al., 2018)

Figure 3

Subunits and Binding Sites of the $GABA_A$ Receptor Complex



Note. Overview of the $GABA_A$ receptor complex, which is constituted by 5 binding sites and 19 subunits. It encloses a chloride (Cl^-)-permeable channel that opens in response to binding of an agonist. On the right extracellular binding sites for GABA, benzodiazepines and etifoxine are shown**.** From "Anxiolytics targeting GABAA receptors: Insights on etifoxine," by P. Poisbeau, G. Gazzo, and L. Calvel, 2018, *The World Journal of Biological Psychiatry*, *19*(Suppl. 1), p. S37. Copyright 2018 by the authors.

The distinct distribution of the subunits related to the complex within the central nervous system suggests that they have specific functions leading to different effects of respective binding ligands (Sieghart & Sperk, 2002). With respect to that, the α1-subunit has been shown to be particularly related to sedation, anticonvulsant effects as well as dependence liability, while anxiolytic effects are mostly mediated by the α2- and α3-subunits (Möhler, 2012).

1.3.2.2 Relation of the GABAergic system to measures of stress and anxiety

The $GABA_A$ receptor has been intensively studied in the context of stress and anxiety disorders, as GABAergic transmission in the central nervous system is critically involved in the regulation and the pathological manifestation of related states (Goddard, 2016). The connection to stress related physiological systems becomes obvious by the fact that there exists a dense network of GABAergic neurons in the hypothalamus, especially around the PVN (Decavel &

van den Pol, 1990; Miklós & Kovács, 2002). Many of those neurons vice versa project to neurons that are related to the release of CRH and were shown to exert a tonic inhibitory effect on their activity. The PVN is further strongly innervated by the medial prefrontal cortex and different limbic brain structures including the amygdala or the septum (Herman, Tasker, Ziegler, & Cullinan, 2002; Ulrich-Lai & Herman, 2009). This led to the assumption that GABAergic neurons in the PVN might filter information which is sent from those regions and thereby strongly control their impact on the activity of the HPA axis. High density of GABAergic neurons was further located within and surrounding the locus coeruleus, which plays a crucial role in the control of the second major stress system, the sympathetic-adrenal-medullary axis (Tsigos & Chrousos, 2002). Together with opioids, GABA mediates local influx to the locus coeruleus and inhibits its neuronal activity, thereby supposing a role of this transmitter in the way this system responds to stressors (Benarroch, 2009).

In fact, research has shown that GABA levels change due to stress. Thereby, the direction of the change seems to depend on the kind and duration of the stressor as well as the respective brain structure in which the measurements are taken. A microdialysis study in rats revealed a significant and fast increase of GABA levels in the hippocampus after introduction to a novel cage – a situation that constitutes psychological stress for the animals (Groote & Linthorst, 2007). In contrast, more physical stress induced by forced swimming resulted in decreased GABA levels in this brain structure. Comparison of acute and repeated immobilization stress in rats revealed reductions of GABA content in the striatum after one hour, whereas in the frontal region such an attenuation was only observable after repeated exposure to the stressful situation for two weeks (Otero Losada, 1988). The authors concluded that the cortical GABAergic system might be crucial for adaptive responses that are required for the adverse conditions during chronic stress. In addition to the measurement of GABA levels, the determination of binding site expression together with the binding affinity of several ligands, which indicate function of the $GABA_A$ receptor, have been shown sensitive to stress (Skilbeck, Johnston, & Hinton, 2010). Rapid changes of the complex after acute stress were shown in animals with the respective direction being equally mediated by the applied stress paradigm as well as gender.

The hypothesis that alterations of GABA levels depend on the nature of stressors was further underlined by magnetic resonance spectroscopy studies in healthy humans. One study revealed a significant decrease of GABA concentration in the medial prefrontal cortex in response to acute stress elicited by electric shocks (Hasler, van der Veen, Grillon, Drevets, & Shen, 2010). However, another spectroscopy study that investigated the effects of acute psychosocial stress

induced by the Trier Social Stress Test found no changes of GABA levels in the medial prefrontal cortex and no associations of this parameter to subjective stress or cortisol release (Houtepen et al., 2017). Interestingly, GABA content in the ventromedial prefrontal cortex was shown to be positively correlated with trait anxiety (Delli Pizzi et al., 2016). High levels of the inhibitory neurotransmitter within this cortical region could be related to reduced excitatory glutamatergic top-down modulation on GABAergic interneurons in the amygdala, resulting in greater activity of this structure and thereby higher anxiety.

Changed levels of GABA and altered expression of the related receptor have also been reported for several anxiety disorders. In patients with generalized anxiety disorder, localized reduction of GABA binding in the temporal lobe was reported (Tiihonen et al., 1997). In patients suffering from panic disorder, binding potential of the $GABA_A$ receptor has been found reduced in frontal, temporal and parietal brain regions, whereas it was heightened in (para-) hippocampal structures compared to healthy controls (Hasler et al., 2008). Those deficits of the $GABA_A$ receptor were further correlated to the severity of pathological symptoms. Reduced distribution volume of the $GABA_A$ receptor within the prefrontal cortex was also found in patients suffering from combat-related posttraumatic stress disorder (Bremner et al., 2000). In conclusion, those findings underline the assumption of disrupted frontal-limbic communication as a major aspect for the development of pathological states with the GABAergic system being crucially involved in the modulation of this communication.

A useful and often applied tool to gain further insights into the role of the GABAergic system in stress and anxiety is constituted by the administration of drugs that are known to modulate the receptor (Arvat, Giordano, Grottoli, & Ghigo, 2002). Partial inverse agonists of the benzodiazepine site, which decrease the chloride channel opening in response to GABA, lead to an attenuated transmission of the inhibitory transmitter and promote anxiety (Horowski & Dorow, 2002). In contrast, enhancement of GABAergic activity is anxiolytic (Möhler, 2012; Rupprecht et al., 2009). The most prominent class of substances in that context is constituted by benzodiazepines, which will be described in the following with a focus on alprazolam, which was administered in the previous work.

1.3.2.3 Benzodiazepines: focus on alprazolam

Benzodiazepines were first discovered in 1956 and since approval for clinical use in 1960 they have continuously been among the most widely prescribed anxiolytic substances (Lader, 2011). They bind to a specific site of the $GABA_A$ receptor, which is distinct from that of GABA. In contrast to other ligands, benzodiazepines do not directly activate the chloride

channels but allosterically change the receptor structure and lower the concentration of GABA required for opening the channel, thereby increasing its efficiency (Nutt & Malizia, 2001). Benzodiazepines are mainly characterized by a fast onset of their anxiolytic, sedating and anticonvulsive effects, which are mediated by the specific subunits and regions they bind to. While the anxiolytic effects are suggested to be mostly mediated by receptors of the limbic system, the muscle relaxing effects are mainly contributed to the modulation of receptors at the spinal cord (Lader, 2011). The anticonvulsive effects, vice versa, seem to arise due to binding at the brain stem and the cerebellum. Especially the binding to the α1- and α5-subunits of the $GABA_A$ receptor seems to be responsible for their unwanted effects including withdrawal symptoms, sedation, amnesia, and cognitive impairment (Rudolph & Knoflach, 2011).

Benzodiazepines are divided into two major subclasses: those that mainly act anxiolytic (e. g. lorazepam, diazepam or alprazolam) and the other class that is most of all used to induce hypnotic states (e. g. triazolam or zolpidem). However, most of the different substances act quite similar and differences mainly raise due to the respective metabolic half-life and the resulting action duration as well as the presence / absence of metabolites that are psychotropically active. Thereby, especially for short-acting benzodiazepines, there is a considerable risk of adverse symptoms after withdrawal. (Starcevic, 2014)

Besides those mentioned agonists of the benzodiazepine receptor, there exist antagonists that equally bind to the complex (e.g. flumazenil). As they modulate its activity in the opposite direction, they are mostly used to reverse adverse side effects of benzodiazepines or to gain further knowledge on processes underlying their therapeutic effects. (Lader, 2011)

In the following, we will focus on the triazolobenzodiazepine alprazolam, which, in contrast to other benzodiazepines, was shown to selectively act via the central GABAergic system with only little affinity for the peripheral benzodiazepine receptor also known as translocator protein (Schmoutz, Guerin, & Goeders, 2014) (see 1.3.3). Alprazolam is a short-acting benzodiazepine that belongs to the most commonly prescribed agents for the treatment of panic and general anxiety disorder (Stahl, 2002). It is usually well absorbed after oral administration with an elimination half-life of eight to 15 hours and an absolute bioavailability between 80 and 100 % (Altamura et al., 2013). Besides anxiety disorders alprazolam has also been shown effective for the treatment of depression and exhibits the most remarkable inhibitory influence on the release of stress related transmitters like ACTH and cortisol (Arvat et al., 2002).

Alprazolam: Preclinical studies on stress and anxiety related parameters

Animal studies quite consistently showed attenuating effects of alprazolam on stress and anxiety related parameters. A study in rhesus monkeys concluded suppression of the HPA axis by alprazolam, as they showed dose-dependent inhibition of ACTH and cortisol secretion after administration of the benzodiazepine (Kalogeras et al., 1990). The authors hypothesized that those effects could be mediated by CRH-related neurons in the hypothalamus, as further in vitro studies of the rat hypothalamus yielded inhibition of basal and serotonin-induced release of CRH in this region due to alprazolam. Another study in rats confirmed the lower concentrations of ACTH in plasma after administration of alprazolam in comparison to control (Owens, Bissette, & Nemeroff, 1989). They further found increased concentration of CRH in the hypothalamus, whereas it was markedly reduced in the locus coeruleus - effects that are opposite to changes of CRH found after stress. Subsequent work that focused on the time course of those effects showed that CRH concentration started to decrease 30 minutes after injection and persisted up to 240 minutes afterwards (Owens, Vargas, Knight, & Nemeroff, 1991). In contrast to previous work, within this study there were no dampening effects of alprazolam on measures related to the basal activity of the HPA axis. In conclusion, these findings suppose that the inhibitory action of alprazolam on the HPA axis might take place at a (supra-) hypothalamic level mediated by local suppression of CRH and is more strongly exhibited during stressed states of the system.

Besides decreases of CRH level in the locus coeruleus after single doses, also chronic administration of alprazolam exerted such a dampening effect (Owens et al., 1991). Interestingly, withdrawal of the benzodiazepine was related to a decrease of CRH receptor concentrations in the anterior pituitary accompanied by increased plasma levels of ACTH and corticosterone what indicates an activation of the HPA axis.

Studies on stimulated states of the organism revealed that pretreatment with alprazolam markedly attenuates the ACTH response to stress, for example, induced by injection of insulin in healthy animals or vice versa hyperglycemia in obese animals (Surwit et al., 1986; van Vugt, Washburn, Farley, & Reid, 1997). However, while one of those studies reported only moderate effects of alprazolam on the cortisol response, the other one found a significant reduction of corticosterone release in mice and rats during rest as well as following stress (Surwit et al., 1986). Equally, in paradigms that investigate more social components of stress like human handling of rats who were not habituated to such treatment, alprazolam was shown to attenuate mild increases in plasma corticosterone levels in the locus coeruleus (Owens, Ritchie, & Nemeroff, 1992). With respect to adrenaline and noradrenaline, there were no effects of

alprazolam in unstressed animals, whereas it significantly reduced the rise of those catecholamines in response to immobilization stress (Vogel, Miller, DeTurck, & Routzahn, 1984). In addition, animals that were treated with the benzodiazepine were overall calmer showing an effect of the treatment on experienced stress and anxiety. In rhesus monkeys bond-related stress, which was induced by separation of the tested animals from their mothers, was attenuated after the administration of alprazolam in a dose-dependent manner (Kalin, Shelton, & Turner, 1991). The treatment further reduced fear related behavior like freezing or cooing induced by staring of a human.

It can be concluded that animal studies quite consistently confirmed dampening effects of alprazolam on the release of glucocorticoids and related compounds, while the effects on catecholamines partly seem to depend on the presence of any stressor. However, the consequences of the inhibited secretion of glucocorticoids are still unclear. One study that applied an animal model of posttraumatic stress disorder suggested that the inhibited response of the HPA axis due to alprazolam might trigger unfavorable outcomes during repeated exposure to a stressor (Matar, Zohar, Joseph, Kaplan, Zeev, & Cohen, 2009). While immediate anxiety and corticosterone levels were attenuated in this study, the fear-related response was even higher during repeated stress-exposure to the trauma related cues.

Alprazolam: Clinical studies on stress and anxiety related parameters

Human studies on the effects of alprazolam on stress and anxiety related parameters have so far yielded inconsistent results for the different implicated systems. Attenuating effects on plasma ACTH and cortisol levels in healthy humans after oral or intravenous administration of alprazolam at single doses of 1.5 up to 3 mg have been shown quite consistently (Charney, Breier, Jatlow, & Heninger, 1986; Risby, Hsiao, Golden, & Potter, 1989; Zemishlany, McQueeney, Gabriel, & Davidson, 1990). The effects on cortisol levels even lasted up to six hours after administration (Zemishlany et al., 1990). Noradrenaline, the major catecholamine related to activity of the SAM system, however, was either only transiently reduced or with a delay of several hours (Risby et al., 1989; Zemishlany et al., 1990). Based on that, the authors concluded that the effects of alprazolam in healthy humans might not be mediated by the central noradrenergic system and possible delayed effects might raise due to peripheral effects of increased sedation and a subsequent lower activity level of the individual. In accordance, almost all of those studies noted increased reports of sedation and drowsiness, whereas effects on peripheral parameters including systolic and diastolic blood pressure and cardiovascular measurements were only modest (Charney et al., 1986; Risby et al., 1989).

Interestingly, the HPA response to the administration of exogenous human CRH seems to be unaffected by alprazolam as there were no attenuating effects on subsequent ACTH or cortisol secretion (Rohrer, Richthofen, Schulz, Beyer, & Lehnert, 1994). Another study confirmed those effects, as they equally did not find an influence of alprazolam on the HPA axis response to administration of vasopressin or CRH (Grottoli et al., 2002). It was concluded that the central inhibitory effects of alprazolam on the HPA axis might be mediated by mechanisms that are controlled by CRH and further vasopressin.

Attenuation of parameters that are related to the HPA axis by alprazolam was also found by studies that investigated the effects after prior exposure to a stressor. A single dose of 1.5 mg alprazolam significantly reduced the cortisol response to metabolic stress induced by acute glucoprivation (Breier, Davis, & Buchanan, 1991). However, this study reported no effects on any of the physiological indices that had increased due to the stress, including diastolic blood pressure, heart rate and body temperature. Studies on the effects of alprazolam on stress due to insulin-induced hypoglycemia in healthy humans have yielded contrasting results. One study reported inhibition of the ACTH peak response but no significant effects on cortisol (Giordano et al., 2003). Further, while alprazolam significantly reduced basal levels of noradrenaline, it did not significantly affect level changes in response to stress. Another study reported significant reduction of peak electromyography and galvanic skin response during rest as well as in response to acoustic startle due to alprazolam (Patel et al., 2014). However, they found no effects on increases of pulse rate, plasma cortisol, ACTH as well as concentrations of adrenaline and noradrenaline. After physical stress induced by a treadmill test, alprazolam taken over three days was shown to reduce adrenaline already at rest, while noradrenaline was just attenuated in response to the stress (Stratton & Halter, 1985). While blood pressure was not affected by the treatment, heart rate at least slightly differed between the groups. Accordingly, increases of heart rate and blood pressure in response to a mental arithmetic stress task were not significantly attenuated after single dose of 0.5 mg alprazolam in comparison to placebo (McCann, Goldfarb, Frisk, Quera-Salva, & Meyer, 1992). After pharmacological induction of panic like symptoms using cholecystokinin-tetra-peptide (CCK-4), 1 mg of alprazolam reduced self-reported panic symptoms as well as related release of ACTH and cortisol (Zwanzger et al., 2003). However, it must be noted that slight reduction of those parameters was also found for the placebo group what could be partly explained by habituation to the applied pharmacological challenge. In a study that investigated the effects of alprazolam on acute stress induced by a parachute jump, prior administration of alprazolam significantly reduced the increase of cortisol and adrenaline, whereas there were no effects on changes of noradrenaline or heart rate (Benschop et al., 1996).

The authors thereof concluded inhibitory effects of alprazolam on activity of the HPA axis but only selectively on the response of the SAM system.

Studies that investigated the effects on paradigms, which are related to psychosocial stress, consistently confirmed effects of alprazolam on parameters related to activity of the HPA axis. A single dose of 0.5 mg alprazolam significantly reduced the increase of ACTH and cortisol in comparison to placebo (Rohrer et al., 1994). Furthermore, they observed effects on physiological parameters including systolic / diastolic blood pressure and heart rate. In a similar study that administered the Trier Social Stress Test effects of alprazolam were only shown for ACTH and cortisol as well as systolic blood pressure (Fries, Hellhammer, & Hellhammer, 2006). They did not report any effects of the benzodiazepine on the secretion of adrenaline, noradrenaline and heart rate. While alprazolam significantly exerted sedative effects, reports of state anxiety and subjective stress were not affected.

It must be concluded that research on the effects of alprazolam on markers of the stress response still yield inconsistencies with the highest correspondence for substances related to the HPA axis. Thus, further clarification of the molecular mechanisms that underlie the effects of alprazolam is needed. Besides that, research should target the investigation of favourable alternatives to overcome the adverse side effects of alprazolam ranging from sedation and impairment of psychomotor or cognitive abilities to dependence, abuse liability and withdrawal symptoms (Golombok, Moodley, & Lader, 1988; Lader, 2011). A promising candidate in that context is the translocator protein, as ligands binding to it were shown to exert comparable efficacy as benzodiazepines, thereby possessing a preferable profile of side effects (Rupprecht et al., 2009).

1.3.3 Peripheral benzodiazepine receptor: Translocator Protein (TSPO)

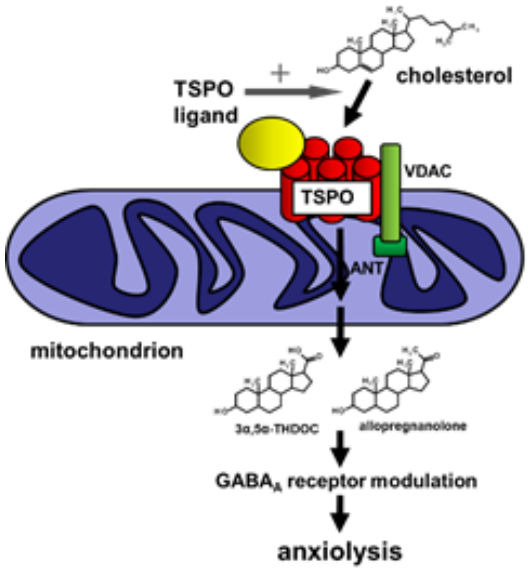
The translocator protein (TSPO) with a molecular mass of 18kDa was detected as a binding site for the benzodiazepine diazepam in the kidney and since then has become a further anxiolytic target (Braestrup & Squires, 1977). Because of its binding affinities, it was initially named peripheral benzodiazepine receptor (PBR). However, due to its function of modulating cholesterol transport into mitochondria it was renamed in 2006 (Papadopoulos et al., 2006).
Following, an overview of the structure and function of TSPO and insights to the research on the role of this protein in stress and anxiety disorders as well as the anxiolytic TSPO ligand etifoxine, which we administered in the previous work, will be provided.

1.3.3.1 Structure and function of the Translocator Protein

The translocator protein consists of five transmembranes with 169 amino acids and is mainly located at the outer membrane of mitochondria (Jaremko, Jaremko, Giller, Becker, & Zweckstetter, 2014; Lacapere & Papadopoulos, 2003). It is expressed in several body parts but mostly in tissues that are involved in the synthetization of steroid hormones like gonads and adrenals in the periphery and (micro-)glia and reactive astrocytes in the central nervous system (Anholt, Pedersen, Souza, & Snyder, 1986; Cosenza-Nashat et al., 2009). Together with a voltage-dependent anion channel (VDAC), the adenine nucleotide transporter (ANT) and the steroidogenic acute regulatory protein (STAR), it forms a complex that serves as bridge from the outer to the inner of mitochondria. (Rone et al., 2012; Zamzami & Kroemer, 2001) (see figure 4).

Figure 4

Structure of the Translocator Protein 18 kDa (TSPO)



Note. Overview of the TSPO complex located at the outer membrane of mitochondria including the voltage-dependent anion channel (VDAC) and the adenine nucleotide transporter (ANT). After binding of cholesterol, TSPO mediates its transport to the inner mitochondrial membrane where it is converted to pregnenolone and in further steps into neurosteroids like allopregnanolone, which exerts anxiolytic effects mediated by positive allosteric modulation of the $GABA_A$ receptor. From "Translocator protein (18 kDa) (TSPO) as a therapeutic target for anxiety and neurologic disorders," by C. Nothdurfter, T. C. Baghai, C. Schüle, and R. Rupprecht, 2012, *European Archives of Psychiatry and Clinical Neuroscience*, *262*(Suppl. 2), p. S108. Copyright 2012 by Springer-Verlag.

TSPO is involved in a row of functions including programmed cell death (apoptosis), mitochondrial respiration and cell proliferation (Gavish et al., 1999; Veenman & Gavish, 2012). However, one of the most crucial functions is the regulation of cholesterol transport into

mitochondria where it is further synthesized to pregnenolone by the cytochrome P450 side chain cleavage enzyme (CYP11A1) (K. E. Krueger & Papadopoulos, 1990; W. L. Miller & Auchus, 2011). After leaving the mitochondria towards the endoplasmic reticulum, pregnenolone undergoes further enzymatic transformations, which result in the formation of neurosteroids like allopregnanolone or tetrahydrodeoxycorticosterone (THDOC) (Lacapere & Papadopoulos, 2003). Those neurosteroids were shown to play an important role in anxiolysis (Rupprecht, 2003; Schüle, Nothdurfter, & Rupprecht, 2014; Stoffel-Wagner, 2001).

The role of TSPO as major player in the transport of cholesterol has recently been questioned by studies that did not find reductions of steroid synthesis after knockout of TSPO but rather observed other dysfunctions like reduced metabolic activity (Banati et al., 2014; Selvaraj & Stocco, 2015). However, most of the research has been underlining its importance, thereby acknowledging the existence of redundant mechanisms, which might replace the functions of this protein under circumstances when it has been removed (Papadopoulos, Fan, & Zirkin, 2018). Within scientific research and clinical work, TSPO has become a crucial biomarker for inflammation, reactive gliosis as well as neurological and mental disorders (Rupprecht et al., 2010). In the following, we will focus on its specific role in stress and anxiety disorders.

1.3.3.2 Relation of TSPO to measures of stress and anxiety

Especially due to the modulation of biosynthesis of steroids, TSPO seems to be crucially involved in the regulation of the major stress systems including the HPA axis, the SAM system, and the neuroendocrine-immune axis (Gavish et al., 1999). Evidence for that mainly stems from preclinical studies, which showed that ligands binding to TSPO with high affinity increased plasma levels of glucocorticoids in normal and hypophysectomized rats (Calogero et al., 1990; Cavallaro, Korneyev, Guidotti, & Costa, 1992). They further blocked ACTH-induced glucocorticoid production in hypophysectomized animals (Cavallaro et al., 1992).

Alterations in the density of TSPO seem to be a sensitive indicator of acute and chronic stress as well as regarding several mental disorders. This was first concluded from a study in rats, which revealed significant reduction of TSPO expression in central and peripheral tissues including the kidney, cerebral cortex, heart, and pituitary after administration of a high number of inescapable tail shocks (Drugan, Basile, Crawley, Paul, & Skolnick, 1986). Expression in other tissues like lung, adrenal or hippocampus, however, was unaffected. For renal tissue, bidirectional effects were reported with increased TSPO after a low number of shocks, while it decreased according to heightening the number.

Studies in humans that determined the density of TSPO by binding of the specific ligand PK 11195 in blood platelets showed upregulated levels three hours after acute stress, which was elicited by an examination situation (Karp, Weizman, Tyano, & Gavish, 1989). Those effects even persisted up to ten days with slightly higher TSPO expression in the experimental group compared to the unstressed control group, which might indicate long-term effects of stress on TSPO expression. Interestingly, they did not find any significant group differences for other stress hormones like cortisol what might be due to the time that had passed until measurement during which those parameters might have declined to their baseline level. Another study that examined the effects of acute stress induced by a PhD examination confirmed increased levels of TSPO expression in response to the stress situation, which were further correlated to heightened levels of allopregnanolone (Fortuyn et al., 2004). As in the study mentioned before, there was no coherence between changes of TSPO density and cortisol levels. The authors concluded that changes in cortisol concentrations after acute psychosocial stress might not depend on rapid biosynthesis. To get more insights on the relation of TSPO and allopregnanolone they further recommended to investigate the effects of TSPO ligands on the synthesis of allopregnanolone in humans. Besides neurosteroids, TSPO expression in healthy people was shown to be associated with self-reported trait anxiety, while there was no coherence with state anxiety (Nakamura, Fukunishi, Nakamoto, Iwahashi, & Yoshii, 2002). Density of TSPO could therefore serve as a marker for anxiety sensitivity and be used for the prediction of responsivity to stressful conditions.

Thus, while acute stress has mostly been found related to increased TSPO expression, repeated exposure to stress, for example, induced by several parachute jumps led to significant decreases (Dar et al., 1990). This fits well with the fact that expression of TSPO has quite consistently been found to be reduced in patients suffering from mental disorders in comparison to healthy control samples. This was, for example, shown for bipolar disorder in combination with adult separation anxiety disorder (Abelli et al., 2010), posttraumatic stress disorder (Gavish et al., 1996), generalized social phobia (Johnson et al., 1998), panic disorder (Marazziti et al., 1994), and depression with comorbid adult separation anxiety disorder (Chelli et al., 2008). In schizophrenic patients such a reduction was found to be associated with current aggression, hostility and anxiety (Ritsner et al., 2003). Most of the studies found those differences only when the respective disorders where combined with certain symptoms related to anxiety (Pini et al., 2005). It has been suggested that such reduction of TSPO expression might reflect a nonspecific response to stress, which aims at protection from chronic overproduction of glucocorticoids, rather than a pathophysiological unique aspect, which is

specific for certain disorders (Gavish et al., 1996; Johnson et al., 1998). The specific link to stress and anxiety related symptoms was further strengthened by a study that found no changes of TSPO expression in patients suffering from depression without comorbid anxiety disorder in response to antidepressant therapy (Sarubin et al., 2016). In conclusion, alterations in the expression of TSPO seem to be affected by stress in a bidirectional manner with up-regulation after acute stress and down-regulation after repeated stress as well as in pathological states.

Further aspects linking TSPO to pathological symptoms target structural changes of specific binding domains of the receptor, which have been related to reduced binding of cholesterol with subsequent disruption of steroid synthesis like pregnenolone and dysregulation of cortisol release (Lacapere & Papadopoulos, 2003). Such alterations of the protein structure and the related functional changes are associated with the single nucleotide polymorphism rs6971, which leads to a substitution from alanine to threonine at position 147 in the transmembrane of TSPO (Owen et al., 2012). It has been suggested that this common polymorphism is associated with dysregulated steroid production in humans (Owen et al., 2017). Reduction of cholesterol binding and transport and subsequently lowered cortisol production due to this functional polymorphism might be associated with enhanced susceptibility to environmental stress and thereby facilitate the development of stress related mental disorders like bipolar disorder (Colasanti et al., 2013). Accordingly, differences in diurnal cortisol rhythm, which were dependent on the presence / absence of that polymorphism, were shown in patients suffering from bipolar disorder with or without alcohol dependence as well as in a healthy control sample (Prossin et al., 2018). Importantly, this polymorphism further determines the affinity with which ligands bind to TSPO resulting in three different patterns (low- / medium- / and high-affinity binders) that might even influence therapeutic effects (Berroterán-Infante, Tadic, Hacker, Wadsak, & Mitterhauser, 2019; Owen et al., 2011). Therefore, it has been suggested to control for the respective genotype in studies that use TSPO ligands to increase their statistical power (Kreisl et al., 2013). However, the impact of this polymorphism on binding properties of therapeutic TSPO ligands like etifoxine and possible changes of their effects remain to be further clarified.

1.3.3.3 TSPO ligands: focus on etifoxine

The most important endogenous ligand of TSPO is cholesterol, which binds to a specific cholesterol recognition domain of the cytosolic carboxy-terminus (H. Li & Papadopoulos, 1998). Further natural ligands comprise porphyrins, phospholipase and the diazepam binding inhibitor with its derivatives (Lacapere & Papadopoulos, 2003).

Several synthetic ligands of TSPO are used to gain further insights to the function of the protein and are, for example, applied within the context of neuroimaging and for the determination of TSPO expression. The most common ones are the isoquinoline carboxamide PK 11195, which exclusively binds to TSPO, and the benzodiazepine Ro5-4864, which further binds to the central benzodiazepine receptor, although with a lower affinity (Le Fur et al., 1983; Papadopoulos, 1993). Studies with these ligands showed that besides the prominent binding site of TSPO located at the outer membrane of mitochondria, there further exist specific binding sites in plasma membranes of human erythrocytes (Olson, Ciliax, Mancini, & Young, 1988). Those high affinity ligands further underlined the role of TSPO in steroidogenesis, as they increased plasma levels of corticosteroids in rats (Calogero et al., 1990).

Other synthetic ligands are promising candidates in the search for novel anxiolytic agents with XBD-173 and etifoxine currently being the most prominent (Nothdurfter, Rammes et al., 2012; Rupprecht et al., 2009). Both substances were shown to increase TSPO expression and neurosteroids levels in preclinical studies (Liere, Pianos, Oudinet, Schumacher, & Akwa, 2017; Lin et al., 2019; Wolf et al., 2015). The fact that XBD-173 binds to TSPO with higher affinity than etifoxine but the latter being the more potent enhancer of neurosteroidogenesis indicates that the modulation might not primarily depend on binding affinity (Wolf et al., 2015).

Within this work, we will focus on etifoxine, which binds to TSPO with a long residency time at the Ro5-4864 binding site (Costa et al., 2017), thereby yielding low binding affinity (Schlichter, Rybalchenko, Poisbeau, Verleye, & Gillardin, 2000; Verleye et al., 2005). In addition to TSPO, etifoxine modulates GABAergic neurotransmission by binding to β2 or β3 subunits of the $GABA_A$ receptor complex – sites that are different from those of benzodiazepines (Bouillot, Bonnefoi, Liger, & Zimmer, 2016; Hamon, Morel, Hue, Verleye, & Gillardin, 2003) (see also figure 3). A recent study found that etifoxine further interacts with α2- and α3-subunits of the $GABA_A$ receptor what might explain its anxiolytic effects with little sedative properties (Mattei et al., 2019). In comparison to benzodiazepines, which bind to the $GABA_A$ receptor with an affinity lying in the nanomolar range, etifoxine yields a binding affinity in the micromolar range (Verleye, Schlichter, & Gillardin, 1999). However, the twofold action of etifoxine on the GABAergic system through direct binding to the $GABA_A$ receptor and indirect effects via the synthesis of neurosteroids mediated by TSPO supposes an important role in the regulation of stress and anxiolysis. In the following, available preclinical and clinical research that investigated the effects of etifoxine on stress and anxiety related parameters will be described.

Etifoxine: Preclinical studies on stress and anxiety related parameters

Efficacy of etifoxine has been proven within the scope of several animal models including neuropathic pain (Aouad, Petit-Demoulière, Goumon, & Poisbeau, 2014), multiple sclerosis (Daugherty et al., 2013), alcohol withdrawal syndrome (Verleye, Heulard, & Gillardin, 2009) or traumatic brain damage (Shehadeh, Palzur, Apel, & Soustiel, 2019), to name only a few. Respective effects were mostly attributed to reduced production of reactive oxygen species, increased antioxidant capacity or reduced release of pro-inflammatory cytokines following administration of the TSPO ligand (Biswas, Farhan, Reilly, Bartholomew, & Shu, 2018). Most of the research on etifoxine, however, targeted the treatment of anxiety related disorders. Thereby, effects were most of all contributed to the modulation of neurosteroids like allopregnanolone (Ugale et al., 2007). Studies in rats showed that etifoxine and allopregnanolone bind to different putative sites at the chloride channel site of the $GABA_A$ receptor and enhance inhibitory transmission of the complex in an additive manner (Verleye, Schlichter, Neliat, Pansart, & Gillardin, 2001). Efficacy of etifoxine on steroids, however, seems to depend on the respective steroid as well as the steroidogenic organ. While etifoxine caused increases of steroids like pregnenolone, progesterone and allopregnanolone in adrenal glands, brain tissue and plasma, no changes were shown in the testis (Liere et al., 2017). Other steroids like corticosterone, however, where increased in the testis, whereas testosterone levels significantly decreased in that region and did not change in brain tissue. One study suggested that the etifoxine-induced stimulation of neurosteroid production might even be mediated through a mechanism that is independent of any membrane receptor (do Rego, Vaudry, & Vaudry, 2015). This was concluded from experiments at the frog hypothalamus, in which the effects of etifoxine were neither affected by antagonists of the central benzodiazepine receptor nor by antagonists targeting the translocator protein. In contrast, an additive effect of another TSPO agonist was observed indicating distinct action mechanisms of the two compounds.

On a behavioral level, anxiolytic-like properties of etifoxine were shown in the scope of classical paradigms like the Vogel's conflict test, in which medicated animals showed significantly increased punished licking of water (Schlichter et al., 2000). Likewise, within the elevated plus maze test etifoxine led to a higher number of entries to the open arms as well as longer time spent within this situation, which is known to induce anxiety in the animals (Ugale et al., 2007). This was further strengthened by a study that found decreased CRH-induced grooming and reversed delay in gastric emptying of a solid meal due to administration of etifoxine (Verleye, André, & Gillardin, 2006). Those effects seemed to be related to GABAergic neurotransmission rather than an interaction with CRH receptors. Involvement of

etifoxine in the attenuation of behavioral and autonomic manifestations of anxiety was further concluded from a study in rats that showed reduced stress-related hyperthermia, freezing behavior and colonic motor activation in response to electric foot shocks (Verleye & Gillardin, 2004). The attenuation of stress-induced CRH activation was thereby attributed to the positive modulation of the inhibitory GABAergic system. In a four-plate test, in which animals received electric shocks when crossing between different plates, etifoxine was shown as efficient as other compounds including gabapentin, diazepam and a selective serotonin-reuptake-inhibitor (Bourin & Hascoët, 2012).

Etifoxine: Clinical studies on stress and anxiety related parameters

The first clinical studies on etifoxine were conducted with small samples in healthy subjects, administered quite high doses of the substance and yielded rather inconsistent results. One study administered a single dose of about 4 mg / kg body-weight in healthy subjects and observed self-reported anxiolytic effects, lowered performance in perceptual tests and reduced susceptibility to extrinsic arousal measured by galvanic skin response and evoked cortical potentials (Sartory & Rust, 1973). However, there were no effects on motor performance. Another study confirmed the reported reduction of the magnitude of galvanic skin response and further found improvement of motor skills in a pursuit rotor task in comparison to placebo after a single dose of 300 mg etifoxine (Córsico, Moizeszowicz, Bursuck, & Rovaro, 1976). As the effects resembled those of the stimulant d-amphetamine, the authors suggested possible psychostimulatory effects of etifoxine under certain circumstances.

Studies that investigated the anxiolytic effects of etifoxine in patient samples so far consistently included patients suffering from adjustment disorder with comorbid anxiety – a condition which has strongly been related to antecedent occurrence of stressful live events (Vanin & Helsley, 2008). In comparison to the azapirone buspirone (15 – 20 mg / day), administration of etifoxine (150 – 200 mg / day) for a period of four weeks yielded greater improvement with regard to clinical global impression (Servant, Graziani, Moyse, & Parquet, 1998). Furthermore, etifoxine showed a preferable ratio between wanted anxiolytic and unwanted side effects, while the total number of adverse events did not differ between the two groups. Comparison of 150 mg etifoxine per day with a daily dose of 1.5 mg lorazepam for a period of 28 days also showed similar reduction of anxiety by both substances (Nguyen et al., 2006). However, within the etifoxine group more responders to the treatment were recorded. Although there was no significant difference concerning the number of adverse events during the study, rebound of anxiety after discontinuation of intake was less remarked within the group

that was allocated to receive the TSPO ligand. Similar results were obtained within a study that compared the same dosage of etifoxine as applied in the previous study to a daily dose of 1.5 mg alprazolam (Stein, 2015). While anxiety was slightly more improved in the alprazolam group, the respective score kept decreasing after the end of treatment in the etifoxine group only. The two groups did not differ with respect to global improvement or responder rates, whereas more adverse events were rated to be treatment-related for the alprazolam group. This was accompanied by a higher rate of withdrawal symptoms. A further study compared the efficacy of etifoxine (150 mg / day) to phenazepam (0.5 mg / day) – a benzodiazepine that is mainly marketed in Russia (Alexandrowsky, Krasnov, Neznanov, & Romasenko, 2010). After six weeks of treatment, both compounds significantly reduced anxiety in patients. However, more patients of the etifoxine group responded to the treatment with respect to general improvement. Additionally, less discontinuation of the study and rebound anxiety after withdrawal were reported for this group.

Research that focused on the comparison of unwanted side effects after single doses of either etifoxine or a benzodiazepine in healthy subjects consistently yielded results which are in favor of the TSPO ligand. While a single dose of 2 mg lorazepam significantly impaired vigilance, psychomotor performance and free recall in a group of healthy subjects, neither 50 mg nor 100 mg etifoxine caused such detrimental effects (Micallef et al., 2001). Likewise, in a sample of elderly, a single dose of 100 mg etifoxine yielded no effects on alertness and cognitive performance (Deplanque et al., 2018). Reported adverse events did not differ from the placebo group, while the rate was 3-fold higher in the group that had received 2 mg lorazepam. Impairment of alertness and a high number of reports related to drowsiness were only found in response to administration of the benzodiazepine.

In conclusion, for the treatment of adjustment disorder with comorbid anxiety etifoxine was shown to be as effective as other substances including azapirones or benzodiazepines (Stein, 2018). With regard to tolerability measures, it even seems superior, especially when data from the phase after ceasing the treatment are taken into account.

1.4 Aims of the thesis

Based on the stated literature, it can be concluded that the benzodiazepine alprazolam is well established for the treatment of anxiety disorders, though still controversial, while the TSPO ligand etifoxine has so far been mainly investigated within the scope of preclinical studies and clinical studies focusing on adjustment disorder with comorbid anxiety. Studies on

healthy subjects were restricted to comparisons to other compounds with a focus on the appearance of side effects including the impairment of cognitive or motor functions. Those were mostly in favor of the TSPO ligand. However, less is known on the impact of etifoxine on parameters related to stress in humans, although findings from animal studies suppose attenuating effects. For alprazolam a number of studies investigated its efficacy on stress, however, so far yielding inconsistent results. Since especially the repeated exposure to stressors is known to be crucial for the development and maintenance of mental disorders, further clarification of the role of those two compounds in the regulation of stress would be of great importance. Thereby, it is of special interest if the divergent modulation of the GABAergic system by the two anxiolytic agents implies different effects on stress and anxiety related parameters or if there still exists substantial overlapping.

To address those questions, we conceptualized a pharmacological intervention study that compared the effects of alprazolam and etifoxine on stress and anxiety related parameters in a multimodal manner. Therefore, three groups of healthy male subjects received either one of the two active substances or placebo for a period of five days. Within the course of the study, they underwent different experimental procedures including the assessment of self-reports, physiological parameters, salivary and blood markers, brain imaging as well as the determination of genetic variants.

Chapter 2 provides an **overview of the clinical trial.** It describes the general study design, characteristics of the study sample, provides information on the investigational medical products as well as on methods and procedures that are relevant for all study parts.

Chapters 3 – 6 describe the four main topics of the present work**.** Each of those chapters is structured equally and starts with a short theoretical introduction, which yields the basis for the research questions and hypotheses that were investigated in the respective part. This is followed by a description of the specific methods and procedures as well as results and discussion.

Chapter 3 focuses on the effects of alprazolam and etifoxine taken over several days on **acute psychosocial stress**. Subjects were exposed to the Trier Social Stress Test in Virtual Reality on day 5 of treatment. Efficacy on the acute stress response was quantified by the magnitude of self-reported stress and other sensations, heart rate and skin conductance as well as endocrine markers like cortisol, allopregnanolone and TSPO expression. We further included

speech-based analyses and performance measures into our analyses and specifically investigated the influence of the TSPO polymorphism rs6971 on the efficacy of etifoxine.

Chapter 4 compares the acute and short-term effects of the two investigational medical products on **fear and anxiety**. Therefore, we administered the NPU Threat Test, which yields a well-validated paradigm to induce and differentiate those emotional states in the laboratory, on day 1 and 5 of treatment. We assessed startle reactivity in response to white noise as well as self-reports of fear and anxiety and further checked for a possible influence of the TSPO polymorphism rs6971 on the efficacy of etifoxine.

As different neuronal circuits are involved in the regulation of stress and anxiety, it seems obvious to investigate the effects of anxiolytic medication on the activity of those brain systems. Therefore, **chapter 5** presents results of resting state functional magnetic resonance imaging (rs-fMRI) that was conducted on day 4 of treatment with the study medication. To determine changes of **functional connectivity in the brain** we performed independent component analysis.

Within **chapter 6**, we compared the effects of alprazolam and etifoxine on **attention, alertness and general mental state**. Therefore, subjects performed a commonly used vigilance task, the Continuous Performance Test, and parameters were compared between the groups on day 1 and 5 of treatment. In addition, we administered several questionnaires and assessed changes of blood parameters to monitor adverse events and further side effects.

Chapter 7 incorporates the findings of the single parts into a **general discussion**, provides implications of the results as well as overall methodological criticism regarding the clinical trial. It concludes with an outlook for future studies.

CHAPTER 2: Overview of the clinical trial

Within this chapter, the general design of the clinical trial with details of overall relevance for the four investigational parts will be described. This includes the study design, recruitment of participants with inclusion and exclusion criteria, drug treatment as well as the general study procedure. Methods and procedures related to the different investigational parts will be described in the respective chapters.

2.1 General study design

The study was realized within the scope of the graduate school *GRK 2174 Neurobiology of emotion dysfunctions* (spokesperson: Prof. Inga Neumann) funded by the German research foundation (DFG; subproject 8). The clinical trial was conducted monocentric at the University of Regensburg (principal investigator: Priv.-Doz. Caroline Nothdurfter; project co-investigator: Prof. Andreas Mühlberger; medical co-investigator: Prof. Thomas Baghai) in a cooperation between the Chair of Psychiatry and Psychotherapy (Prof. Rainer Rupprecht) and the Chair of Clinical Psychology and Psychotherapy (Prof. Andreas Mühlberger). Collection and analysis of the functional imaging data were conducted in collaboration with Prof. Jens Schwarzbach. Study monitoring was conducted by Prof. Thomas Wetter.

The clinical trial was randomized in a block-wise and double-blind manner comprising three treatment arms (two investigational medicinal products and placebo). The study plan was proved by the ethics committee of the University of Regensburg (vote # 17-746-111) and the Federal Institute for Drugs and Medical Devices (BfArM) (vote # 4042553). It was conducted according to the guidelines of the International Conference on Harmonization (ICH, 2006) and Good Clinical Practice (GCP) as well as the German Medicines Act (AMG, 13th amendment) and the Declaration of Helsinki (World Medical Association, 2004). The clinical trial was registered at the Clinical Trials Register (EudraCT number: 2016-004254-15) and the regional authorities (government of Upper Franconia). The period used to conduct the clinical trial was from July 2018 to November 2019.

2.2 Participants

2.2.1 Recruitment

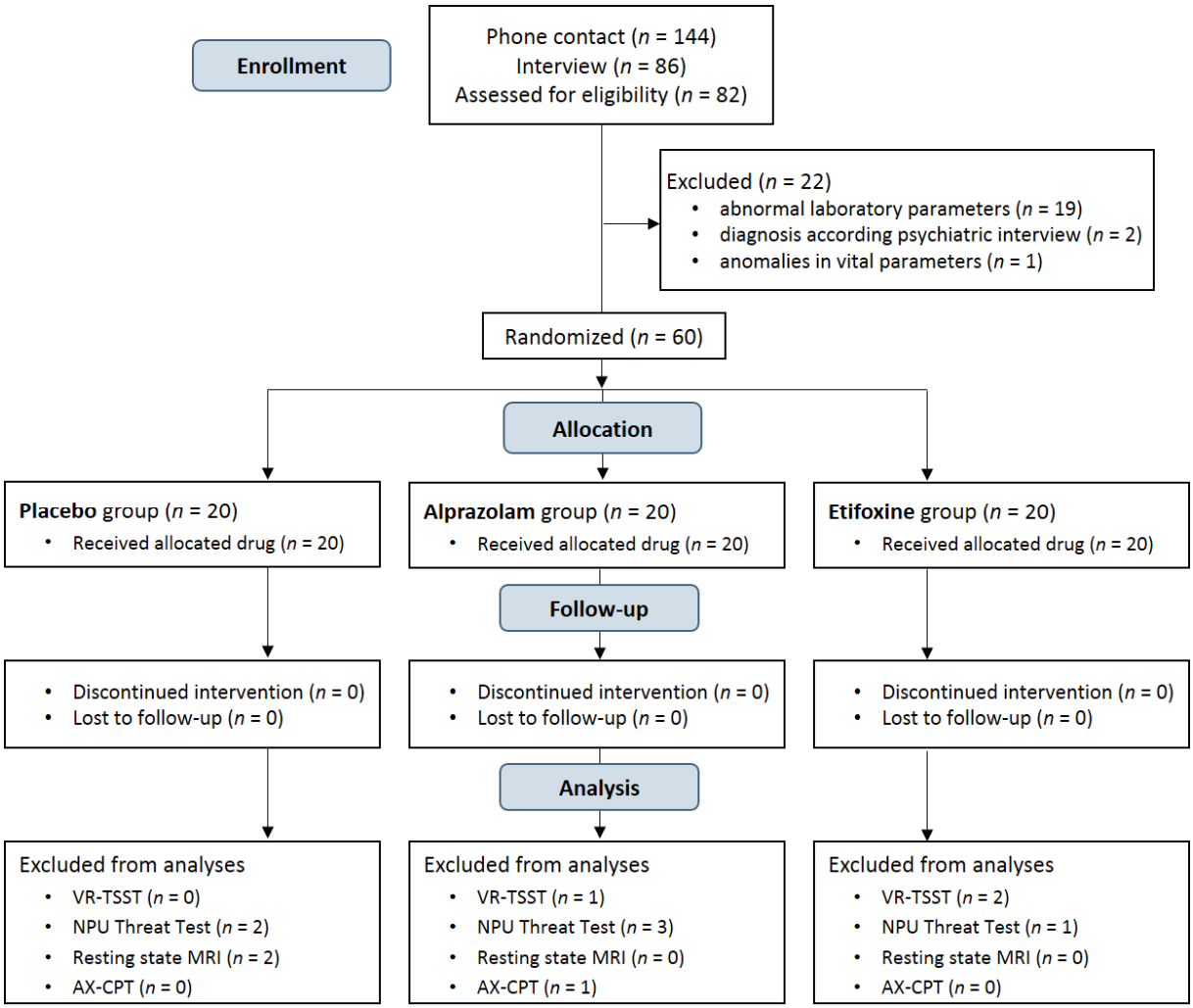
Male healthy participants aged between 18 and 55 years were recruited via advertisements on bulletin boards and in job portals for students as well as announcements in lectures (see appendix A). The required sample size of $N = 60$ was calculated for the primary outcome parameter of the clinical trial – the subjective stress level in response to the psychosocial stress task (see chapter 3). Determination of the expected effect size was based on a study that investigated the effects of alprazolam on psychosocial stress induced by the TSST and reported an effect size of $\eta 2 = .17$ (Fries et al., 2006). Setting the probabilities for an α-error at $\alpha = .05$, for a ß-error at $ß = .20$ (corresponding to a test power of $1 – ß = .80$) and including three groups, computations with G*Power (Faul, Erdfelder, Lang, & Buchner, 2007) yielded a required total sample size of $N = 51$ ($n = 17$ per group). Considering possible drop-out rates we recruited 20 subjects per group ($N = 60$).

Study prospects were contacted by phone and given general information on the study, especially about the medication and crucial criteria for participation. In case of persistent interest, we made an appointment for the informative interview (see 2.4.1) with a medical investigator. The initial phone call was conducted with 144 prospects. In total, we invited 86 subjects for the interview of which 82 underwent the initial screening. We could not include 22 persons because of negative results at the screening. For an overview of the recruitment see the CONSORT statement (figure 5) (Schulz, Altman, & Moher, 2010). According to the ICH guideline E9 (European Medicines Agency, 1998), analysis of the complete trial was planned for the “intention-to-treat” (IIT) population comprising all participants that were included into the study after fulfilling the criteria for participation. As all included participants completed the study, this sample equals the per-protocol (PP) population.

Source data of the participants were collected in case report forms. Participants were paid 500 euros for the complete participation. In case of exclusion after the screening, they received a compensation of 20 euros.

Figure 5

CONSORT Flowchart of Participants



Note. VR-TSST = Trier Social Stress Test in Virtual Reality (see chapter 3), NPU Threat Test (see chapter 4), resting state MRI = magnetic resonance imaging (see chapter 5), AX-CPT = Continuous Performance Test, AX-version (see chapter 6).

2.2.2 Inclusion criteria

The most crucial criterion for participation was signing of the consent form after oral and written information. Since gender and menstrual cycle phase are known to have an influence on stress related parameters (Childs, Dlugos, & de Wit, 2010; Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999), only male participants were included. Because age was also shown to affect those parameters (Kudielka, Buske-Kirschbaum, Hellhammer, & Kirschbaum, 2004), we defined a range from 18 to 55 years. Subjects had to be able to conceive nature and meaning of the clinical trial, to understand and realize the demands of the physician and to show adequate personal maturity. By signing of the consent form, they declared the willingness to forgo the consumption of alcohol, driving a car and the operation of heavy

machines during intake of the study medication. To avoid any transmission of the active substances, participants with a partner in a reproductive age had to assure the use of an adequate prevention method during study participation (Pearl-Index > 1 %). In case of pregnancy of the partner, it was compulsory to use a condom.

2.2.3 Exclusion criteria

Subjects were not included if they had an actual diagnosis of a mental disorder, especially abuse of alcohol / drugs within the preceding 12 months, alcohol / drug dependency in history or increased risk of suicidality or endangerment of self and others ascertained within the M.I.N.I. Neuropsychiatric Interview (Sheehan et al., 1998). Further, subjects should not suffer from a chronic somatic or neurological disease or regularly take any medication. For vital parameters, the following phenomena led to an exclusion: heart rate (HR) of < 50 or > 90 bpm, cardiac function constraints (except AV-block of 1st grade if HR was in normal range), blood pressure of < 90 or > 140 mmHg (systolic) or < 50 or > 90 mmHG (diastolic) and body temperature < 35 ° C or > 37.5 ° C (Breithaupt-Groegler et al., 2017). For blood parameters related to liver and kidney function the following thresholds must not be exceeded: > 10 % for GPT, > 20 % for GOT, > 20 % for bilirubin and > 0.1 mg / dl for creatinine. For other blood parameters resembling those of a full blood count, deviations of more than double of the upper limit or more than half of the lower limit were not accepted. Current drug consumption had to be ruled out for the following substances: amphetamines, benzodiazepines, cannabis, opiates, cocaine, ethylglucuronide, ethanol, fentanyl, pregabalin, buprenorphine, and methadone.

Further exclusion criteria were contraindications against the study medication (see 2.3.1 and 2.3.2) or known allergies / hypersensitivity against etifoxine hydrochloride, alprazolam or one of the other active contents. Accordingly, subjects were not included if they showed intolerance of galactose, lack of lactase or a glucose-galactose-malabsorption. They had to assure that they did not participate in another study according to the AMG concurrently and did not take psychotropic medication within the last six months. It was necessary that subjects had sufficient knowledge of German language and yielded no contraindications against the implementation of functional imaging. We did not include persons that already participated in a study using the Trier Social Stress Test to prevent influences of possible habituation (Jönsson et al., 2010; Klumpers et al., 2010; Wüst, Federenko, van Rossum, Koper, & Hellhammer, 2005). Because of known effects of smoking on cortisol levels (Childs & de Wit, 2009), we only included subjects who smoked less than five cigarettes per day.

2.2.4 Demographics

In total, we included 60 healthy male participants (20 per group) to the study. They were aged between 18 and 48 years (M = 27.77, SD = 6.92). A univariate ANOVA showed that the three treatment groups did not differ concerning age (see table 1). Most of our participants were students (n = 38, 63.6 %); 11 of them were employed (18.3 %), seven stated to be a student and employed (11.7 %), two were in vocational training (3.3 %), and two stated "other" (3.3 %). Almost all subjects have acquired Abitur (n = 55, 91.6 %), one subject finished secondary modern school, two had a secondary school certificate and two stated "other". Because of the fixed dosage scheme of the study medication, we checked that the groups did not differ concerning height, weight and body mass index (BMI) (see table 1).

Table 1

Means, Standard Deviations and Statistics of Physical Parameters of the Study Sample

Variable	Placebo	Alprazolam	Etifoxine	Statistics		
	M ± *SD*	*M* ± *SD*	*M* ± *SD*	*F* ratio	*df*	*p*
Age (years)	26.05 ± 4.71	27.55 ± 6.51	29.70 ± 8.78	1.43	2,57	.249
Height (cm)	180.55 ± 5.67	179.30 ± 7.66	179.0 ± 5.80	0.27	2,57	.762
Weight (kg)	77.50 ± 11.83	77.55 ± 16.17	77.41 ± 10.07	0.00	2,57	.999
BMI (kg / m^2)	23.75 ± 3.13	23.96 ± 3.51	24.07 ± 2.65	0.05	2,57	.949

The analyses of the TSPO polymorphism rs6971 (see 3.3.2.5) for the subjects of the etifoxine group (n = 20) yielded that n = 12 were high-affinity-binders (homozygous with GCG / GCG), while n = 5 were mixed-affinity-binders (heterozygous with GCG / ACG) and n = 3 were low-affinity-binders (homozygous with ACG / ACG).

2.3 Drug treatment

Participants were randomly assigned to one of three groups and received either 150 mg etifoxine per day, 1.5 mg alprazolam per day or placebo for five days in a double-blind manner. The respective dosages resemble the initial dosages used for treatment in patients and were further oriented on a previous study that compared the two compounds and reported comparable anxiolytic effects (Stein, 2015). Medication was provided as capsules (cps.) that were taken orally at three time points at 8.00 AM., 12.00 PM and 6.00 PM (for an overview see table 2).

Table 2

Dose Regimen of the Study Medication per Treatment Group

Group	Treatment	8.00 AM / 12.00 PM / 6.00 PM				
		Day 1	Day 2	Day 3	Day 4	Day 5
1	Etifoxine (mg)	50 / 50 / 50	50 / 50 / 50	50 / 50 / 50	50 / 50 / 50	50 / 50
2	Alprazolam (mg)	0.5 / 0.5 / 0.5	0.5 / 0.5 / 0.5	0.5 / 0.5 / 0.5	0.5 / 0.5 / 0.5	0.5 / 0.5
3	Placebo (cps.)	1 / 1 / 1	1 / 1 / 1	1 / 1 / 1	1 / 1 / 1	1 / 1

2.3.1 Etifoxine

Subjects of the etifoxine group received a daily dose of 150 mg of the substance ($C_{17}H_{17}ClN_2O$), which belongs to the group of benzoxazine derivatives. It was developed in the 1960s by Hoechst (1971). Because of its anxiolytic and anticonvulsive properties, it is indicated for the treatment of psychosomatic manifestations of anxiety (Biocodex, 2015) and, for example, approved in France (Stresam®, Biocodex, France; ATC code: N05BX03; marketing authorization number: 7560/2006/01-02). The usual dose according to the summary of product characteristics (SmPC) consists of three to four capsules per day, which are administered in two to three divided doses and taken from several days to weeks.

The anxiolytic effects of etifoxine are caused partially by directly binding to the β2 or β3 subunits of the $GABA_A$ receptor at binding sites distinct to those of benzodiazepines (Hamon et al., 2003). Furthermore, there is an indirect modulation of the GABAergic system by stimulating the synthesis of neurosteroids like allopregnanolone via binding to the mitochondrial translocator protein (Verleye et al., 2005).

Etifoxine is absorbed rapidly after oral administration. Maximal concentration in the blood is reached after two to three hours. It is metabolized via the liver and the biological half-time is about two to six hours, for the active metabolite up to 20 hours. (Choi & Kim, 2015)
For an overview of the possible side effects see table 3.

Table 3

Side Effects of Etifoxine as Listed in the SmPC

Frequency	Symptoms
Rare (≥1/10000 to <1/1000)	Slight drowsiness at the beginning of treatment, maculopapular rash, erythema multiforme, facial edema
Very rare (<1/10000)	Urticaria, Quincke's edema
Not known (cannot be estimated from the available data)	Anaphylactic shock, DRESS syndrome, Stevens-Johnson syndrome, leukocytoclastic vasculitis, hepatitis, hepatic cytolysis syndrome, metrorrhagia in women taking oral contraceptives, lymphocytic colitis

Contraindications listed in the SmPC comprise hypersensitivity against etifoxine hydrochloride or any of the excipients, shock state, severely impaired liver and / or renal function, and myasthenia. Because of possible interactions, the consumption of alcohol or any other medication, especially CNS depressants, is not recommended. Furthermore, driving a car or operating heavy machines should be avoided whilst intake. A large French pharmacovigilance study, which reflected a period of 12 years, reported no case of abuse or dependence and only a very rare occurrence of serious adverse reactions mainly comprising dermatological or hypersensitivity reactions (Cottin et al., 2015).

2.3.2 Alprazolam

Subjects of this group received a daily dose of 1.5 mg alprazolam ($C_{17}H_{13}ClN_4$), which belongs to the group of benzodiazepines. It was first marketed in 1984 by Pfizer and is indicated for the symptomatic treatment of acute and chronic states of tension, agitation and anxiety (ratiopharm GmbH, 2015). For the previous work, alprazolam was obtained from ratiopharm (ATC code: N05BA12; marketing authorization number: 37734.00.00). The usual initial dose comprises 0.25 to 0.5 mg three times a day, which can be increased up to 0.5 to 3 mg per day (divided into single deliveries). The period of intake may not exceed eight to 12 weeks.

Alprazolam directly binds to the $GABA_A$ receptor in the brain and thereby modulates the release of the inhibitory neurotransmitter GABA. It is absorbed rapidly after oral administration with peak concentrations in plasma after one to two hours. The biological half-time lies between nine to 16 hours. Taking alprazolam over a longer period can result in development of tolerance or physical / mental dependence (Verster & Volkerts, 2004).
For an overview of the possible side effects see table 4.

Table 4

Side Effects of Alprazolam as Listed in the SmPC

Frequency	Symptoms
Very common (≥ 1/10)	Sedation, sleepiness*
Common (≥ 1/100 to < 1/10)	Loss of appetite, confusion*, depression, ataxia*, coordination disturbances, limited memory function, slow speech, concentration difficulties, dizziness*, headache*, blurred vision*, obstipation, nausea, asthenia, irritability
Occasional (≥ 1000 to < 1/100)	Hyperprolactinemia, hallucinations, fits of rage, aggressive behavior, hostility, anxiety, agitation, changes of libido, sleeplessness, abnormal thinking, nervousness, stimulation, amnesia, dystonia*, tremor, vomiting, liver dysfunctions, icterus, dermatitis, musculoskeletal weakness*, incontinence, urinary retention, sexual dysfunction, menstrual irregularity, weight chance, increase of intraocular pressure
Not known	Increase of appetite, disturbances of the vegetative nervous system, emotional apathy*, reduced attention*, double vision, tachycardia, hypotonia, stuffy nose, diarrhea, xerostomia, increased salivation, dysphagia, hepatitis, angioedema, skin reactions, peripheral edema, exhaustion*

Note. * appear mostly at the beginning of treatment or at higher dosages.

Contraindications listed in the SmPC comprise myasthenia gravis, hypersensitivity against the active substance, severe respiratory insufficiency, sleep apnea, severe dysfunctions of liver or acute intoxication through alcohol or other CNS active substances. Because of possible interactions it should not be consumed with alcohol or any other medication, especially other CNS depressants. Furthermore, recipients should forgo driving a car or operating heavy machines whilst intake.

2.3.3 Placebo

Subjects in this group received identical looking pills that were also manufactured by the pharmacy in Erlangen and contained only filler mixture (components: 99.5 % mannitol, 0.5 % silicon dioxide) and no active substances.

2.3.4 Manufacturing and labelling of the study medication

The investigational medicinal products (IMPs) were repackaged into hard gelatin capsules that looked identical for each of the three treatment groups by the pharmacy of the University Hospital of Erlangen (filler mixture+: 99.5 % mannitol, 0.5 % silicon dioxide).

Blinding of the medication was realized with a randomization list, which was generated by an independent biometrician of the Centre for Clinical Studies of the University Hospital of Regensburg using the software SAS 9.4 (SAS institute Inc., Cary, NC, USA) and directly sent to the pharmacy. In addition to repackaging the medication according to this list, they set up the Investigational Medicinal Product Dossiers, envelopes for unblinding in case of emergency and labels for the medication containers. The medication was retrieved in three charges. Upon receiving, it was stored in a locker at the research station 18 D at room temperature below 25 °C. Distribution and redemption were noted in a medication log that was stored in the same locker.

2.4 General study procedure

Following, an overview of the time schedules and measurements of the single study days is given. Detailed information on the methods will be provided in the corresponding chapters. For an overview of the complete study procedure see table 6.

2.4.1 Informative interview

Interested persons were invited to an interview at the Bezirksklinikum Regensburg, in which they were informed about all relevant details of the study (planned inquiries, time schedule, study medication, related risks, rights and obligations) by a medical investigator. After clarifying possible questions, subjects received the written information document, the consent form and the conditions of the probands insurance. In case of further interest, they were invited for the screening after at least one night of reflection.

2.4.2 Screening

The screening had to be scheduled within no longer than seven days before the planned start of participation. Upon arrival at the research station 18 D of the Bezirksklinikum Regensburg, an investigator first checked the dated signature of the consent form and signed it as well. Participants were provided a copy of the documents. The order of the subsequent parts of the screening could vary according to the temporal circumstances of the clinical day-to-day.

Usually, the screening started with the physical examination, which was conducted by an investigator who assessed the general physical state and checked for the presence of any common chronic disorder. Further, the use of stimulants, regular medication intake and

intolerances were explored. This was followed by the first blood sampling (sober) following standard precautions under sterile conditions. Screening blood for the assessment of safety parameters (see 2.2.2) was analyzed at the laboratory of the Bezirksklinikum Regensburg (responsible person: Georg Weinfurtner). The study blood for the efficacy analyses was sent to the laboratory of the Chair of Psychiatry and Psychotherapy located at the University Hospital Regensburg (responsible person: Priv.-Doz. Caroline Nothdurfter (for an overview see table 5).

Table 5

Overview of Blood Samples Taken for the Assessment of Safety and Efficacy Parameters

Purpose	Tubes (Sarstedt, Nürmbrecht)	Study day (time)
Screening blood (safety)	1 x 7.5 ml serum-gel	Screening (flexible)
	1 x 2.7 ml EDTA	Day 5 (9 AM)
	1 x 3 ml citrate	
Study blood (efficacy)	1 x 9 ml citrate	Screening (flexible)
	2 x 9 ml EDTA	Day 1 (9 AM)
	1 x 9 ml serum	Day 4 (9 AM)
		Day 5 (9 AM)
		Day 5 (~ 3 PM)

At each blood sampling, participants completed a questionnaire, which comprised statements on feeling sick, sad or anxious, on sleep quality and duration of the preceding night, physical exercise the day before as well as on intake of any medication, food, nicotine or caffeine (see appendix B). Afterwards, height and body weight of the subjects were noted, and blood pressure, pulse and body temperature were measured. This was followed by the recording of an electrocardiogram. After passing of a urine sample for the drug screening, participants completed the trait and state version of the Positive and Negative Affect Schedule (Krohne, Egloff, Kohlmann, & Tausch, 1996), the trait and state version of the State-Trait Anxiety Inventory (Laux, Glanzmann, Schaffner, & Spielberger, 1981), Visual Analogue Scales (VAS) on general mood, calmness, wakefulness, anxiety and concentration (see appendix C) as well as a sociodemographic questionnaire (see appendix D). Mental health was assessed by a psychologist using the German version of the Mini-International Neuropsychiatric Interview (M.I.N.I.) (Ackenheil, Stotz, Dietz-Bauer, & Vossen, 1999; Sheehan et al., 1998).

At the end of the screening, subjects moved to the Chair of Clinical Psychology and Psychotherapy where the Continuous Performance Test (AX-CPT, see chapter 6) was administered for the first time. After that, they completed the Anxiety Sensitivity Index (ASI-3) (Kemper, Ziegler, & Taylor, 2009), the Intolerance of Uncertainty Scale (IUS-18) (Gerlach,

Andor, & Patzelt, 2008), the Stress Coping Questionnaire (SVF 120) (Erdmann & Janke, 2008), and the Brief Fear of Negative Evaluation Questionnaire (BFNE) (Reichenberger et al., 2015).

All inclusion and exclusion criteria were checked by an investigator and subjects were informed about their eligibility the latest one day after the screening. If they did not fulfill the study criteria, they were paid the compensation and handed out a copy of the laboratory results if wished. In case of abnormal values, they were suggested to consult a general physician.

2.4.3 Day 1 of treatment

Subjects who fulfilled the inclusion criteria returned to the research station to start participation in the study either on a Monday or on a Friday. There, they received the first capsule of the medication by a physician at 8.00 AM and were instructed about the correct intake. Half an hour later, subjects completed the state version of the PANAS, the VAS on their current mood and questionnaires on possible side effects (see appendix E) and the general mental state (see appendix F). They were handed out a folder for day 2 and 3 of treatment, which contained the questionnaires just mentioned, a diary for documenting the time points of medication intake and a card containing information on persons and facilities to contact in case of emergency. One hour after taking the first medication, blood samples were collected, and the accompanying questionnaire was handed out.

Subsequently, the AX-CPT was administered for a second time at around 9.30 AM. After a short break, participants continued with the NPU Threat Test (see chapter 4). At the end of day 1, participants were handed out the remaining medication for the present day as well as for days two and three of treatment (in total eight capsules).

2.4.4 Days 2 and 3 of treatment

On day 2 and day 3 of treatment there were no planned study visits. Participants were instructed to take the study medication autonomously at the given time points and to complete the questionnaires half an hour after intake of the morning medication.

2.4.5 Day 4 of treatment

On day 4 of treatment study medication for the complete day was provided at the Bezirksklinikum at 8 AM. Half an hour later, participants completed the questionnaires

concerning their mood (PANAS state, STAI state, VAS) and on potential side effects. This was followed by the third blood collection at 9 AM.

At 12.30 PM the subjects were expected at the premises of the Neuroradiology Department where the MRI scan took place (see chapter 5). After the scan, participants received instructions with respect to the stress test on the following day (see chapter 3). On day 5 of treatment they should not drink any caffeinated drinks after 9 AM, not consume any food after 11 AM, not drink anything else than water and not chew a gum or brush their teeth after noon.

2.4.6 Day 5 of treatment

On day 5 of treatment subjects were provided the medication at 8 AM at the Bezirksklinikum and half an hour later completed the same questionnaires as on the days before. This was followed by another blood taking at 9 AM, which additionally comprised the safety parameters that were already determined at the screening. Likewise, vital parameters were checked for possible changes with respect to the screening day, which could be due to the treatment.

Paralleling day 1 participants completed the AX-CPT for a third time and subsequently the NPU Threat Test. After a break and the intake of the study medication at noon, participants returned for the Trier Social Stress Test in Virtual Reality (VR-TSST) which also took place in the premises of the Chair of Clinical Psychology and Psychotherapy at 1 PM (see chapter 3).

Following this task, the last planned visit took place at the research station at around 3 PM with a last blood sample and completion of the questionnaire concerning possible side effects. Participants returned the empty medication containers and the diary of medication intake. In a final meeting with an investigator, subjects were informed about their present blood values and vital parameters. In case of abnormal values in comparison to the screening, they were requested for a follow-up investigation of the relevant parameter after a period that was determined by the investigator. They were reminded of the post phone call after seven days and thanked for participation in the study.

2.4.7 Post phone call

After a period of seven days, subjects were interviewed by phone concerning the course of their physical and mental state and on possible side effects after completion of the study using a self-designed questionnaire (see appendix G).

Table 6

Overview of the General Study Procedure

Study day	Time	Investigational part
Screening	7.45 AM	Checking dated signature of written consent form
	8.00 AM	Physical examination
	8.30 AM	Blood taking (sober) + questionnaire "blood taking" • study blood • screening blood
	8.40 AM	Measurement of vital parameters
	8.50 AM	Urine sample
	8.55 AM	PANAS trait/state, STAI trait/state, VAS
	9.15 AM	Psychiatric interview (M.I.N.I.)
	10.00 AM	Continuous Performance Test (AX-CPT)
	10.30 AM	ASI-3, IUS-18, SVF 120, FNE-K
Day 1 of treatment (Monday / Friday)	8.00 AM	1st distribution of study medication
	8.30 AM	PANAS state, VAS, questionnaire mental state & side effects
	9.00 AM	Blood taking + questionnaire "blood taking" • study blood
	9.30 AM	Continuous Performance Test (AX-CPT)
	10.30 AM	NPU Threat Test
	12.00 noon	2nd distribution of study medication
	6.00 PM	Intake of study medication
Day 2 of treatment (Tuesday / Saturday) **+ Day 3 of treatment** (Wednesday / Sunday)	8.00 AM	Intake of study medication
	8.30 AM	PANAS state, VAS, questionnaire mental state & side effects
	12.00 noon	Intake of study medication
	6.00 PM	Intake of study medication

Study day	Time	Investigation part
Day 4 of treatment (Thursday / Monday)	8.00 AM	3rd distribution of study medication
	8.30 AM	PANAS state, VAS, STAI state, questionnaire mental state & side effects
	9.00 AM	Blood taking + questionnaire “blood taking“ • study blood
	12.00 noon	Intake of study medication
	1.00 PM	Functional imaging
	6.00 PM	Intake of study medication
Day 5 of treatment (Friday / Tuesday)	8.00 AM	4th distribution of study medication
	8.30 AM	PANAS state, VAS, STAI state, questionnaire mental state & side effects
	8.45 AM	Physical inspection
	9.00 AM	Blood taking (sober) + questionnaire “blood taking“ • study blood • screening blood
	9.30 AM	Continuous Performance Test (AX-CPT)
	10.45 AM	NPU Threat Test
	12.00 noon	Intake of study medication
	1.00 PM	Trier Social Stress Test in Virtual Reality (VR-TSST)
	~ 3.00 PM	Blood taking + questionnaire “blood taking“ • study blood
	~ 3.15 PM	Questionnaire on side effects
Post enquiry (7 days after the end of participation)	flexible	Post enquiry concerning possible side effects and mental state during the days after the study by phone

CHAPTER 3: Effects of etifoxine & alprazolam on acute psychosocial stress

3.1 Theoretical background

Stimuli that evoke anxiety and presuppose pathological states like generalized anxiety disorder are often of psychological nature and do not directly threaten the survival of an organism on the physiological level. Amongst others, the experience of stressful life events during infancy or adulthood turned out as one remarkable risk factor for their development and maintenance (Moreno-Peral et al., 2014). To clarify the physiological and molecular processes of those associations and be able to examine action mechanisms underlying the effects of anxiolytics, standardized research in the laboratory is inevitable. A good starting point for that is constituted by acute psychosocial stress, which can be reliably evoked in laboratory settings and effectively leads to an activation of central physiological systems like the HPA axis and the SAM system (Allen, Kennedy, Cryan, Dinan, & Clarke, 2014; Kudielka, Hellhammer, & Kirschbaum, 2007).

Acute psychosocial stress mainly raises in situations when an individual is evaluated by others implying the risk of negative evaluation and further by experienced uncontrollability within the respective situation (Dickerson & Kemeny, 2004). A standardized paradigm that incorporates these components and was shown to reliably induce psychosocial stress in the laboratory is the Trier Social Stress Test (TSST) (Kirschbaum, Pirke, & Hellhammer, 1993). Within the TSST, participants are instructed to imagine they have applied for their dream job. Therefore, they are asked to give an interview speech and solve arithmetical tasks in front of a committee consisting of trained psychologists that are judging the performance. Participants are not given any feedback except being prompted to go on with the speech in case they stop talking or make a mistake during the arithmetic task. Typical results that mark the stress response comprise increases of physiological parameters like heart rate or skin conductance, endocrine parameters like cortisol or ACTH as well as perceived stress levels (Allen et al., 2014; Dickerson & Kemeny, 2004). Recently, virtual reality based versions of the TSST were established to overcome stated issues like the high amount of personnel needed for its implementation and necessary efforts to keep the experimental conditions standardized across participants (Jönsson et al., 2010). Thereby, studies using head-mounted displays (HMDs) (Santl et al., 2019; Shiban et al., 2016; Zimmer, Buttlar, Halbeisen, Walther, & Domes, 2018)

or Cave Automatic Virtual Environments (Wallergard, Jönsson, Österberg, Johansson, & Karlson, 2011) revealed results that are comparable to those shown for in vivo studies.

Besides variations to refine methodological premises, the identification of additional stress related markers was targeted by researchers to promote multimodal investigation. This led, for example, to an inclusion of vocal measurements like mean or variation of fundamental frequency, which indicates stretching and tension of the vocal folds as well as vocal intensity and sub-glottal pressure and has been shown to change in response to psychosocial stress (Pisanski et al., 2018; Pisanski, Nowak, & Sorokowski, 2016). For some parameters like the neuroactive metabolite allopregnanolone, which is known to be a positive modulator of the $GABA_A$ receptor (Majewska, Harrison, Schwartz, Barker, & Paul, 1986), evidence has been contradictory. While studies on rats noted an increase of the allopregnanolone level in response to stress elicited by CO_2-inhalation or forced swimming (Barbaccia et al., 1996; Purdy, Morrow, Moore, & Paul, 1991), some studies in humans applying the TSST could not confirm that (Altemus et al., 2001; Childs & de Wit, 2009). However, another study that examined the effects of a doctoral defense on endocrine parameters did not only report an increase of cortisol but also of allopregnanolone and further expression of TSPO (Fortuyn et al., 2004). This indicates a link between this anxiolytic target protein and psychosocial stress, which is further underlined by studies revealing a relationship between the presence of the TSPO related gene polymorphism rs6971 and mental disorders like bipolar disorder (Colasanti et al., 2013) or alcohol use disorder (Wiers et al., 2019). As this polymorphism, which affects the binding of ligands to the protein, was further shown to have an effect on diurnal cortisol release (Prossin et al., 2018), a relation to acute psychosocial stress seems obvious (Papadopoulos et al., 2018). We are not aware of any study that investigated the effects of a TSPO ligand on stress-related parameters in humans. However, promising evidence stems from studies in animals, which showed stress-reducing effects of etifoxine indicated by measurements of body temperature, freezing behavior and colonic motility (Verleye & Gillardin, 2004). The authors attributed these effects to an attenuation of the stress-induced activation of CRH through enhancement of the inhibitory GABAergic system. This modulation, vice versa, is known to be mediated by stimulation of the synthesis of anxiolytic neurosteroids like allopregnanolone (do Rego et al., 2015).

The pharmacological modulation of the GABAergic system was indeed shown to affect psychosocial stress in human studies. One of the first studies that examined the effects of a single dose of 0.5 mg alprazolam on acute stress reported an attenuation of ACTH and cortisol release as well as decreased heart rate in response to a stressful interview situation (Rohrer et

al., 1994). Another study that investigated the effects of alprazolam on acute stress induced by a parachute jump confirmed the results for cortisol and extended them to adrenaline but did not find any effects on heart rate (Benschop et al., 1996). Missing effects on heart rate were also reported by another study investigating the effects of lorazepam in a public speaking task, which did, however, find attenuated subjective stress accompanied by sedative effects (Guimarães, Zuardi, & Graeff, 1987).

We are aware of one study that combined the investigation of a single dose of 1 mg alprazolam with the standardized protocol of the Trier Social Stress Test (Fries et al., 2006). They underpinned preceding literature by finding a dampening effect of alprazolam on stress-induced release of ACTH and cortisol. However, they did not observe any effects of the benzodiazepine on autonomous parameters like heart rate or blood pressure and hardly found an influence on self-reports. One hypothesis used for explanation concerned the period of intake of the medication. All mentioned studies applied only a single dose of an anxiolytic reflecting an acute effect. However, it might be possible that effects on physiological and subjective parameters in healthy subjects would just arise after intake of several doses.

3.2 Research questions and hypotheses

Based on the stated literature, we investigated the effects of etifoxine and alprazolam on acute psychosocial stress in healthy male subjects. As the two anxiolytics affect the GABAergic system in a different manner, we wanted to check if the dampening effects that were shown for alprazolam and other benzodiazepines can be transferred to the TSPO ligand etifoxine. For that, one group was administered etifoxine, which acts twofold on the $GABA_A$ receptor, once by directly binding to it and additionally via modulation of TSPO and subsequent synthesis of neurosteroids. The other subjects received either the benzodiazepine alprazolam or placebo for a period of five days. On the last day of treatment all subjects were exposed to the Trier Social Stress Test in Virtual Reality (VR-TSST). To obtain a multimodal evaluation of the effects we included common parameters like subjective ratings, heart rate, skin conductance level, cortisol release, voice-based parameters, allopregnanolone measurements and TSPO expression into our analyses. In contrast to previous studies, we did not test the effects after intake of a single dose but after several days, thereby examining more short-term than acute effects.

The primary endpoint was the subjective stress level reported after the VR-TSST. According to the anxiolytic properties of both substances, we hypothesized that the increase in subjective stress in response to the task would be similarly reduced by etifoxine and alprazolam.

This effect was further expected to be reinforced by other self-reports on state anxiety, general mood and calmness. However, given the known sedating effects of alprazolam we expected more reports of increased fatigue and lessened concentration in that group compared to placebo and etifoxine.

Although data on the effects of benzodiazepines on autonomous measurements are contradictory, we expected an influence of both anxiolytic agents on physiological parameters due to the longer duration of intake compared to previous studies. We expected alprazolam and etifoxine to reduce the stress-induced increase of heart rate and skin conductance level in comparison to placebo.

For the vocal parameters, we first aimed to replicate findings of prior work, which showed an increase of F0 *Mean* and a decrease of F0 *SD* in response to stress. Further, we expected a voice-based emotion classifier to identify higher arousal and more negative valence during stress in comparison to baseline. Since voice parameters were shown to be associated with stress-induced cortisol release and due to possible muscle-relaxing effects of the treatment, we suggested modulating effects of the anxiolytic agents also on those parameters.

Research has shown an attenuation of the stress related increase of cortisol release due to alprazolam. As etifoxine also partly acts via the GABAergic system, we expected to extend the finding of inhibited activity of the HPA axis to the TSPO ligand. Since etifoxine acts mainly via the modulation and synthesis of neurosteroids like allopregnanolone, we expected increased levels in this group compared to the groups receiving alprazolam and placebo. In general, we supposed an increase of this neurosteroid due to the stress task. TSPO expression was shown to be increased after acute stress and due to administration of etifoxine. As for allopregnanolone, we expected an increase of TSPO expression in response to the VR-TSST with the highest expression within the etifoxine group.

Since benzodiazepines are known to possess sedative effects, we expected participants of the alprazolam group to perform worse during the speech and the math task. This meant less correct answers in the math task as well as decreased speech rate in front of the committee in comparison to etifoxine and placebo.

Due to the influence of the gene polymorphism rs6971 on the binding affinity of TSPO ligands the analyses for the common stress markers were repeated after exclusion of participants of the etifoxine group that were identified as low-affinity binders. Since there is little known on the specific influence of the polymorphism on binding and efficacy of etifoxine, we checked rather exploratory if parameters of the stress reaction were changed in subjects that show the genetic variant.

3.3 Methods and procedure

3.3.1 Trier Social Stress Test in Virtual Reality (VR-TSST)

The Trier Social Stress Test administered in this study was a virtual reality based version (VR-TSST) (Shiban et al., 2016) that was generated with the Steam Source game engine (Valve Corporation, Bellevue, Washington, USA) and controlled / synchronized by a script based on Cybersession (VTplus GmbH, Würzburg, Germany). During the experiment, participants wore a head mounted display (HMD; HTC, Vive, Taoyuan, Taiwan) and headphones (Sennheiser, HD 215, Sennheiser electronic GmbH & Co. KG, Wedemark-Wennebostel, Germany).

After entering the virtual reality, participants started with a short habituation to the environment by moving in the virtual room with a joystick that was positioned in front of them. After that, they were asked to enter a hall where they met a female investigator who gave them further instructions. She told them to imagine that they have applied for their dream job and are therefore asked to mentally prepare a speech of five minutes in which they should convince a psychologically trained committee of their suitability for the job. They were further told that the committee members were going to make notes concerning the speech and would analyze the behavior. Thereby, within the speech subjects should not focus on their curriculum vitae but more on their strengths and weaknesses. With the additional information that a second task will follow, which they would be told about later, they were then left alone for three minutes to prepare the speech without knowing how long the preparation phase would last. At the end of the preparation phase subjects were prompted by a loudspeaker announcement to go into the next room where they were awaited by the committee. This was constituted by two males and one female sitting on a table and wearing laboratory coats (see figure 6).

Figure 6

Virtual Committee During the Trier Social Stress Test

In the lecture room all participants were teleported to stand centrally in front of the committee. Upon that, they were asked by one of the males to take a step back and to stand upright for the sake of the video recording – that was actually not taken. The female member prompted them to begin with their speech and caused them to go on if they paused for 20 seconds by asking pre-recorded questions (see appendix H). After five minutes, participants were thanked and told about the second task in which they had to count back from the number 2023 in steps of 17. They were asked to count faster betweenwhiles or restart after making a mistake. In total, participants spent about 15 minutes in the virtual reality.

3.3.2 Outcome measures

3.3.2.1 Primary endpoint - subjective stress level (SUD)

The primary endpoint of the total study was the subjective stress level accompanying the stress task. This was stated by the subjects verbally (*How stressed do you feel at the moment?*) on a scale ranging from 0 = not stressed at all (*überhaupt nicht gestresst*) to 100 = extremely stressed (*extrem gestresst*) at eight time points (T-30, T-10, T-1, T+1, T+10, T+20, T+30, T+45) throughout the study (for an overview see figure 7).

3.3.2.2 Further subjective measurements

State-Trait Anxiety Inventory (STAI)

To measure trait anxiousness as well as changes of state anxiety, we administered the German version (Laux et al., 1981) of the State-Trait Anxiety Inventory (STAI) (Spielberger, Gorsuch, & Lushene, 1970). The trait version assesses relatively stable interindividual differences in evaluating situations as threatening and was administered at the screening day when all participants were medication-free. The state version inquires the extent of current anxiety resembling an emotional state that is characterized by symptoms like nervousness, agitation, concerns, and increased activity of the autonomous nervous system (Spielberger, 1972). This form was administered directly before and after the VR-TSST.

Both versions consist of 20 items that are stated on a four-point scale from 1 = almost never (*fast nie*) to 4 = almost always (*fast immer*) for the trait-version and from 1 = not at all (*überhaupt nicht*) to 4 = very much so (*sehr*) for the state-version. Seven items of the trait-version and ten items of the state-version are formulated towards lack of anxiety and were coded inversely before computing the sum score (range 20 to 80). Both forms show high internal consistency with Cronbach's α of about .90. While retest-reliability for the trait scale is

satisfying to good with r_{retest} ranging from .68 to .96, more sensitivity to change is shown for the state-scale with r_{retest} between .03 and .76 (Laux et al., 1981).

Visual analogue scales (VAS)

For the measurement of further subjective state variables, we administered VAS (see appendix C) in which we asked participants to rate their current mood (very bad – very good), calmness (very excited – very relaxed), wakefulness (very tired – very awake), anxiety (very calm – very anxious), and concentration (very confused – very concentrated) on bipolar scales ranging from 1 to 10. The VAS were completed directly before and after the VR-TSST.

3.3.2.3 Physiological measurements

Heart rate (HR)

Heart rate was recorded using the Brain Vision Recorder (Brain Products GmbH, Gilching, Germany). For that, we attached self-adhesive pre-gelled surface electrodes (Ag/AgCl, Ø = 40 mm) that were additionally prepared with Signa electrode gel (Parker Laboratories, Fairfield, USA). One electrode was placed at the mid-section of the sternum. Another electrode serving as reference was placed on the upper ribs left side and a third one serving as ground electrode was attached at the abdomen on the right body site. HR was recorded continuously with a sampling rate of 1000 Hz.

Subsequent pre-processing with the Brain Vision Analyzer (Version 2.1, Brain Products GmbH, Germany) comprised low-pass-filtering (cut-off: 30 Hz, slope: 12dB / oct), high-pass-filtering (cut-off: 0.16 Hz, slope: 12 dB / oct) as well as a Notch filter at 50 Hz. R-waves were detected using an inbuilt solution and further checked by visual inspection.

For the analyses, we separated the data into the following segments: baseline_rest (BL_rest: 2min.), baseline_spoken (BL_spoken: 1 min.), preparation (3 min.), speech (5 min.), math (3 min.), post (2 min.). Since physiological parameters are known to increase in response to speaking aloud, we included the BL_spoken as reference for further analyses to avoid that otherwise increases might have been erroneously attributed to stress (Grimley et al., 2018).

Skin conductance level (SCL)

Skin conductance was continuously recorded using the Brain Vision Recorder with a sampling rate of 1000 Hz. For that, we used two surface electrodes (Ag/AgCl, Ø = 8 mm) that were prepared with isotonic electrode cream (TD-246, PAR Medizintechnik GmbH, Germany)

and were attached at the thenar eminence of the non-dominant hand after participants had washed their hands without soap.

During pre-processing with Brain Vision Analyzer, we applied a low-pass-filter (cut-off: 1 Hz, slope: 12db). Data segmentation was performed analogous to the heart rate measurements with baseline_rest (BL_rest: 2 min.), baseline_spoken (BL_spoken: 1 min.), preparation (3 min.), speech (5 min.), math (3 min.), and post (2 min.). As for the heart rate analyses, we chose BL_spoken as reference baseline period for the computations on SCL.

Vocal indices

For the analysis of speech-based parameters, we recorded baseline measurements and the speech during the stress task using an USB microphone (MeteorMic, Samson, New York, USA) that was positioned in front of the participants. To obtain the neutral voice baseline, we asked the participants to describe a picture of a house and numbers hanging on a wall in an anteroom of our virtual scenario for one minute. The stress recordings were gathered during the five minutes of oral presentation in front of the committee. The three minutes of the mathematical task were not included into these analyses, as the way of speaking differed too much from the other two episodes.

Analyses were performed with a modified component of the software openSMILE (Eyben, Wöllmer, & Schuller, 2010), which enables speech classification analyses based on an algorithm that was trained with huge datasets of recordings from professionally acted emotions. This component was provided to our chair by the company audEERING (Gilching, Germany) within the scope of another project (OPTAPEB, funded by the BMBF). Permission was obtained to use it for this study as well. The component was controlled via Anaconda Python (version 3 5.0.1, Anaconda Inc., Austin, Texas, US).

First, based on an inbuilt voice activity detector all segments containing voiced speech were extracted. We included the first 20 seconds for the baseline and the stress measurement into our analyses, as no commentaries of the committee that could have disrupted the speaking were inserted for any of the subjects up to this time point. This yielded segments with a mean duration of 20.93 seconds for the baseline (*SD* = 1.47 s) and 20.75 seconds for the presentation (*SD* = 1.08 s) that were achieved after a total frame duration of 28.92 seconds (*SD* = 5.44 s) for the baseline and 33.67 seconds (*SD* = 9.02 s) for the presentation. Of those, we exported the mean and the standard deviation of the fundamental frequency (F0 *Mean* and F0 *SD*) as well as values reflecting arousal and valence. Those ranged on a scale from -1 to 1 and were computed

based on 6375 single parameters. For arousal and valence, we included only those values that were allocated by the algorithm with a probability of greater than .4.

The selection of the included parameters was based on a pre-study that was conducted at our chair to test the software. Within this study, 33 healthy females underwent the VR-TSST with half of the group being exposed to an essential oil (unpublished work). Amongst other stress parameters, we recorded speech before and during the stress task. We found an increase of the mean fundamental frequency (F0 *Mean*) during stress, whereas the opposite was shown for the respective standard deviation (F0 *SD*). For the emotion classifications, we found an increase of arousal during stress, while valence was more negative in comparison to baseline.

For the analysis of the objective performance, we calculated the number of correctly stated digits (math_correct) as well as the proportion of false answers with respect to the total number of stated digits (math_false rate) during the math task and the total length of spoken segments during the speech in front of the committee (speech_duration) for each subject.

3.3.2.4 Endocrine measurements

Salivary cortisol

Salivary samples to determine free cortisol were taken in parallel to the subjective stress ratings at eight time points (T-30, T-10, T-1, T+1, T+10, T+20, T+30, T+45) throughout the experiment using salivette collection tubes (Sarstedt, Nümbrecht, Germany). For that, participants had to remove a swab from the tube, put it in their mouth and leave it there for 60 seconds. We instructed them to slightly move their jaw to stimulate salivation. After putting the swab back into the tube, they were closed and frozen at -20 ° C until further processing.

Biochemical analyses were carried out at the laboratory of the Chair of Psychiatry and Psychotherapy of the University of Regensburg. Frozen saliva samples were brought to room temperature (RT) and analyzed with an enzyme-linked immunosorbent assay (ELISA) kit (IBL, Hamburg, Germany; batch number: RE52611) according to the manufacturers protocol. The assay sensitivity was 0.08 nmol / l. Intra-assay variation ranged between 3.2 % and 6.1 %, inter-assay variation between 4.2 % and 17.0 %.

Allopregnanolone

For the analysis of allopregnanolone we used the blood samples that were taken in the morning of day 5 one hour after medication intake and in the afternoon of the same day about one and a half hours after the beginning of the VR-TSST. Uninterrupted refrigeration was

ensured for the tubes. They were first centrifuged at 3700 rpm (Megafuge 2.0 R, Heraeus, Thermo Scientific) for ten minutes at 4 °C. The remaining serum was pipetted into five tubes of 0.1 ml and four epicups of 0.5 ml and frozen at -80°C until further analyzing.

Determination of allopregnanolone was carried out at the Leibniz Research Centre for Working Environment and Human Factors in Dortmund (responsible person: Priv.-Doz. Jörg Reinders). They applied gas chromatography-mass-spectrometry with prior derivatization.

TSPO expression

For the analysis of TSPO expression, blood samples of the same time points as for the allopregnanolone analysis were used. After centrifuging the full blood samples (9 ml citrate blood tubes) at 3200 rpm (Megafuge Heraeus® 1.0 – 2100 x g, Thermo Scientific) for three minutes at RT, we placed 2400 µl of the platelet rich plasma and 400 µl acid-citrate-dextrose solution (Sigma-Aldrich, Taufkirchen, Germany) into 15 ml centrifuge tubes and homogenized the components. Following further centrifugation at 3200 rpm for three minutes at RT, the supernatant was decanted and the pellets containing platelets were frozen at -80 °C until determination of TSPO expression using Western blots.

Protein concentrations of lysates were quantified using the Bradford method (M. M. Bradford, 1976) with the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Munich, Germany). First, 50 µg of protein lysates were separated by sodium dodecyl sulfate gel electrophoresis with 3.5 % stacking and 15% running in 4 µl buffer and water. After incubating the lysates at 95° C for five minutes, molecular weight markers of 3 µl were applied and the first gel run started (160 V, 50 min., RT). After transferring the lysates onto a nitrocellulose membrane (0.7 A /100 V, two hours, RT), immune detection was accomplished on the membranes that were first colored with Ponceau S (AppliChem GmbH, Darmstadt, Germany). Separation of the membrane was performed from left to right at 25 kDa and 35 kDa. This was followed by further incubation with 5 % non-fat dry milk in 10 ml PBS-T (phosphate-buffered saline with Tween 20) for two hours at RT. TSPO was detected with a rabbit-monoclonal-anti-TSPO antibody (Davids Biotech, Regensburg, Germany) (Milenkovic et al., 2018) and ß-actin with a rabbit-anti-ß-actin (Sigma-Aldrich) overnight at 4°C. On the following day, the blots were washed with PBS-T three times for ten minutes, followed by incubation with secondary horseradish peroxidase-conjugated antibodies for one hour at RT and again washed 3 x 10 minutes with PBS-T.

Bands were detected using chemiluminescence with a digital imaging system (Image Quant LAS 4000, GE Healthcare Europe, Freiburg, Germany). Densitometric analyses were

performed with ImageJ Software (Wayne Rasband, National Institute of Health, USA). TSPO values were normalized to ß-actin values of the same sample and to an untreated control sample.

3.3.2.5 Possible covariates and manipulation check

TSPO gene polymorphism rs6971

The TSPO gene polymorphism rs6971 was only determined for the subjects of the etifoxine group using 4 ml full blood of the screening day when all participants were unmedicated. The samples were frozen at -80 ° C until further analysis.

After extraction of genomic DNA from the whole blood samples with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), quality was assessed with optical absorbance and gel electrophoresis. Following, polymerase chain reaction was used to amplify exon 4 of the TSPO gene containing the polymorphism rs6971 (alanine or threonine at position 147). After that, sequencing was accomplished according to the Sanger method (Sanger, Nicklen, & Coulson, 1977) with the following primers: hTSPO-exon4-F (5'-AGT TGG GCA GTG GGA CAG-3'), hTSPO-exon 4-R (5'-GCA GAT CCT GCA GAG ACG A-3') (Metabion, Planegg, Germany). The respective data were analyzed using SnapGene software (GSL Biotech, Chicago, IL, USA).

Stress Coping Questionnaire (SVF 120)

The Stress Coping Questionnaire (Erdmann & Janke, 2008) assesses coping strategies in demanding situations, which were shown to be related to the cortisol response within the Trier Social Stress Test (Janson & Rohleder, 2017). A trait version, the SVF-120, includes 120 items that resemble 20 different strategies (e.g. trivialization, distraction, flight) to cope with being affected, aroused or upset by someone or something. Subjects completed this form at the screening day stating on a 5-point scale from 0 = not at all (*gar nicht*) to 4 = most likely (*sehr wahrscheinlich*) how strongly the respective strategy resembles their usual reaction. Item scores were summed up to positive and negative coping strategies. Internal consistency of the normative sample was about $\alpha = .95$ for both the positive and negative strategies, while retest-reliabilities were .84 and .82, respectively.

Brief Fear of Negative Evaluation Scale (BFNE)

To assess the experience of distress and fear in relation to being negatively evaluated by others, we administered the German version (Reichenberger et al., 2015) of the Brief Fear of Negative Evaluation Scale-Revised (BFNE-R) (Carleton, McCreary, Norton, & Asmundson,

2006). It consists of 12 items (e.g., “I am afraid that others will not approve of me”; German: “*Ich habe Angst, dass andere sich nicht positiv über mich äußern*”) that are rated on a 5-point Likert-scale from 1 = not at all characteristic of me (*überhaupt nicht charakteristisch für mich*) to 5 = extremely characteristic of me (*äußerst charakteristisch für mich*). For evaluation, we computed the sum score, which ranges from 12 to 60 with greater fear of negative evaluation indicated by a higher score. The scale shows very good internal consistency (α = .94) and an acceptable 2-week test-retest reliability.

Self-Statements During Public Speaking Scale (SSPS)

The Self-Statements During Public Speaking (SSPS) scale (Hofmann & DiBartolo, 2000) is based on ten items with one half related to positive self-statements (e. g. “I can handle everything”; German: “*Ich kann mit allem umgehen*”) and the other half to negative self-statements (e. g. “I’m a loser”; German: “*Ich bin ein Versager*”). Subjects should imagine a typical public speech situation and rate on a scale from 0 = do not agree at all (*stimme überhaupt nicht zu*) to 5 = agree extremely (*stimme sehr zu*) how much they agree with the different statements. Internal consistency is acceptable with Cronbach’s α = .75 for the positive scale and good with α = .86 for the negative statements. We administered this questionnaire after the VR-TSST and not at the screening day to avoid prior anticipation of the speaking task.

Igroup Presence Questionnaire (IPQ)

The Igroup Presence Questionnaire (IPQ) (Schubert, 2003) retrospectively measures the feeling of being present in an virtual reality environment using 14 items that are stated on a seven-point scale ranging from -3 to +3 (transformed to range from 1 to 7 for further analyses). It comprises four subscales assessing the following aspects: spatial presence (SP; feeling of physically being in the VR), involvement (I; attention paid to the VR), experienced realism (ER; subjectively experienced reality) and one item assessing the sense of being there (G). All scales show good internal consistency with Cronbach’s α of .80 for spatial presence, .76 for involvement, .68 for experienced realism and .85 for sense of being there.

3.3.3 Procedure

To control for circadian fluctuations of cortisol release (Kudielka, Hellhammer, & Wüst, 2009) and standardize the time interval between medication intake and the stress test, we scheduled the start of this paradigm around 1 PM for every participant. After arrival in the

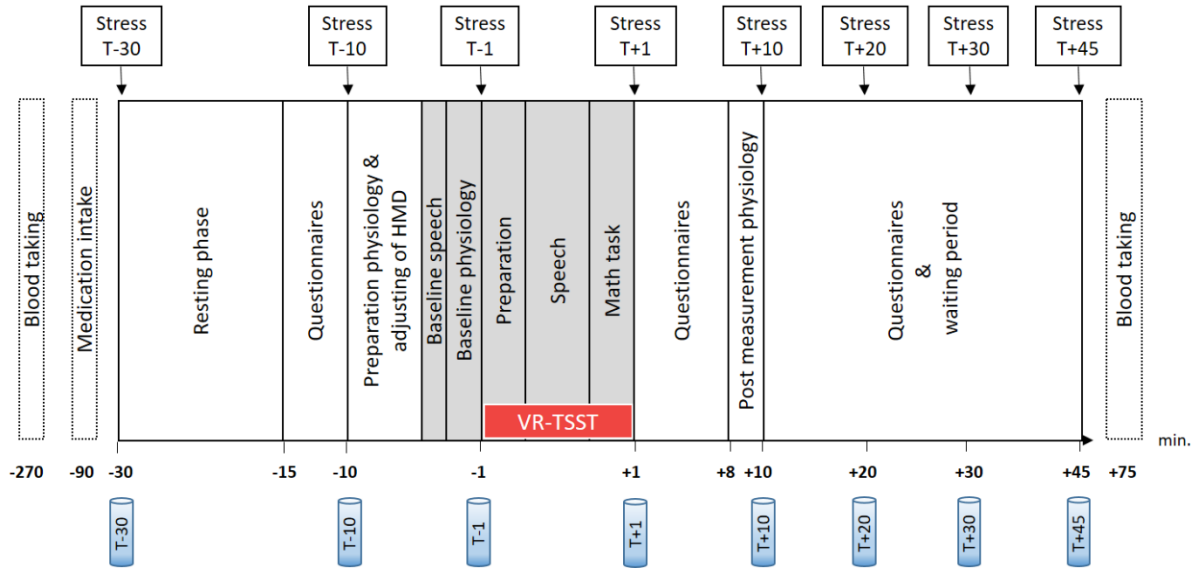
laboratory, participants gave a first saliva sample (T-30) and rated their subjective stress level. Following, they were guided into another room where they sat alone in rest for 15 minutes without handling their phones or doing any other arousing activity. Every participant was provided the same neutral reading material (HÖRZU Wissen; issue 2, April / May 2018). From that time point, subjects were asked not to drink anything more until the end of the experiment to avoid dilution of the saliva samples.

At the end of this resting phase, participants completed the STAI state and the VAS, gave a second saliva sample (T-10) and stated their current stress level. After attachment of the physiological measurements and adjustment of the HMD, we started the virtual reality and took the baseline voice recording that further served as a sight test for the virtual environment. This was followed by a baseline recording of heart rate and skin conductance during which participants stood quiet for a period of two minutes. Before the start of the stress test in virtual reality (see 3.3.1), we took another saliva sample (T-1) and assessed the subjective stress level.

At the end of the task, participants rated their stress level and stated how present they had felt in the virtual reality on a scale from 0 = not present at all to 100 = very present. After removing the HMD, another saliva sample (T+1) was taken. The participants remained in the laboratory for further 45 minutes and completed the STAI state and the VAS for a second time as well as the IPQ and the SSPS. Seven minutes after the end of the stress task, participants were asked to interrupt the questionnaires and get up for the post measurement of the physiological parameters from minute 8 to 10 after the stress test. After that, they finished the questionnaires, gave further ratings of their actual subjective stress level and saliva samples (T+10, T+20, T+30, T+45). For an overview of the procedure see figure 7.

Figure 7

Experimental Procedure of the VR-TSST



Note. Experimental procedure showing the key time points surrounding the VR-TSST including the assessment of the subjective stress level (upper part) and saliva samples (lower part). Experimental parts in virtual reality are marked in grey.

3.3.4 Statistical analysis

Statistical analysis was performed for n = 18 of the etifoxine group, n = 19 of the alprazolam group and n = 20 of the placebo group. One participant could not perform the stress task because of technical problems with the virtual reality system. For two subjects there was an extreme time delay or heavy sight distortions, respectively, due to technical issues. For the heart rate analysis, we further excluded one subject of the alprazolam group and one from the placebo group due to issues with the measurement. Likewise, for the electrodermal measures, one participant from the etifoxine group, two from the alprazolam group and three from the placebo group were excluded. Because of extreme background noise in the baseline measurement, we had to exclude two subjects for the complete analysis of the voice data. One participant of the etifoxine group was additionally discarded from the cortisol analyses because of single missing values.

Analyses concerning the formulated hypotheses were conducted using the statistics software SPSS (version 25, IBM Statistics) with a significance level at α = .05 for all analyses. In accordance to recommendation guidelines we log-transformed the skin conductance data (Boucsein et al., 2012) as well as the cortisol values (R. Miller & Plessow, 2013) before analysis. For TSPO expression we computed percent changes with respect to the values of the screening day for all of the four treatment days.

Comparisons of the three groups with regard to the psychological trait measurements (trait anxiety, stress coping, fear of negative evaluation, self-statements related to public speaking) as well as for the statements concerning presence in the virtual reality were carried out using univariate analyses of variance (ANOVA) with treatment as between subjects factor.

For the analyses concerning the subjective, physiological and endocrine measurements, repeated measures ANOVAs were calculated including the between-subjects factor treatment and the within-subjects factor time. The number of levels of the factor time varied for the different variables. For the subjective stress rating and the cortisol level we included eight time points into the analysis (T-30, T-10, T-1, T+1, T+10, T+20, T+30, T+45). For the other subjective ratings, the voice parameters as well as TSPO expression and allopregnanolone level the within subjects factor time comprised two levels (pre-stress, post-stress). To check for changes of heart rate and skin conductance level in response to the stress task we included five levels (BL_spoken, preparation, speech, math, post).

Beforehand, we tested for homogeneity of variances using Levene's tests. In case of violation of sphericity indicated by a significant p-value of the Mauchly-test, we report Greenhouse-Geisser (GG) corrected values. Effect sizes are reported as partial eta ($\eta 2$). In case of significant main effects or interactions yielded by the ANOVA, we computed post-hoc t-tests or univariate ANOVAs to determine differences between the single groups or time points. To account for multiple comparisons and the concomitant accumulation of the alpha error, Bonferroni correction was applied.

For cortisol, we further checked if the three groups differed with respect to overall "cortisol response" defined as an increase of at least 1.5 nmol / l in response to the VR-TSST (R. Miller, Plessow, Kirschbaum, & Stalder, 2013). This was accomplished using $\chi 2$-tests according to Pearson.

To check for covariation between the most common stress parameters, we computed Pearson correlation analyses including salivary cortisol (log-transformed), subjective stress level, heart rate, skin conductance level (log-transformed), and allopregnanolone level separately for the three groups. Therefore, we identified the maximum increase (baseline to peak) values of the included parameters in response to the VR-TSST individually for each subject. As baseline values for each participant we chose T-1 for cortisol, T-10 for the subjective stress level, BL_spoken for heart rate and skin conductance level, and T-270 for allopregnanolone. The peak values were identified as the highest values for each single participant from the following time points: T+1, T+10, T+20 or T+30 for cortisol, T+1 for

subjective stress level, preparation, speech or math for heart rate and skin conductance, and T+75 for allopregnanolone.

To analyze objective performance throughout the VR-TSST, we computed univariate ANOVAs for each of the three parameters (math_correct, math_false rate, speech_duration) including treatment as between subjects factor.

To examine the impact of the TSPO polymorphism, we repeated analyses for the subjective stress level, heart rate, skin conductance as well as the molecular markers after exclusion of participants of the etifoxine group that were homozygous for the polymorphism.

3.4 Results

First, a description of the trait variables relevant for the analysis of the stress parameters is given. This is followed by the results concerning the effects of etifoxine and alprazolam on psychological, physiological and endocrine measures of the stress reaction. Further analyses included correlations between the different stress related parameters, objective performance markers and the influence of the TSPO gene polymorphism rs6971.

3.4.1 Descriptives

The three groups did not differ concerning trait anxiety, positive / negative coping strategies or fear of negative evaluation measured before intake of the study medication. Furthermore, there were no group differences concerning negative or positive self-statements during public speaking assessed after the stress task (see table 7).

Table 7

Means, Standard Deviations and Statistics for the Psychometric Trait Variables Related to Psychosocial Stress

Scale	Placebo	Alprazolam	Etifoxine	Statistics		
	M ± *SD*	*M* ± *SD*	*M* ± *SD*	*F* ratio	*df*	*p*
STAI trait	34.63 ± 6.60	32.65 ± 6.84	30.70 ± 5.55	1.87	2,57	.164
SVF-120 pos.	12.77 ± 3.12	12.06 ± 2.72	14.06 ± 3.16	2.27	2,57	.112
SVF-120 neg.	7.83 ± 4.19	6.60 ± 3.55	6.47 ± 2.15	0.98	2,57	.382
BFNE	30.35 ± 6.79	31.85 ± 9.46	31.10 ± 8.0	0.17	2,57	.845
SSPS neg.	5.45 ± 6.0	6.79 ± 4.84	6.15 ± 4.90	0.31	2,56	.732
SSPS pos.	16.45 ± 4.63	16.32 ± 3.40	17.05 ± 4.30	0.18	2,56	.840

Note. STAI = State-Trait-Anxiety-Inventory, SVF-120 = Stress Coping Questionnaire, BFNE = Brief Fear of Negative Evaluation Scale, SSPS = Self-Statements during Public Speaking Scale.

The extent to which the subjects felt present or immersed in the virtual reality, experienced realism or had the sense of being in the virtual reality during the VR-TSST did also not differ between the groups (see table 8).

Table 8

Means, Standard Deviations and Statistics for the Measures of Presence in the Virtual Reality

Scale	Placebo	Alprazolam	Etifoxine	Statistics		
	M ± *SD*	*M* ± *SD*	*M* ± *SD*	*F* ratio	*df*	*p*
IPQ_SP	4.34 ± .48	4.25 ± .46	4.34 ± .41	0.24	2,56	.787
IPQ_I	4.09 ± .45	3.95 ± .65	3.90 ± .45	0.68	2,56	.513
IPQ_ER	3.49 ± .59	3.46 ± .57	3.73 ± .54	1.28	2,56	.286
IPQ_G	4.70 ± .73	4.53 ± .77	4.75 ± .72	0.49	2,56	.617

Note. IPQ = Igroup Presence Questionnaire (SP = spatial presence, I = involvement, ER = experienced realism, G = general sense of being in the virtual reality).

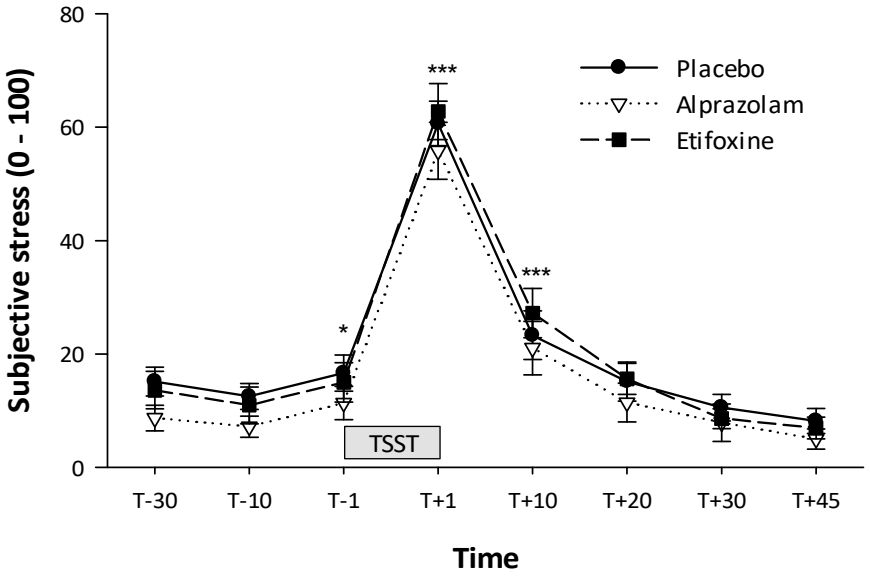
3.4.2 Subjective ratings of stress, anxiety and sedation

The subjective stress level, which was rated by the participants at eight time points significantly changed across the stress task, $F_{3.14,169.68} = 172.21$, $p < .001$, $\eta_p^2 = .76$. Follow-up tests showed that the following time points were marked by a significant increase of subjective stress in comparison to the baseline value that was taken directly after the resting phase (T-10): T-1 (-4.12, 95%-CI[-7.77, -.50], $p = .014$), T+1 (-49.54, 95%-CI[-58.14, -40.93], $p < .001$) and T+10 (-13.64, 95%-CI[-21.59, -5.70], $p < .001$) (see figure 8 for an overview).

The subjective stress level did not significantly differ between the three groups, $F_{2,54} = 0.92$, $p = .404$, $\eta_p^2 = .03$, and the changes in subjective stress throughout the task were not affected by treatment as there was no significant interaction between time and treatment, $F_{6.28,169.68} = 0.35$, $p = .916$, $\eta_p^2 = .01$.

Figure 8

Subjective Stress in Response to the VR-TSST

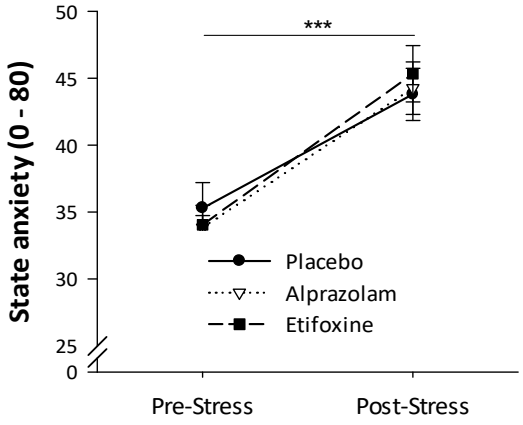


Note. Overview of group differences concerning the subjective stress level (range 0 – 100) in response to the VR-TSST. Error bars show standard errors. Asterisks mark significant difference to T-10, *$p < .05$, **$p < .01$, ***$p < .001$.

State anxiety measured using the STAI questionnaire significantly increased in response to the task, $F_{1,54} = 85.60$, $p < .001$, $\eta_p^2 = .61$ (see figure 9). However, there was neither a general effect of the treatment, $F_{2,54} = 0.05$, $p = .951$, $\eta_p^2 = .002$, nor did the study medication interact with the increase of state anxiety due to the VR-TSST, $F_{2,54} = 0.58$, $p = .577$, $\eta_p^2 = .02$.

Figure 9

State Anxiety in Response to the VR-TSST



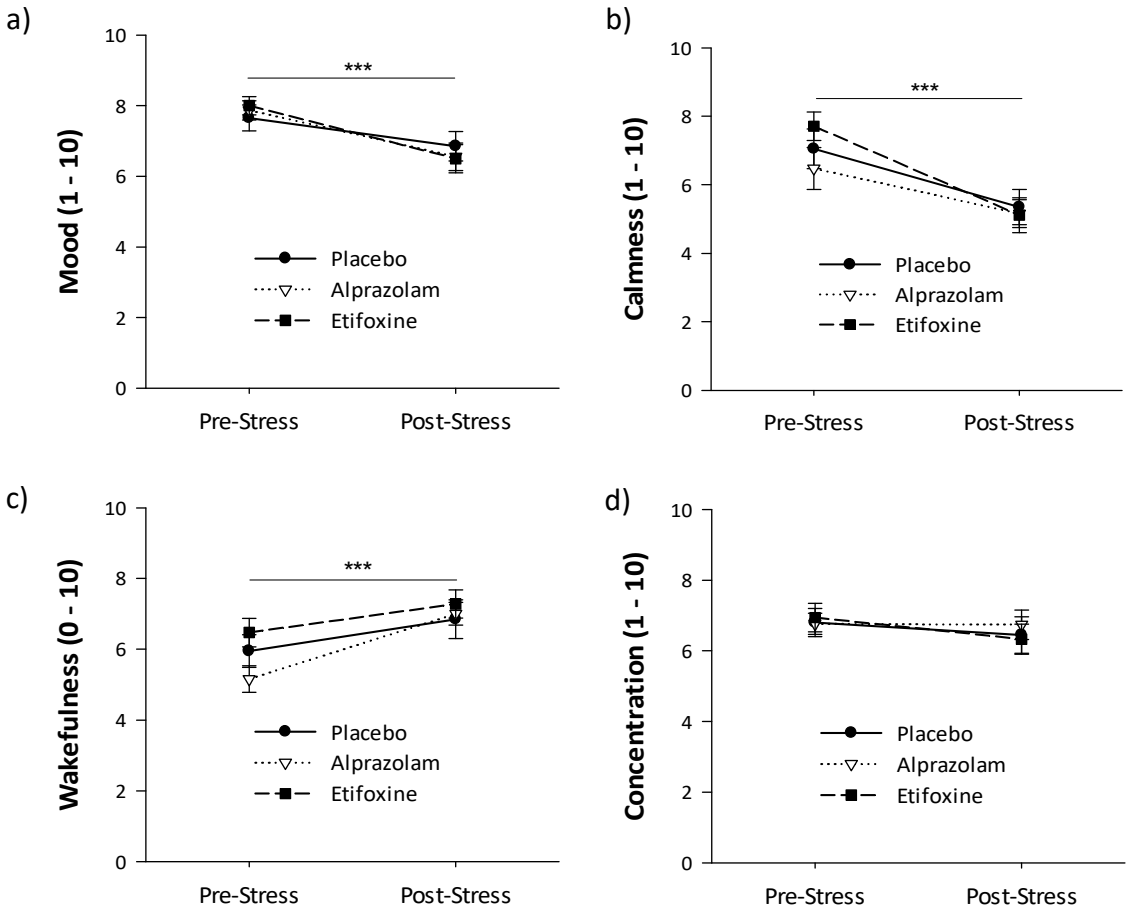
Note. Overview of group differences concerning the state anxiety (STAI sum score: range 20 – 80) in response to the VR-TSST. Error bars show standard errors. ***$p < .001$.

Analyses for parameters on the mental state assessed by visual analogue scales revealed significantly worse mood, $F_{1,53} = 20.33$, $p < .001$, $\eta_p^2 = .28$, and higher arousal after the task, $F_{1,53} = 29.22$, $p < .001$, $\eta_p^2 = .36$, accompanied by an increase of wakefulness, $F_{1,53} = 26.67$, $p < .001$, $\eta_p^2 = .34$, and anxiety, $F_{1,53} = 30.50$, $p < .001$, $\eta_p^2 = .37$. Concerning concentration, no significant changes were yielded, $F_{1,53} = 1.98$, $p = .165$, $\eta_p^2 = .04$ (see also figure 10).

These parameters were not affected by the treatment: mood, $F_{2,53} = 0.02$, $p = .979$, $\eta_p^2 = .001$, calmness, $F_{2,53} = 0.42$, $p = .658$, $\eta_p^2 = .02$, wakefulness, $F_{2,53} = 0.90$, $p = .414$, $\eta_p^2 = .03$, anxiety, $F_{2,53} = 0.22$, $p = .807$, $\eta_p^2 = .01$, and concentration, $F_{2,53} = 0.06$, $p = .946$, $\eta_p^2 = .002$. There were further no significant interactions between time and treatment for any of the variables: mood, $F_{2,53} = 0.55$, $p = .583$, $\eta_p^2 = .02$, calmness, $F_{2,53} = 1.32$, $p = .276$, $\eta_p^2 = .05$, wakefulness, $F_{2,53} = 2.48$, $p = .093$, $\eta_p^2 = .09$, anxiety, $F_{2,53} = 0.94$, $p = .399$, $\eta_p^2 = .03$, and concentration, $F_{2,53} = 0.57$, $p = .572$, $\eta_p^2 = .02$.

Figure 10

Subjective Responses to the VR-TSST



Note. Overview of group differences concerning for the VAS scales (all range 0 – 10): mood (a), calmness (b), wakefulness (c) and concentration (d) in response to the VR-TSST. Error bars show standard errors. ***$p < .001$.

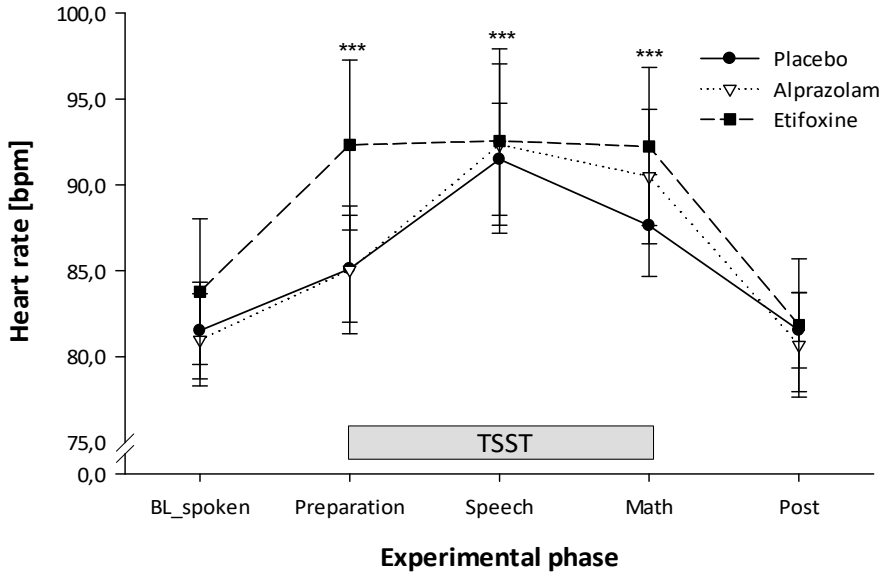
3.4.3 Physiological stress parameters

Heart Rate (HR)

There was a significant increase of heart rate in response to the stress task, $F_{2.70,140.14} = 34.41$, $p < .001$, $\eta_p^2 = .40$. Follow-up tests revealed an increased heart rate during the preparation phase (-5.68, 95%-CI[-8.37, -2.98], $p < .001$), the speech in front of the committee (-10.39, 95%-CI[-14.43, -6.35], $p < .001$) and the math task (-8.36, 95%-CI[-11.66, -5.06], $p < .001$) in comparison to the baseline, while there was no significant difference for the post resting measurement (.74, 95%-CI[-2.30, 3.78, $p = 1.0$) (see figure 11). There was no general effect of treatment on the heart rate, $F_{2,52} = 0.15$, $p = .865$, $\eta_p^2 = .01$, and the changes throughout the experiment were not affected by the anxiolytic substances, $F_{5.39,140.14} = 1.30$, $p = .263$, $\eta_p^2 = .05$.

Figure 11

Heart Rate in Response to the VR-TSST



Note. Overview of group differences for heart rate (bpm) in response to the VR-TSST. Error bars show standard errors. Asterisks mark significant difference to BL_spoken, ***$p < .001$.

Skin conductance level (SCL)

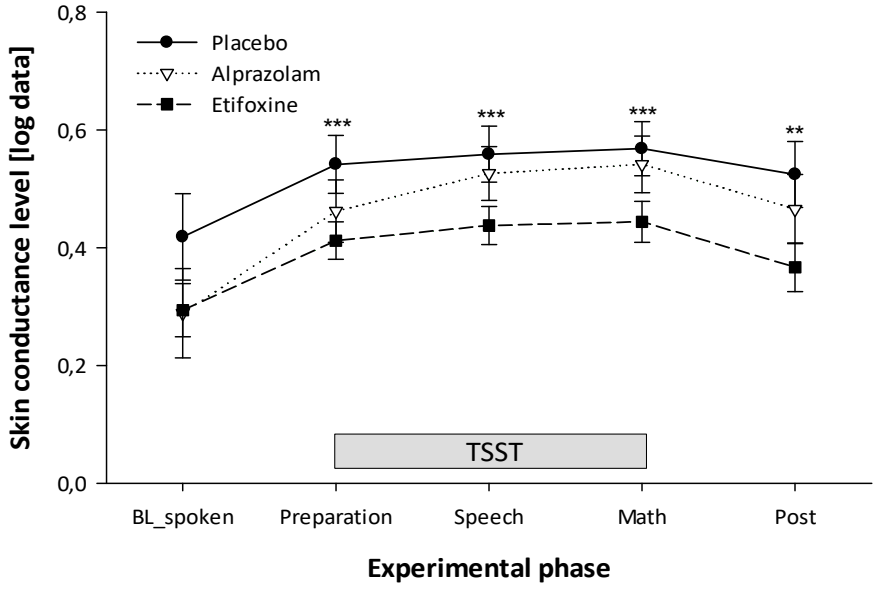
Tonic electrodermal activity was significantly affected by the psychosocial stress induction, $F_{1.41,67.72} = 27.74$, $p < .001$, $\eta_p^2 = .37$. Follow-up tests for the different segments yielded differences between the baseline measurement and all other experimental phases: preparation (-.14, 95%-CI[-.22, -.06], $p < .001$), speech (-.18, 95%-CI[-.26, -.09], $p < .001$),

math task (-.20, 95%-CI[-.27, -.10], $p < .001$), and post resting measurement (-.12, 95%-CI[-.21, -.03], $p = .003$) (see figure 12).

However, neither general skin conductance was significantly affected by the treatment, $F_{2,48} = 1.87$, $p = .165$, $\eta_p^2 = .07$, nor were the stress-induced changes indicated by a missing significant interaction between time and treatment, $F_{2.82,67.72} = 1.12$, $p = .347$, $\eta_p^2 = .04$.

Figure 12

Skin Conductance Level in Response to the VR-TSST



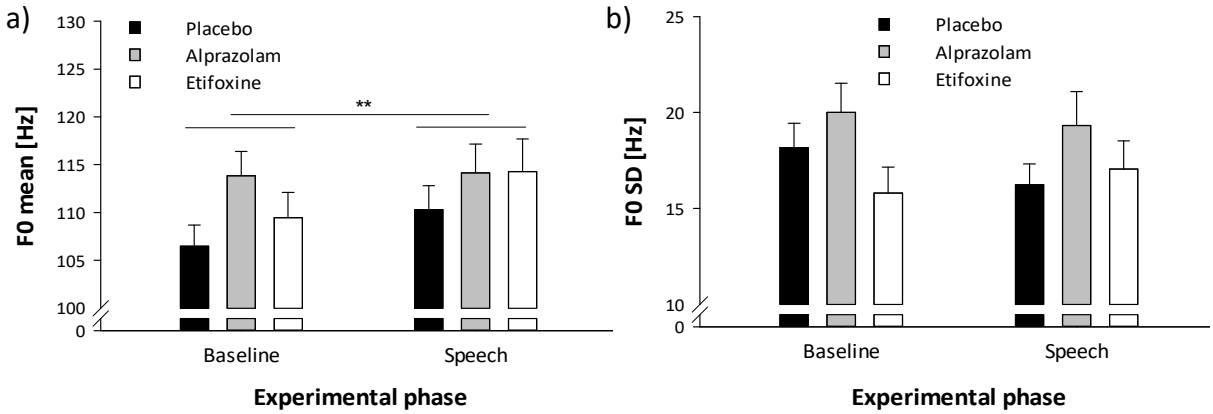
Note. Overview of group differences for skin conductance level changes (log data) in response to the VR-TSST. Error bars show standard errors. Asterisks mark significant difference to BL_spoken, $^{**}p < .01$, $^{***}p < .001$.

Vocal indices

While F0 *Mean* significantly increased in response to the stress task, $F_{1,52} = 9.84$, $p = .003$, $\eta_p^2 = .16$, there was no significant main effect of experimental phase for F0 *SD,* $F_{1,52} = 0.37$ $p = .546$, $\eta_p^2 = .007$ (see figure 13). Neither was there an effect of treatment on F0 *Mean,* $F_{2,52} = 1.18$, $p = .315$, $\eta_p^2 = .04$, nor for F0 *SD,* $F_{2,52} = 1.75$, $p = .184$, $\eta_p^2 = .06$. Furthermore, we found no significant interaction between experimental phase and treatment for F0 *Mean,* $F_{2,52} = 2.06$, $p = .138$, $\eta_p^2 = .07$, or F0 *SD,* $F_{2,52} = 1.58$, $p = .216$, $\eta_p^2 = .06$.

Figure 13

F0 Mean and SD in Response to the VR-TSST

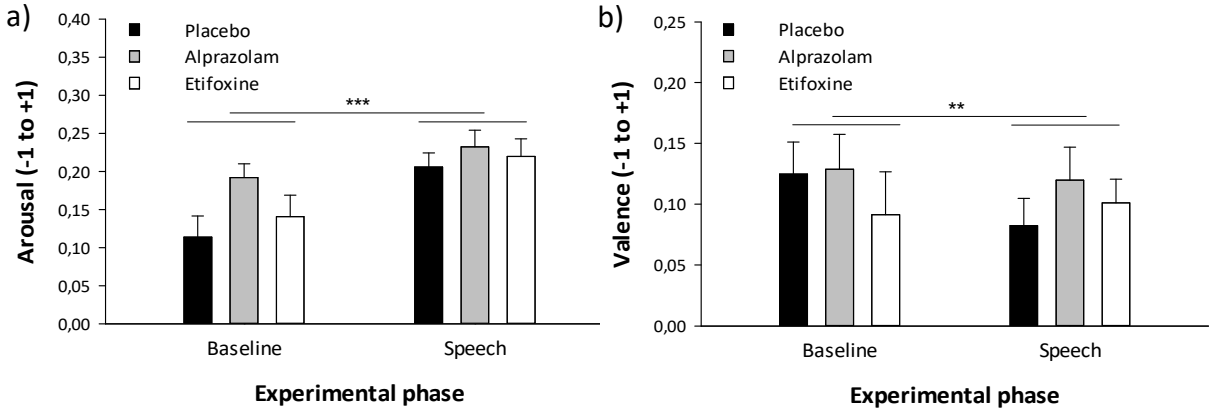


Note. Overview of group differences for changes in voice parameters from the spoken baseline to the speech in front of the committee: F0 *Mean* (a) and F0 *SD* (b) (both in Hz) in response to the VR-TSST. Error bars show standard errors. ***p* < .01.

The voice-based algorithm identified higher arousal during the stressful speech in comparison to baseline, $F_{1,52} = 34.62$, $p < .001$, $\eta_p^2 = .40$, while valence was shown more negative during stress, $F_{1,35} = 7.59$, $p = .009$, $\eta_p^2 = .18$ (see figure 14). There was neither an effect of treatment on arousal, $F_{2,52} = 1.60$, $p = .212$, $\eta_p^2 = .06$, nor for valence, $F_{2,35} = 0.05$, $p = .952$, $\eta_p^2 = .003$. Furthermore, we found no significant interaction between time and treatment for arousal, $F_{2,52} = 1.67$, $p = .198$, $\eta_p^2 = .06$, and valence, $F_{2,35} = 2.14$, $p = .132$, $\eta_p^2 = .11$.

Figure 14

Arousal and Valence Measured in Voice in Response to the VR-TSST



Note. Overview of group differences for voice-based classifications from the spoken baseline to the speech in front of the committee: arousal (a) and valence (b) (both range -1 to 1) in response to the VR-TSST. Error bars show standard errors. ***p* < .01, ****p* < .001.

3.4.4 Endocrine stress- and anxiety-related parameters

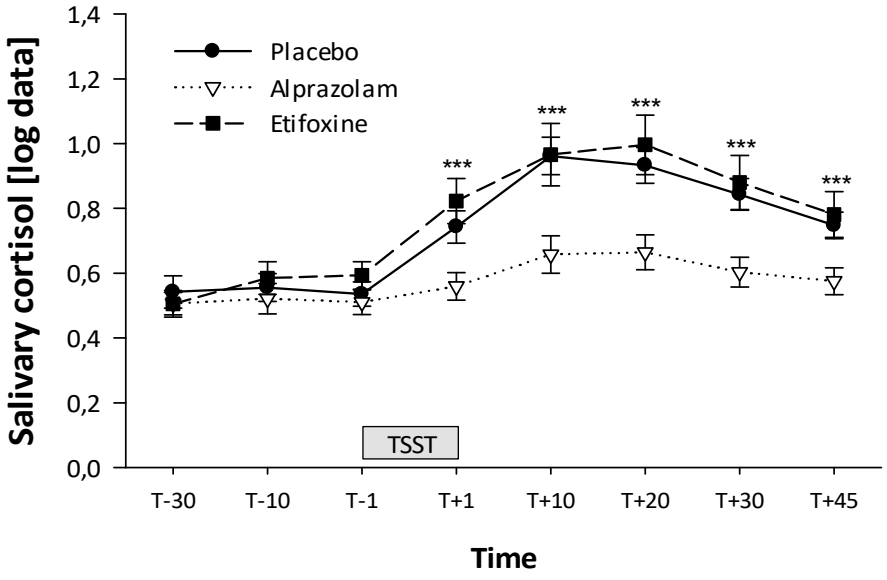
Salivary cortisol

Cortisol release was shown to change significantly across the experiment, $F_{1.85,98.23}$ = 56.59, $p < .001$, $\eta_p^2 = .52$. Follow-up tests showed a higher cortisol level for all time points after the end of the stress test compared to the baseline taken directly after the resting phase (T-10): T+1 (-.16, 95%-CI[-.25, -.06], $p < .001$), T+10 (-.32, 95%-CI[-.45, -19], $p < .001$), T+20 (-.32, 95%-CI[-.45, -.19], $p < .001$), T+30 (-.23, 95%-CI[-.34, -.11], $p < .001$) and T+45 (-.15, 95%-CI[-.24, -.06], $p < .001$) (see figure 15).

Treatment significantly affected overall cortisol release, $F_{2,53} = 5.69$, $p = .006$, $\eta_p^2 = .18$. Follow-up tests revealed significant differences between alprazolam and placebo (-.16, 95%-CI[-.31, -.002], $p = .046$) and between alprazolam and etifoxine (-.21, 95%-CI[-.37, -.05], $p = .007$). This finding was reinforced by a significant interaction between time and treatment, $F_{3.71,98.23} = 5.02$, $p = .001$, $\eta_p^2 = .16$. Follow-up tests showed that alprazolam and etifoxine differed from time point T+1 (-.26, 95%-CI[-.46, -.07], $p = .004$), while the difference between alprazolam and placebo got significant from time point T+10 (-.30, 95%-CI[-.55, -.06], $p = .011$).

Figure 15

Salivary Cortisol in Response to the VR-TSST



Note. Overview of group differences concerning the cortisol level measured in saliva (log data) in response to the VR-TSST. Error bars show standard errors. Asterisks mark significant difference to T-10, ***$p < .001$.

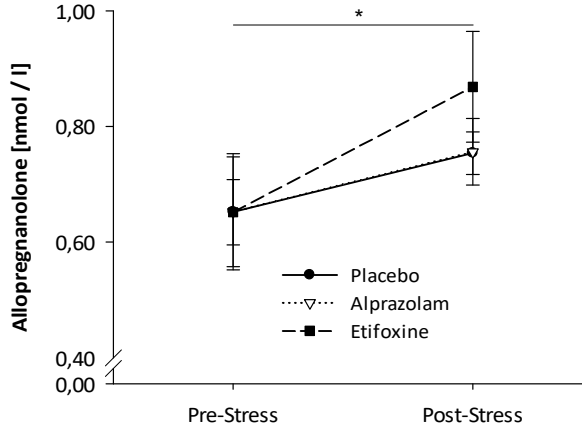
Maximal cortisol values were reached 20 minutes after the VR-TSST in the etifoxine group (M = 13.84 nmol / l) as well as in the alprazolam group (M = 4.36 nmol / l), while for the placebo group (M = 9.86 nmol /l) the maximum was reached ten minutes after the stress task. The χ2-tests to check for cortisol responders according to the 1.5 nmol / l criterion showed that the distribution of responders and non-responders differed significantly between the groups, χ2 = 11.26, p = .004. In the placebo group n = 17 subjects were revealed as responders, while in the etifoxine group we found n = 13 cortisol responders and in the alprazolam group n = 7.

Allopregnanolone

There was a significant increase of the allopregnanolone level due to the VR-TSST, $F_{1,54}$ = 5.12, p = .028, η_p^2 = .09 (see figure 16). However, there were no significant differences between the three treatment groups for the overall allopregnanolone level, $F_{2,54}$ = 0.33, p = .718, η_p^2 = .01, nor was the change of allopregnanolone due to the VR-TSST affected by the active substances as there was no significant interaction between experimental phase and treatment, $F_{2,54}$ = 0.37, p = .694, η_p^2 = .01.

Figure 16

Serum Allopregnanolone in Response to the VR-TSST



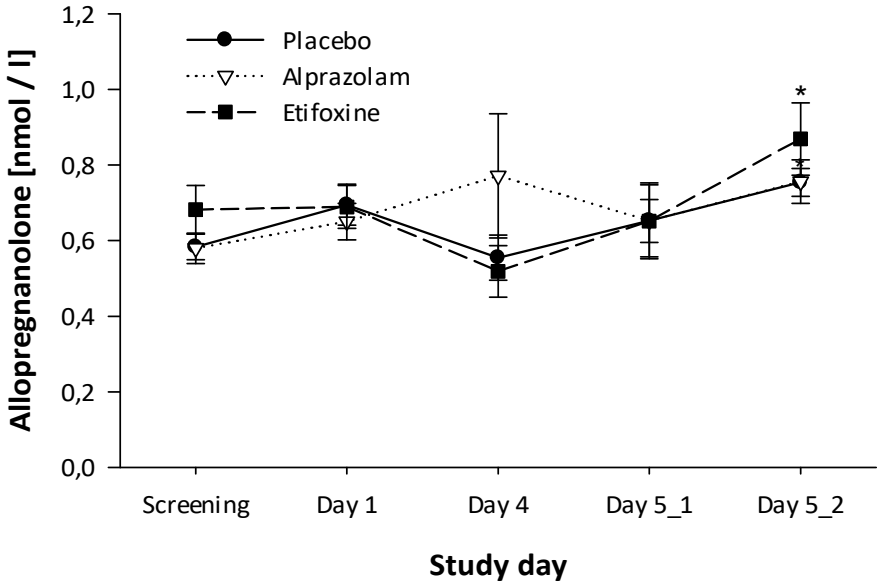
Note. Overview of group differences concerning the allopregnanolone level measured in blood serum (nmol / l) in response to the VR-TSST. Error bars show standard errors. *p < .05.

The allopregnanolone level significantly changed across the study, $F_{2.84,150.59}$ = 3.72, p = .014, η_p^2 = .07 (see figure 17). Follow-up tests showed a significant difference in comparison to the screening day only for day 5 of treatment (-.19, 95%-CI[-.31, -.08], p < .001).

There were no significant differences between the three treatment groups for the overall allopregnanolone level, $F_{2,53} = 0.25$, $p = .783$, $\eta_p^2 = .009$, nor was the change of allopregnanolone due over the study days affected by the treatment as there was no significant interaction between study day and treatment, $F_{5.86,150.59} = 1.26$, $p = .279$, $\eta_p^2 = .05$.

Figure 17

Serum Allopregnanolone over the Course of the Study



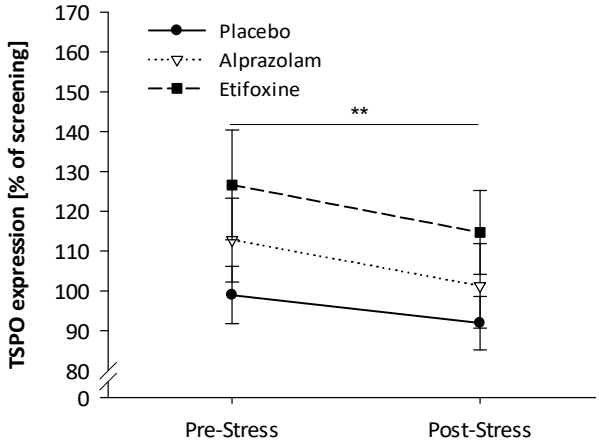
Note. Overview of group differences concerning the allopregnanolone level measured in blood serum (nmol / l) over the course of the study. Error bars show standard errors. Asterisks mark significant difference to the screening, *$p < .05$.

TSPO expression

TSPO expression in platelets significantly decreased in response to the VR-TSST, $F_{1,52} = 7.36$, $p = .009$, $\eta_p^2 = .12$ (see figure 18). However, there were no significant differences between the three groups for the overall expression of TSPO, $F_{2,52} = 1.82$, $p = .173$, $\eta_p^2 = .07$, nor was the change of TSPO expression due to the acute psychosocial stress affected by the active substances as there was no significant interaction between experimental phase and treatment, $F_{2,52} = 0.18$, $p = .833$, $\eta_p^2 = .007$.

Figure 18

TSPO Expression in Platelets in Response to the VR-TSST

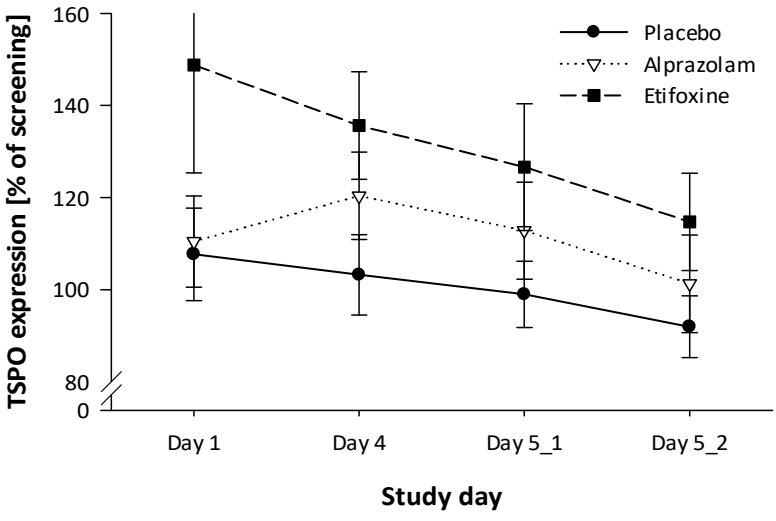


Note. Overview of group differences concerning the TSPO expression in blood platelets (% of screening) in response to the VR-TSST. Error bars show standard errors. $^{**}p < .01$.

TSPO expression did not significantly change across the complete study duration, $F_{1,.56,\ 80.87} = 2.92$, $p = .07$, $\eta_p^2 = .05$ (see figure 19). There was a significant group difference for the overall expression of TSPO, $F_{2,52} = 3.43$, $p = .04$, $\eta_p^2 = .12$. Follow-up tests showed a difference between etifoxine and placebo (30.98, 95%-CI[1.47, 60.50], $p = .037$) but not between alprazolam and placebo (10.78, 95%-CI[-18.29, 39.85], $p = 1.0$) or alprazolam and etifoxine (-20.21, 95%-CI[-50.47, 10.05], $p = .314$). However, there was no significant interaction between study day and treatment for TSPO expression, $F_{3.11,80.87} = 0.50$, $p = .70$, $\eta_p^2 = .02$.

Figure 19

TSPO Expression in Platelets over the Course of the Study



*Note***.** Overview of group differences concerning the TSPO expression in blood platelets (% of screening) over the course of the study. Error bars show standard errors.

3.4.5 Further analyses

3.4.5.1 Correlation between subjective, physiological and endocrine stress markers

As there were inhibiting effects of alprazolam on the release of cortisol but no effects on any of the other parameters, we wanted to check for the covariation of the single stress parameters in the three treatment groups to see if dissociations have arisen due to the anxiolytic treatment. Thereby, correlation analyses for the most common stress markers yielded different results for the three groups. Only in the placebo group there was a positive significant correlation between subjective stress and heart rate (r = .539, p = .021). In the etifoxine group the physiological parameters heart rate and skin conductance level showed a contrary course in response to the stress indicated by a negative correlation (r = -.601, p = .011). While there was a covariation between the stress-induced change of cortisol level and heart rate in the alprazolam group (r = .489, p = .047) and even more strongly in the placebo group (r = .708, p = .001), no considerable correlation of these markers was shown for the etifoxine group. In the placebo group there was further a negative correlation between allopregnanolone and cortisol changes due to the stress task (r = -.607, p = .008). (see also table 9).

Table 9

Correlations of Stress-Induced Changes of Subjective Stress, Heart Rate, Skin Conductance, Cortisol, and Allopregnanolone

		1.	2.	3.	4.	5.
1. Subjective stress	Placebo	-				
	Alprazolam	-				
	Etifoxine	-				
2. Heart rate	Placebo	**.539***	-			
	Alprazolam	.013	-			
	Etifoxine	.194	-			
3. Skin conductance	Placebo	-.012	.201	-		
	Alprazolam	.021	.043	-		
	Etifoxine	-.368	**-.601***	-		
4. Salivary cortisol	Placebo	.356	**.708****	-.064	-	
	Alprazolam	-.040	**.489***	-.218	-	
	Etifoxine	.114	.435	.039	-	
5. Allopregnanolone	Placebo	-.465	-.380	.177	**-.607****	-
	Alprazolam	-.275	.264	-.357	.210	-
	Etifoxine	.028	.154	-.058	.245	-

Note. *p < .05, **p< .01.

3.4.5.2 Objective performance

Univariate ANOVAs to assess the effects of the anxiolytic treatment on the performance during the stress task in front of the committee yielded no significant effects for any of the objective parameters including the number of correctly stated numbers during the math task (math_correct), the proportion of mistakes (math_false rate) and the total duration of spoken segments during the speech (speech_duration) (see table 10).

Table 10

Means, Standard Deviations and Statistics for the Objective Performance Parameters of the VR-TSST

Scale	Placebo	Alprazolam	Etifoxine	Statistics		
	M ± *SD*	*M* ± *SD*	*M* ± *SD*	*F* ratio	*df*	*p*
Math_correct	16.42 ± 20.65	12.63 ± 6.46	13.88 ± 6.92	0.36	2,49	.701
Math_false rate	17.09 ± 12.23	17.85 ± 14.24	17.36 ± 11.62	0.02	2,49	.984
Speech_duration	166.71 ± 50.24	175.09 ± 68.79	161.02 ± 46.06	0.27	2,49	.766

Note. Math_correct = number of correctly stated numbers (*n*), math_false rate = proportion of false answers (%), speech_duration = total time of spoken segments during the speech (s).

3.4.5.3 TSPO polymorphism

We repeated the analyses for the subjective stress level, the physiological parameters heart rate and skin conductance level as well as the endocrine markers salivary cortisol, allopregnanolone and TSPO expression after exclusion of three participants of the etifoxine group that were homozygous for the polymorphism rs6971 (see 2.2.3). For none of those parameters main effects or interactions were altered after exclusion of those participants. For an overview of the results see table 11.

Table 11

Results of the Repeated Measures ANOVAs for the Main Stress Parameters after Correction for the TSPO Polymorphism

Stress parameter	Source	*F ratio*	*df*	*p*	η_p^2
Subjective stress	Phase	156.60	3.02,153.85	**< .001**	.75
	Treatment	0.88	2,51	.420	.03
	Phase x treatment	0.25	6.03,153.85	.958	.01
Heart rate	Phase	28.91	2.95,141.62	**< .001**	.38
	Treatment	0.27	2,48	.761	.01
	Phase x treatment	1.23	5.90,141.62	.296	.05
Skin conductance	Phase	24.36	1,40,63.04	**< .001**	.35
	Treatment	1.68	2,45	.197	.07
	Phase x treatment	0.93	2.80,63.04	.425	.04
Salivary cortisol	Phase	49.30	1.93,96.31	**< .001**	.50
	Treatment	4.55	2,50	**.015**	.15
	Phase x treatment	4.08	3.85,96.31	**.005**	.14
Allopregnanolone	Phase	4.77	1,51	**.034**	.08
	Treatment	0.36	2,51	.700	.01
	Phase x treatment	0.37	2,15	.695	.01
TSPO expression	Phase	8.42	1,49	**.006**	.16
	Treatment	1.81	2,49	.175	.07
	Phase x treatment	0.37	2,49	.693	.02

3.5 Discussion

3.5.1 Overview

Within this part of the work, we examined the effects of alprazolam and etifoxine on acute psychosocial stress in healthy subjects. Therefore, we administered the VR-TSST on day 5 of treatment with either one of the two substances or placebo. Subjective stress and anxiety as well as heart rate and skin conductance significantly increased in response to the task. The same pattern was shown for salivary cortisol and allopregnanolone levels in serum, whereas TSPO expression in platelets was decreased and some of the voice-based parameters were affected in different directions.

None of the two anxiolytic substances had attenuating effects on any of the subjective and physiological parameters. Activity of the HPA axis indicated by salivary cortisol was blunted by alprazolam but not by etifoxine. Stress-induced changes of allopregnanolone and TSPO expression were not affected by any of the two compounds. Covariation amongst the stress-related parameters differed between the three groups. Repetition of the main analyses after excluding participants of the etifoxine group that were homozygous for the TSPO polymorphism rs6971 yielded no remarkable changes.

3.5.2 No effects of alprazolam and etifoxine on subjective stress and anxiety

The examination of possible stress reducing effects of alprazolam and etifoxine required the generation of a considerable response to the applied laboratory paradigm. Indeed, the subjects in this study felt subjectively stressed by the task to an extent that is comparable to other studies applying this paradigm in virtual reality or in vivo (Santl et al., 2019; Shiban et al., 2016; Zimmer et al., 2018). Likewise, ratings of feeling present in the virtual scenario were similar to those reported by a previous study that was conducted in our laboratories (Shiban et al., 2016).

Against our hypotheses, we found no group differences implying that neither anticipation stress, nor the reports directly after the VR-TSST or at any of the other time points were affected by the anxiolytic substances. Thus, this study ties up with prior research, which reported inconsistencies on the effects of single doses of benzodiazepines on acute stress. While some studies observed reduction of subjective anxiety before and during public speaking after 10 mg diazepam (McNair et al., 1982; Zuardi, Cosme, Graeff, & Guimarães, 1993) or 1 mg lorazepam (Guimarães et al., 1987), this was not confirmed by other researchers that applied

the same substances in equal dosage (Graeff, Zuardi, Giglio, Filho, & Karniol, 1985). Discrepancies between those early studies were traced to the fact that they did not further control for changes of cortisol release and applied unstandardized protocols impeding direct comparisons (van Hedger, Bershad, & Wit, 2017). We are aware of one study that applied the standardized protocol of the Trier Social Stress Test to investigate the effects of alprazolam in healthy men (Fries et al., 2006). In line with our findings, they did not report any effects on ratings of state anxiety although mood was slightly improved in the group that received a single dose of 1 mg alprazolam. With respect to standardization, the present work might have even added up as the virtual reality based version warranted a highly controlled stress situation for every participant (Jönsson et al., 2010).

A crucial difference to the mentioned study of Fries and colleagues (2006) is constituted by the fact that subjects of that study were administered double the dose as applied within the present work. Indeed, dose-dependency was, for example, concluded from a study on diazepam which reported clear anxiolytic effects within the scope of a public speaking task after 10 mg but only a marginal effect for 5 mg (McNair et al., 1982). Increasing the dose, especially in case of benzodiazepines, is often accompanied by sedating effects, which might on the one hand account for altered subjective experience including calmness but otherwise also impede positive effects of the medication (Fries et al., 2006). An influence of sedation can be excluded in the present work, as the groups neither differed concerning reported wakefulness or concentration before and after the task nor with respect to more objective performance measures like correct math and speech fluency in front of the committee.

However, although we applied a lower dose in contrast to recent research, subjects of our study were exposed to the stress task after five days of medication intake resembling a more chronic use as in patients. Nevertheless, we could not confirm our hypothesis that a constant level built over days would be sufficient to exert alleviating effects of the two substances on the subjective stress response. While the assumptions on the effects of alprazolam were mostly based on findings after single administration in healthy subjects, for etifoxine we relied on studies that reported calming effects in animals (Verleye & Gillardin, 2004) as well as anxiolytic effects in patients (Nguyen et al., 2006; Stein, 2015). The latter ones were primarily specified by changes of the clinician-rated Hamilton Rating Scale for Anxiety (Hamilton, 1959) after treatment of at least seven up to 42 days with the effects getting more remarkable with increased duration. Hence, even a period of five days might have been too short to identify effects of etifoxine on subjective measurements. Furthermore, attenuating effects could be restricted to highly or even pathologically anxious subjects. The nature of a pharmacological

trial might rather attract subjects that exhibit low general anxiety what seems true for the present sample, as the trait variables related to stress and anxiety scored on average in the lower or medium range, respectively. Overall, subjects stated more positive than negative stress coping strategies, low fear of negative evaluation and moderate fear of speaking in public. Thus, although state anxiety assessed with the STAI (Laux et al., 1981) increased due to the VR-TSST and exaggerated the cut-off of 39 to 40, which marks a clinically relevant level (Knight, Waal-Manning, & Spears, 1983), it still might have been too small to detect effects of the anxiolytic treatment.

Concerning the assessment of subjective experience, it was further shown that the variability of administered scales or questionnaires might account for differences between studies (van Hedger et al., 2017). Besides the adjectives used for description of the respective state (e.g., stressed, anxious, aroused), the time points at which the ratings were taken must be considered. Except for the STAI, all ratings were based on single items and were inquired either before or retrospectively after the task. Future studies might profit from scales that provide a more differentiated picture and assess online reports during the stress situation. Furthermore, the VAS that were administered within the present work must be interpreted with caution, as the dimensions of the five scales were inconsistent with the positive and negative statements not always being placed at the same end. Therefore, we cannot rule out that some items were rated different than intended by single subjects.

3.5.3 No effects of alprazolam and etifoxine on the physiological stress response

Heart rate as well as skin conductance level significantly increased in response to the psychosocial stress. In comparison to baseline higher values were measured during the preparation phase of the experiment resembling anticipation anxiety and even to a higher extent during the speech and processing of the math task in front of the committee. Increases of electrodermal activity even lasted up to the post measurement ten minutes after the task.

Neither alprazolam nor etifoxine attenuated the stress-induced changes of the physiological parameters. Thus, we could not replicate findings of preclinical studies that showed effects of both compounds on stress-induced changes of parameters related to activity of the sympathetic-adrenal-medullary axis (Verleye & Gillardin, 2004; Vogel et al., 1984). From a clinical viewpoint, our hypotheses concerning heart rate were based on research on benzodiazepines, as we are not aware of studies on etifoxine which included that parameter.

Available studies for benzodiazepines, however, differ with respect to the administered dosage as well as experimental stimulation. During rest, effects on cardiovascular measurements after intravenous or oral administration of alprazolam were only modest and delayed (Risby et al., 1989; Zemishlany et al., 1990). The authors identified the ascertained delay as possible indicator that attenuating effects on peripheral parameters might be attributable to increased sedation and a subsequent lower activity level. The fact that subjects in the present work did not report high levels of sedation before or after the stress task might therefore at least partially account for the missing findings.

Potential dependence on the applied dosage must be concerned with respect to the nature and intensity of the stressor. While administration of 1.5 mg alprazolam for three days decreased heart rate changes in response to physical stress on the last day of treatment (Stratton & Halter, 1985), a single dose of equal amount exerted no such effects on metabolic stress (Breier et al., 1991). One study reported significant reductions of blood pressure and heart rate in response to public speaking after a single dose of 0.5 mg alprazolam (Rohrer et al., 1994). However, this was not confirmed by other studies after application of the same dosage (McCann et al., 1992) or only partially for basal blood pressure after application of 1 mg (Fries et al., 2006). Heterogeneous susceptibility to anxiolytic substances might be explained by differences in the activation of central pathways in response to varying stressors and experimental protocols. Doubts in the involvement of the noradrenergic system in the exhibition of the GABA-mediated effects of benzodiazepines and similarly etifoxine arise from studies that included the measurement of catecholamines. While some showed an impact of alprazolam on noradrenaline levels during rest but not in response to metabolic stress (Giordano et al., 2003) or acute stress induced by a parachute jump (Benschop et al., 1996), others found no effects on the secretion of catecholamines at all (Fries et al., 2006). Nevertheless, the additional measurement of those markers might be included in future studies to further clarify a possible effect of etifoxine.

Concerning the less-studied parameter skin conductance, we could not replicate early studies that reported attenuating effects of etifoxine (Córsico et al., 1976; Sartory & Rust, 1973). However, both studies administered only one capsule and a much higher dose of about 300 mg. For benzodiazepines a possible influence of gender was summoned up within the scope of an aversive conditioning paradigm as attenuation of skin conductance response after administration of diazepam was only shown in women (Hellewell, Guimarães, Wang, & Deakin, 1999).

Furthermore, for parameters that are related to stress-induced activity of the SAM effects of anxiolytic treatment might just become consistently measurable in patients suffering from anxiety disorders or at least showing hyperactivity of the noradrenergic system. A connection was, for example, drawn in a study that reported higher increase of heart rate as well as release of noradrenaline induced by the TSST in patients with generalized or separation anxiety disorder in comparison to healthy controls (Gerra et al., 2000).

In addition to the well-established parameters in stress research, we were able to replicate findings of increased mean fundamental frequency (F0) during the stressful speech in front of the committee in comparison to the baseline recordings (Pisanski et al., 2016; Pisanski et al., 2018). However, standard deviation of that vocal parameter was unaffected. Notably, the applied voice-based algorithm was able to detect higher arousal and negative valence during the stressful task even though for the latter a high number of subjects had to be excluded due to the a priori defined criterion to only include assignments that are met with a confidence greater than 0.4. None of the voice-based parameters was affected by the anxiolytic treatment. This is especially surprising for alprazolam, as changes of F0 were shown to be related to cortisol release (Pisanski et al., 2016), which was blunted in that group (see 3.5.4). To our knowledge, this was the first controlled trial that investigated the effects of anxiolytic treatment on vocal measures and future research might follow up. Studies might, for example, include further parameters like mimics or body language, improve recording equipment and apply more realistic speech passages as baseline measurement.

3.5.4 Attenuation of stress-induced cortisol release only by alprazolam

While cortisol measured in saliva significantly increased in the placebo and the etifoxine group throughout the stress task, we found a blunted response due to administration of alprazolam. Interestingly, in contrast to other studies, we found no effects of the benzodiazepine on baseline cortisol levels (Fries et al., 2006; Rohrer et al., 1994). While the difference to the etifoxine group was measurable directly after the stress task, differences in comparison to the placebo group only emerged ten minutes later.

The inhibition of the stress-induced cortisol release by alprazolam is in line with prior human research that investigated effects of the benzodiazepine, mostly after administration of a single dose, on markers related to activity of the HPA axis in response to various stressors (Benschop et al., 1996; Breier et al., 1991; Fries et al., 2006; Rohrer et al., 1994; Zemishlany et al., 1990). It seems obvious that the yielded effects are mediated by binding of alprazolam to the $GABA_A$ receptor, as there exists a dense network of GABAergic neurons in the

hypothalamus, especially around the PVN, which plays a crucial role in the regulation of the HPA axis (Decavel & van den Pol, 1990; Miklós & Kovács, 2002). In vivo and in vitro studies supposed the effects of alprazolam to be mediated by suppression of neurons that are involved in the secretion of CRH thereby inhibiting the release of corticosteroids (Kalogeras et al., 1990).

Additional mediation of the effects shown for alprazolam by peripheral benzodiazepine receptors also known as translocator protein seems unlikely, as it shows only little affinity for that complex (Schmoutz et al., 2014). Yet, a possible role of TSPO in the attenuation of stress-induced CRH activation was supposed by studies that showed reduction of CRH-induced behavior in rats after administration of the TSPO ligand etifoxine (Verleye et al., 2006; Verleye & Gillardin, 2004). In vitro studies yielded that etifoxine does not directly modulate activity of CRH-receptors and supposed that attenuating effects might rather be based on its GABAergic properties (Verleye et al., 2006). However, within the present work we could not confirm any effects of etifoxine on basal or stress-induced release of cortisol in humans. This might be attributable to the twofold action mechanism of the compound. Preclinical research has shown that besides direct facilitation of GABAergic inhibition, etifoxine mainly acts by increasing neurosteroid production after binding to TSPO (Schlichter et al., 2000; Verleye et al., 2005). Neurosteroids, such as allopregnanolone, vice versa, are known to be potent agonists of the $GABA_A$ receptor (Majewska et al., 1986) and were shown to exert anxiolytic effects through interactions with hypothalamic CRH. Levels of allopregnanolone, however, were not affected within the present study (see 3.5.5) and might have been too small to modulate subsequent higher-level processes. Since also studies on benzodiazepines sometimes only reported inhibition of the ACTH peak response but not of cortisol (Giordano et al., 2003), it would be interesting to further include the assessment of precursors like ACTH to check if effects of etifoxine might become measurable on a lower level.

In general, it needs to be clarified if the dampening effects of alprazolam on stress-induced cortisol release are rather beneficial or detrimental. In an animal model of posttraumatic stress disorder it was shown that the inhibitory effects of alprazolam on the HPA axis were associated with an even exaggerated stress response during repeated exposure (Matar et al., 2009). Focusing on the combination of pharmacotherapy and psychotherapy it was even argued that the suppression of stress-induced cortisol release by anxiolytics like benzodiazepines might interfere with extinction learning in exposure-based cognitive behavioral therapy and thereby hamper its effects (Otto, McHugh, & Kantak, 2010). Such interdependencies should either way be considered by clinicians when combining different options for the treatment of mental disorders and require further research.

3.5.5 Stress-induced increase of allopregnanolone independent of treatment

Allopregnanolone measured in serum increased in response to the stress task. This is in accordance with findings from preclinical studies, which assessed cerebral levels of that neurosteroid after stress induction by CO_2-inhalation (Barbaccia et al., 1996) or forced swimming (Purdy et al., 1991). Studies in humans, however, were so far inconsistent with some showing increased allopregnanolone levels after a PhD examination (Fortuyn et al., 2004) but missing effects in response to the TSST (Altemus et al., 2001; Childs & de Wit, 2009). However, a compensatory and therefore vital role of allopregnanolone seems obvious as, for example, decreased levels in response to an induced panic attack were shown in patients suffering from panic disorder suggesting distorted reactivity compared to healthy controls (Ströhle et al., 2003).

With respect to the mentioned TSST studies, it must be noted that they included rather specific samples with investigating postpartum women (Altemus et al., 2001) or comparing male smokers and non-smokers (Childs & de Wit, 2009). However, also the subgroup of non-smokers resembling the subjects of the present sample showed no significant stress-induced increase of allopregnanolone. Since both studies found increased cortisol levels due to the stress, it was suggested that the threshold for induced secretion of allopregnanolone in humans might just be higher. Besides sample characteristics and amount of the stress level, time points of measurement might account for differences between studies. Within the present work, the baseline blood measurement was taken at 9 AM, while the other studies assessed their reference values ten minutes (Altemus et al., 2001) or 30 minutes (Childs & de Wit, 2009) before the stressor. At that time, some anticipation anxiety might already have been present impeding further measurable increases in response to the task. Furthermore, there exist variations concerning the post measurement, which was taken about 60 minutes after the stress task within the present study. It might be hypothesized that ten minutes (Altemus et al., 2001) up to 30 minutes (Childs & de Wit, 2009) after termination of the stress might have been too early to find any changes, although another study even reported increased levels right during the stress situation (Fortuyn et al., 2004). However, to rule out that other factors in between the blood samplings in the morning and in the afternoon might have influenced our findings, future research should finally include several time points of measurement.

Surprisingly, in contrast to preclinical studies, which demonstrated a significant increase of neurosteroids after application of etifoxine (Verleye et al., 2005; Wolf et al., 2015), we could not replicate those effects in our sample of healthy male subjects. Although the allopregnanolone level after the VR-TSST seemed slightly higher in the etifoxine group

compared to alprazolam and placebo, this difference did not reach statistical significance. However, it can be speculated that the overall increased level after the task might mainly be traceable to changes in the etifoxine group. It is possible that the applied dose of etifoxine was too small to exert measurable effects in healthy subjects. Even in cell culture increased levels of pregnenolone, which precedes the synthesis of allopregnanolone, were only shown in response to higher doses (Wolf et al., 2015). It is further possible that effects in humans are restricted to subjects suffering from anxiety or other mental disorders as patients often show disrupted levels of neurosteroids (Schüle et al., 2014). Hence, they might be more susceptible to stimulatory effects of the TSPO ligand.

3.5.6 Stress-induced decrease of TSPO expression independent of treatment

In contrast to recent studies that showed increased levels of TSPO in blood platelets after oral examinations (Fortuyn et al., 2004; Karp et al., 1989), expression was significantly decreased after the stress task in the present work. Interestingly, the mentioned studies did either report no changes of stress hormones like cortisol or absent coherence between changes of TSPO density and cortisol levels. Alas, we could not perform correlation analysis between TSPO expression and any of the other measures, as we computed the percent change within the subject referencing to the baseline measurement of the screening day. With that we accounted for the need of different gels and reference measures within the western blot analysis impeding a direct comparison of raw values between different subjects. Although the use of western blotting to determine expression of TSPO is a common approach (Wolf et al., 2015), other studies determined density of TSPO based on binding of the specific ligand PK 11195 (Fortuyn et al., 2004; Karp et al., 1989) what might account for the exerted differences.

In general, it must be regarded that we did not assess the pure effect of acute stress on TSPO expression as two thirds of the subjects have already been taking one of two anxiolytic substances for five days at the time the VR-TSST was administered. Although there were no effects of etifoxine or alprazolam on stress-induced changes of TSPO density, the overall level throughout the course of the study was significantly more increased by etifoxine in comparison to the two other groups. This increase was the most prominent on day 1 of treatment followed by a slight decrease over the following days in all three groups. Hence, it is possible that the changes on day 5 are not solely attributable to an impact of stress but rather to a general decrease over the longer intake of the medication.

With regard to research that ascribed a major role to TSPO in regulating the synthesis of neurosteroids (Gavish et al., 1999), it is conspicuous that expression of the protein was found

to be decreased after the VR-TSST, whereas allopregnanolone levels were increased. However, also studies in cells revealed some kind of dissociation, as TSPO expression, in contrast to allopregnanolone, was increased after administration of etifoxine independent of the applied dose (Wolf et al., 2015). For example, other components related to the mitochondrial complex might be additionally involved in the synthesis of neurosteroids, as has already been suggested by some researchers (Banati et al., 2014; Selvaraj & Stocco, 2015).

3.5.7 Different covariation between stress markers due to the treatment

The different parameters that were chosen for the quantification of stress, especially those related to the activity of the SAM system and the HPA axis, have often been only weakly associated or even dissociated within laboratory settings (Ali, Nitschke, Cooperman, & Pruessner, 2017; Campbell & Ehlert, 2012). Interestingly, within the present work changes of cortisol and heart rate in response to the VR-TSST were positively associated to a high extent in the placebo group and at least weakly for the alprazolam group indicating similar activation of the two axes. With respect to the blunted cortisol response shown for subjects that had received alprazolam, it might be speculated that the benzodiazepine also affected heart rate at least to some amount in individual subjects.

Within the placebo group, higher levels of subjective stress in response to the task were accompanied by increased heart rate. Alterations of cortisol and allopregnanolone level, however, were negatively associated in that group. The fact that allopregnanolone in blood was measured about one hour after salivary cortisol might indicate that extensive increase of cortisol in response to the stress task inhibited the subsequent release of allopregnanolone to a certain amount. Previous research reported correlations between allopregnanolone and noradrenaline supposing a connection of the neurosteroid to activity of the SAM axis (Childs et al., 2010). In contrast, the present results introduce a possible antagonistic connection to the HPA axis which warrants further investigation.

For subjects of the etifoxine group no covariations pointing to the same direction between any of the stress markers were shown while heart rate and skin conductance level were even negatively associated. Fractionation of electrodermal and cardiovascular measures has already been reported in the past suggesting that those parameters must be accounted for independently in research on psychosocial stress (Croft, Gonsalvez, Gander, Lechem, & Barry, 2004). It remains unclear why this finding was restricted to the etifoxine group. It is possible that interfering actions like higher movement or touching of the electrodes in that group might explain the observed dissociation. However, as anomalies of the physiological data were

carefully controlled during the preprocessing, it is worth pursuing a possible effect of etifoxine that unfolds selectively for separate autonomic measures.

In sum, the stress-related parameters that were assessed in the present study showed differential relations in the three experimental groups what could be traced to the pharmacological manipulation. For the further clarification of causalities, future research might explicitly manipulate specific parts of the stress systems and investigate the influence of alprazolam and etifoxine.

3.5.8 TSPO gene polymorphism rs6971 and efficacy of etifoxine

As it was shown that the TSPO gene polymorphism rs6971 exerts restrictive effects on the binding affinity of ligands (Owen et al., 2011), we repeated some of the analyses after excluding subjects of the etifoxine group that were homozygous for that genetic variant. None of the analyses yielded different results after exclusion of the respective three participants. It must be noted that the overall sample size and especially the little number of affected subjects does not even approximately achieve the large sample sizes required for the investigation of genetic associations (Hong & Park, 2012). Nevertheless, it is worth investigating if very strong effects might become visible even in a small sample. To our knowledge, this is the first study that investigated the effects of etifoxine in humans taking that polymorphism into account. Since studies in cell culture revealed comparatively low affinity of etifoxine for TSPO, its pharmacological efficacy might less be determined by its binding affinity to the respective complex (Wolf et al., 2015). This might imply low susceptibility to the polymorphism in comparison to other TSPO ligands and be taken as a sign for the twofold action mechanism of etifoxine with additional modulation of the GABAergic system (Mattei et al., 2019).

3.5.9 Conclusion

The application of the VR-TSST on day 5 of treatment with either the benzodiazepine alprazolam, the TSPO ligand etifoxine or placebo revealed differential effects for the substances in healthy male subjects. The most prominent finding was an inhibition of the cortisol response to stress by alprazolam but not by etifoxine. Distinct modulation of HPA axis activity might indicate the different action mechanisms of the two anxiolytic compounds with a specific focus on the strength of GABA modulation. Besides replication and further clarification, future research should address the question of pharmacologically blunted activity of the HPA axis

being promoting or unfavorable with respect to repeated stress exposure and long-term outcome.

In general, in conjunction with the applied experimental paradigm, we were only able to detect molecular changes due to the anxiolytic medication, as there were no effects on any of the subjective or physiological parameters. Nevertheless, distinct relation of the individual stress markers within the three groups suggests that they might be differentially susceptible to the medication. Regarding etifoxine future research should follow up on our findings that did not show stimulating effects on the synthesis of allopregnanolone in healthy subjects, although TSPO expression was increased directly after the first intake.

Based on the given findings, subsequent clinical trials might target the administration of graded doses of alprazolam and etifoxine, online assessment of subjective experience, within-subjects comparisons using stress tasks that are suitable for repetitive testing as well as variations of intake duration. Furthermore, as only healthy men were included into the present study, future studies should consider the inclusion of women as well as patient samples to check the generalizability of the reported results. Clarification of a possible influence of the TSPO related gene polymorphism rs6971 on general stress reactivity and efficacy of etifoxine, might be followed-up within clinical studies that include larger samples and form their experimental groups based on the presence or absence of the genetic variant.

CHAPTER 4: Effects of etifoxine & alprazolam on fear and anxiety

4.1 Theoretical background

Investigating the anxiolytic effects of pharmacological compounds in patient samples is mostly preceded by studies based on animal models or including healthy subjects. A validated and standardized approach in this field that has been applied in animal and human research is the measurement of startle reactivity in response to an abrupt, intense noise along with the presentation of aversive stimuli like electric shocks, light pulses or aversive pictures (Grillon, 2008). The so-called fear-potentiated startle paradigm was first devised for research in rats to pursue indices that humans frighten more to loud noise when being afraid (J. S. Brown, Kalish, & Farber, 1951). Analogue studies in humans mostly rely on the comparison of the startle response quantified by the magnitude of eye blinks during instructed threat and safe conditions (Grillon, Ameli, Woods, Merikangas, & Davis, 1991).

Startle based paradigms have frequently been used in research investigating the anxiolytic effects of different compounds - notably substances that act via the GABAergic system. In animals rather consistent effects of various compounds in reducing the magnitude of fear-potentiated startle were reported (Davis, Falls, Campeau, & Kim, 1993). However, in human studies pharmacological validation of this paradigm has so far yielded contradictory results. One of the first studies that investigated the effects of a single dose of diazepam in a paradigm based on affective pictures reported a specific reduction of the fear potentiated startle response due to diazepam (Patrick & Berthot, 1996). Subsequent studies either applying negative pictures or electric shocks confirmed this effect for diazepam as well as alprazolam and lorazepam in a dose-dependent manner (Bitsios, Philpott, Langley, Bradshaw, & Szabadi, 1999; Graham et al., 2005; Riba et al., 2001). In contrast, others only reported reduction of overall startle reactivity without specificity to the valence of the stimuli after administration of various benzodiazepines (Acheson et al., 2012; Baas et al., 2002; Murphy, Downham, Cowen, & Harmer, 2008). At first, this was attributed to resemble rather non-specific effects of sedation than actual anxiolysis (Abduljawad, Langley, Bradshaw, & Szabadi, 1997; Baas et al., 2002; Scaife, Langley, Bradshaw, & Szabadi, 2005). This was contradicted by authors arguing that the mere negative context constituted, for example, by a dark surrounding or simply the experimental threat-related instructions applied in respective experiments could have produced anxiety itself (Baas et al., 2002; Guscott, Cook, & Bristow, 2000). Therefore, it was suggested that the effect of

benzodiazepines in humans could be restricted to contextual anxiety while not affecting cued fear (Grillon et al., 2006).

While the mentioned studies in humans relied on the comparison between threat and safe conditions only, newer research revealed the need of further differentiation between phasic fear related to specific cues and sustained anxiety depending on the respective context (Davis, Walker, Miles, & Grillon, 2010; Grillon, 2008). Confirmation of this assumption stems from studies in rats that compared specific agonists and antagonists of different systems (Miles, Davis, & Walker, 2011). Likewise, imaging studies in humans revealed differences in neural activation linking the central nucleus of the amygdala to phasic fear and the bed nucleus of the stria terminalis to sustained anxiety (Walker, Toufexis, & Davis, 2003). In accordance, fear has been more related to specific phobias and panic, while anxiety is more connected to generalized anxiety disorder, posttraumatic stress disorder and depression (R. F. Krueger, 1999).

An experimental paradigm that considers the assumption of fear and anxiety being differentiable states, which vary with regard to the duration (phasic vs. sustained) and predictability of an aversive event, is the so-called NPU Threat Test (Schmitz & Grillon, 2012). Within this task, electric shocks are applied with respect to three different conditions. In the neutral phase no shocks are administered and participants are safe. The previous threat condition was split into two phases. During a predictable phase, administration of the shock is linked to the presentation of a defined cue evoking specific fear. In the unpredictable phase the aversive stimulus can be applied at any time independent of the cue eliciting sustained anxiety throughout this condition. Studies concordantly showed the greatest startle reactivity during the unpredictable condition, while increases of reactivity during the predictable condition were shown to be dependent on the respective cue.

The proposed hypothesis that benzodiazepines might preferably affect contextual anxiety was initially validated in a study from Grillon et al. (2006). While a single dose of 1 mg alprazolam significantly reduced the startle response in the unpredictable condition, there were no effects on cued fear in the predictable condition. This study further invalidated hypotheses that contributed former effects to sedation, as the sedative diphenhydramine, which lacks anxiolytic properties, attenuated baseline startle response equally to alprazolam without specific effects on contextual fear. However, another study that applied the same medication and experimental paradigm could not confirm the effects of alprazolam on anxiety-related startle, as they only found reduction of overall baseline startle due to the medication (Baas et al., 2009). Attempts of explanation for these striking differences concerned factors like gender and methodological issues with respect to the shock and startle noise presentation. It was further

suggested to use multiple measurements and thereby investigate a more chronic use of the anxiolytic agents. A study that addressed the repeatability of currently used paradigms for the investigation of pharmacological compounds showed that the fear-potentiated startle constitutes a suitable tool for repeated designs of drug studies (Klumpers et al., 2010).

As for the effects of anxiolytics on startle measurements, also concerning subjective anxiety ratings research has so far yielded discrepant results. While some studies reported a reduction of subjective anxiety due to drug administration (Baas et al., 2009; Cornwell, Garrido, Overstreet, Pine, & Grillon, 2017; Graham et al., 2005), this was not confirmed by others (Grillon et al., 2006).

While there is emerging although inconsistent research on benzodiazepines on fear potentiated startle, we are not aware of any study that investigated the effects of etifoxine applying an equivalent paradigm. However, XBD-173, another TSPO ligand, was shown to counteract pharmacologically induced panic after administration of CCK-4 in rats and healthy humans (Rupprecht et al., 2009). In general, studies in animals that report anxiolytic effects of etifoxine (Verleye & Gillardin, 2004) or studies in patient samples, which showed comparable efficacy to that of benzodiazepines (Nguyen et al., 2006; Stein, 2015), lead us to the suggestion of similar efficacy of etifoxine with respect to that specific paradigm.

4.2 Research questions and hypotheses

Within this part of the work, we aimed to shed further light on the so far inconsistent findings concerning the effects of benzodiazepines on fear and anxiety in a standardized startle related paradigm – the NPU Threat Test. We further wanted to introduce another anxiolytic compound into this area of research. As the TSPO ligand etifoxine is also prescribed for psychosomatic manifestations of anxiety, we expected a similar profile of efficacy as was found for benzodiazepines. While most of the studies tested the effects after administration of a single dose, we applied the paradigm twice to directly assess acute effects as well as short-term effects after intake of five days.

We expected alprazolam and etifoxine to decrease contextual anxiety indicated by a reduction of the startle magnitude and subjective anxiety in the absence of cues in the unpredictable condition. We anticipated no effects on fear-potentiated startle and subjective ratings to the threat signal in the predictable condition resembling cued fear. We expected the effects to be visible after acute and maybe even stronger after short-term administration. Further analyses concerned the baseline startle reactivity as well as the TSPO polymorphism rs6971.

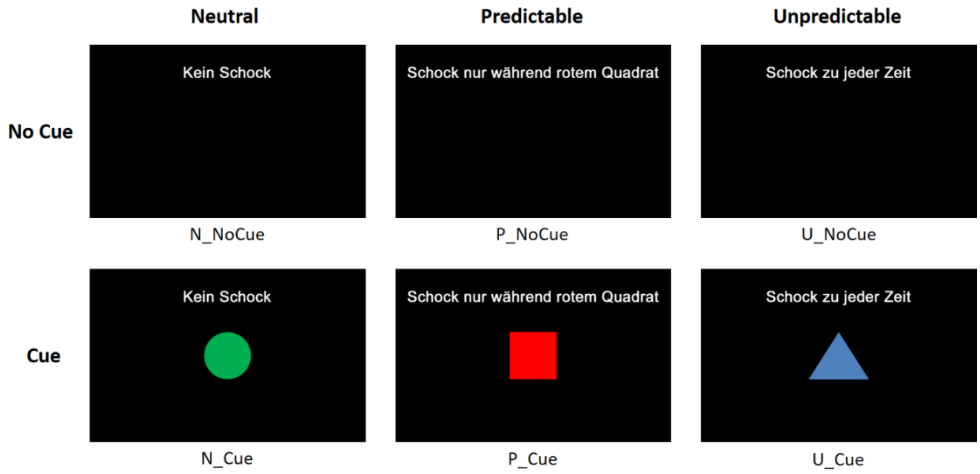
4.3 Methods and procedure

4.3.1 NPU Threat Test

To investigate our questions, we administered a version of the NPU Threat Test that was set up using the software Presentation (version 19.0, Neurobehavioral Systems Inc., Albany, California, US). Within this paradigm, participants were exposed to electrical shocks that lasted about 100 ms and were administered at the upper side of the forearm by electrodes connected to a constant-current stimulator (Digitimer DS7A; Digitimer, Hertfordshire, UK). The strength of the shocks was adjusted individually for each subject using an Bayesian algorithm (Onat & Büchel, 2015) that was adapted at our chair. Starting at a strength of 0.3 mA the participants rated on each shock if it felt unpleasant or painful. In case of unpleasantness, the strength was increased in constant steps with the goal to reach the individual threshold between unpleasantness and pain. After estimation of this threshold, the algorithm automatically determined the strength that was applied during the task by computing a value 1.3 times the threshold. During the experiment, the shocks were applied according to three different conditions at which the probability of their occurrence was indicated by geometrical figures (cues) with the following scheme: in the neutral (N) condition (green circle) participants were „safe“ and received no shock, during the predictable (P) condition (red square), participants could receive a shock only when the cue was present and during the unpredictable (U) condition (blue triangle), the shock could be administered at any time independent of the cue. The respective condition was indicated by a text at the screen for the whole period (see figure 20).

Figure 20

Experimental Conditions of the NPU Threat Test



Throughout the experiment, which was divided into two blocks of 15 minutes, the three conditions were presented alternately according to the following scheme: U N P N P N U or P N U N U N P. Participants were assigned to start with either one or the other order of conditions at the two testing days in a randomized manner. During each condition, which lasted 120 seconds, the respective geometrical cues were presented three times for a period of 8 seconds. Within each P-condition, one to two shocks were administered each time about 0.5 seconds before the cue disappeared. In each U-condition, also one to two shocks were given but only during periods when no cue was present, while in the N-condition no shocks were administered. In total, each subject received 12 electric shocks.

To evoke startle reactivity, bursts of white noise at 103 dB with a duration of 40 ms were presented via headphones (Sennheiser HD 569, Sennheiser electronic GmbH & Co. KG, Wedemark-Wennebostel, Germany) throughout the experiment. Subjects were told that the tone is delivered independently of the electric shocks. To avoid an influence of excessive startle reactivity at the beginning and to obtain a measurement that resembles general startle reactivity, we applied a habituation phase with four presentations of the tone before the start of the actual experiment. During the experiment, participants were not supposed to actively perform any task but were told to watch the screen attentively throughout the whole time.

4.3.2 Outcome measures

4.3.2.1 Physiological measurements

Startle response

For the measurement of startle blink responses, we attached two surface electromyographic electrodes at the left orbicularis oculi muscle and further an isolated ground electrode at the right mastoid and a reference electrode (all Ag/AgCl, Ø = 8 mm) at the left mastoid. Before attaching the electrodes that were filled with Signa electrode gel (Parker Laboratories, Fairfield, USA), we prepared the relevant skin areas by cleaning with disinfectant (Softasept N, B. Braun Melsungen AG, Melsungen, Germany) and rubbing with a peeling gel (skinPure, Nihon Kohden, Tokyo, Japan) to reduce the impedance between the electrode gel and the skin surface. We then checked for the impedances and detectability of eye blinking.

Preprocessing was accomplished with Brain Vision Analyzer by applying the following filters: low cutoff filter of 28 Hz with a slope of 24 dB /Oct, high cutoff filter of 499 Hz with a slope of 24 Hz / Oct and a Notch Filter of 50 Hz to control for the influence of electromagnetic interference. After dividing the data into segments representing the six different conditions

(N_NoCue, N_Cue, P_NoCue, P_Cue, U_NoCue, U_Cue), we rectified it and applied smoothing with a moving average window of 16 ms. Each segment was baseline corrected 50 ms prior to the onset of the startle tone. Startle amplitudes (in microvolt) were defined as peak magnitudes between 20 and 150 ms after the probe onset. Trials with strong baseline noise (> 5 µV before tone onset) were automatically excluded. For the remaining trails, artifact rejection was performed manually for each peak. Amplitudes smaller than 2 µV were defined as non-responses and set to zero. Participants were defined as non-responders if they showed less than 50 % of evaluable startle responses throughout the complete task or less than 6 trials evaluable for each condition (Lieberman et al., 2017).

4.3.2.2 Subjective anxiety

According to the paper outlining the NPU Threat Test (Schmitz & Grillon, 2012), we administered a questionnaire asking for subjective statements according to the task once during the break between the two parts and at the end of the experiment. Subjects were asked to state the level of anxiety they experienced during the three conditions (neutral, predictable, unpredictable) separately for the time windows when the respective cue was present or absent on a scale from 1 = not anxious (*nicht ängstlich*) to 10 = extremely anxious (*sehr ängstlich*). The ratings given between the two blocks and those from the end of the task were averaged for further analysis.

4.3.2.3 Further subjective measurements

In addition to their subjective anxiety during the different conditions, participants were asked to rate the likeliness at which they expected the shock to be applied in the three conditions during presence and absence of the cue by stating a percentage from 0 to 100 %. This was followed by questions on how intense they experienced the electrical shock on a scale from 1 = mild (*schwach*) to 10 = high (*stark*), how much anxiety the shock was provoking on a scale from 1 = low (*wenig*) to 10 = high (*viel*) and how painful the shock felt on a scale from 1 = not painful at all (*überhaupt nicht*) to 10 = very painful (*sehr*). Further, we asked if the participants would prefer to proceed with the “shock at any time”- condition or with the “shock only at red square”-condition and which of the two they experienced more unpleasant. Concerning the white noise, participants were asked on the experienced loudness (1 = low (*leise*) to 10 = loud (*laut*)), unpleasantness (1 = not at all (*überhaupt nicht*) to 10 = very (*sehr*)), anxiety (1 = not at all (*überhaupt nicht*) to 10 = very (*sehr*)), and if they wished to hear the noise again from 1 =

no (*nein*) to 10 = absolutely (*unbedingt*). In parallel to the subjective anxiety ratings, we averaged those ratings prior to analysis.

4.3.2.4 Possible Covariates

Anxiety Sensitivity Index 3 (ASI-3)

The Anxiety Sensitivity Index - 3 (Taylor et al., 2007) assesses the trait variable anxiety sensitivity, which describes the fear of sensations related to arousal and is handled to be a contributor for different anxiety disorders, as it serves as an amplifier of anxiety. This questionnaire was administered at the screening day to control for a possible influence on our outcome measures of the NPU threat test, as it could be shown that the subscales physical and cognitive concerns are associated with the startle response to unpredictable threatening stimuli (Nelson, Hodges, Hajcak, & Shankman, 2015).

We administered the German version (Kemper et al., 2009), which consists of 18 items (e. g. "It scares me when my heart beats rapidly"; German: "*Es macht mir Angst, wenn mein Herz schnell schlägt*") that are rated on a five-point scale ranging from 0 = very little (*stimme gar nicht zu*) to 4 = very much (*stimme völlig zu*). It comprises three scales – physical concerns, social concerns and cognitive concerns – with six items each. In addition to the three scale scores, we computed a sum score for general anxiety sensitivity. For the German version, internal consistency is acceptable to good with Cronbach's α = .75 for physical concerns, α = .77 for social concerns, α = .78 for cognitive concerns, and α = .86 for the general score. In a sample of treatment seeking smokers, retest-reliabilities between r_{retest} = .60 and r_{retest} = .82 were shown for the three subscales.

Intolerance of Uncertainty Scale 18 (IUS-18)

The Intolerance of Uncertainty Scale (IUS) (Dugas, Buhr, & Ladouceur, 2004) assesses the trait intolerance of uncertainty (IU), a factor that is proposed to play a role in the maintenance of different anxiety disorders. We applied the German version, which consists of 18 items (IUS-18) (Gerlach, Heinrichs, Bandl, & Zimmermann, 2007), for example, "Uncertainty makes me uneasy, anxious or stressed" (German: "*Unsicherheit bereitet mir Unbehagen, Angst oder Stress*") that are answered on a five-point scale from 0 = completely disagree (*stimme gar nicht zu*) to 4 = completely agree (*stimme völlig zu*). Based on analyses of different versions, three replicable factors were yielded: reduced ability to act due to IU, burden due to IU and vigilance due to IU. The reliability of the total scale is high with Cronbach's α =

.90, retest-reliability is r_{retest} = .66. Intolerance of uncertainty was assessed at the screening as a potential influence factor on the parameters of the NPU Threat Test, as it was shown to be associated with weakened aversive responses to uncertain threatening stimuli (Nelson & Shankman, 2011).

4.3.3 Procedure

The NPU Threat test was administered on day 1 and 5 of treatment about two hours after the intake of the morning medication. Testing took place at a laboratory located at the Chair of Clinical Psychology and Psychotherapy of the University of Regensburg. Upon arrival, participants were received by one of two experimenters that was male or female, respectively, for half of the subjects at random. They were then seated in front of a desktop PC and handed out a document that contained information on the procedure as well as the presented stimuli and conditions of the task (see appendix I). After clarification of possible open questions by the examiner, we proceeded with the placement of the electrodes for the physiological recordings as well as the electrodes for the electrical stimulation. Before conduction of the shock work-up, the examiner read out a standardized instruction (see appendix J). The adjustment was performed individually for each subject and on each of the testing days.

After the habituation phase, information on the three conditions was presented for a second time on the screen and participants were reminded by the experimenter to move as little as possible during the experiment to avoid artefacts of the physiological measurements. In case of no further questions, the experiment was started. The first block was followed by a break of five minutes during which the subjects completed questionnaires and were given the chance to move a bit. Following, the second block was started. At the end, participants completed the questionnaires for a second time and were disentangled from the electrodes.

4.3.4 Statistical analysis

Statistical analysis was conducted for the data of n = 19 for the etifoxine group, n = 17 for the alprazolam group and n = 18 for the placebo group. The respective participants had to be excluded because of technical issues that caused a lower shock strength during the task compared to the prior work-up. Additionally to the six participants that were excluded due to the different experimental settings, we excluded six further participants (n = 3 from the etifoxine group, n = 1 from the alprazolam group and n = 2 from the placebo group) for the analysis of the startle reactivity, since they were identified as non-responders.

Statistical analysis was carried out using the statistics software SPSS (version 25, IBM Statistics) with a significance level of $\alpha = .05$ for all analyses. Before analysis, startle data were transformed into t vales ([Z scores x 10] + 50) to account for interindividual variability of general startle reactivity (Blumenthal et al., 2005; D. E. Bradford, Starr, Shackman, & Curtin, 2015).

Using univariate ANOVAs, we tested for possible group differences concerning the trait variables related fear and anxiety (anxiety sensitivity and intolerance of uncertainty) with treatment as between subjects factor. To check for variation concerning the settings and ratings of the electrical shock and the white noise, we conducted repeated measures ANOVAs including study day (day 1, day 5) as within subjects factor and treatment as between subjects factor. Before computing repeated measures ANOVAs, we tested for homogeneity of variances using Levene's tests and sphericity using Mauchly-tests. In case of violation of sphericity, we report Greenhouse-Geisser (GG) corrected values. Significant main effects or interactions were followed up by post-hoc t-tests t or univariate ANOVAs to determine differences between the groups or time points. In these cases, we accounted for alpha error accumulation because of multiple testing by Bonferroni correction.

To check for a general suitability of the applied paradigm to induce fear and anxiety, we computed a repeated measures ANOVA comprising the within subject factors day (day 1, day 5), condition (N, P, U) and cue (NoCue, Cue) as well as the between subjects factor treatment. To analyze the effects of the medication on cued fear, we computed a repeated measures ANOVA with the within-subjects factor day (day 1, day 5) and cue (NoCue, Cue) and the between-subjects factor treatment for the values of the predictable (P) condition.

For the analysis of the effects of our treatment on contextual anxiety, we computed a repeated measures ANOVA with the within-subjects factor day (day 1, day 5) and condition (N, U) and the between-subjects factor treatment for the t-standardized scores of the time intervals when no cue was shown. The same analyses as for the startle responses were conducted for retrospective ratings of subjective anxiety.

Further analyses concerned the general startle reactivity. Therefore, we computed a repeated measures ANOVA including the values of the habituation phase that preceded the experiment. To examine the impact of the TSPO polymorphism, we repeated analyses for the startle measurements related to contextual anxiety and cued fear after exclusion of participants of the etifoxine group that were homozygous for the polymorphism.

4.4 Results

First, analysis of group differences concerning the trait variables related to fear and anxiety and concerning settings and ratings of the experimental manipulation are given. Following, the results for the effects of etifoxine and alprazolam on fear and anxiety related startle response and respective subjective ratings will be presented. Further analyses examined effects on baseline startle reactivity and the influence of the TSPO gene polymorphism rs6971.

4.4.1 Descriptives

4.4.1.1 Psychometric variables

The groups did not differ concerning the psychometric scales that were administered at the screening day including anxiety sensitivity in general as well as the subscales physical concerns, social concerns, and cognitive concerns. Further, intolerance of uncertainty (IU) and the subscales burden due to IU, vigilance due to IU, or reduced ability to act due to IU as well as trait anxiety assessed with the STAI did not differ between the groups (see also table 12).

Table 12

Means, Standard Deviations and Statistics for the Psychometric Trait Variables Related to Fear and Anxiety

Scale	Placebo	Alprazolam	Etifoxine	Statistics		
	M ± *SD*	*M* ± *SD*	*M* ± *SD*	*F* ratio	*df*	*p*
ASI-3 total	15.72 ± 9.53	13.29 ± 9.27	17.16 ± 6.95	0.91	2,51	.407
physical	4.78 ± 4.10	3.12 ± 3.43	4.47 ± 2.82	1.13	2,51	.332
social	7.56 ± 3.79	6.65 ± 3.48	9.00 ± 3.62	1.93	2,51	.156
cognitive	3.39 ± 3.03	3.53 ± 3.54	3.68 ± 2.67	0.04	2,51	.958
IUS-18 total	39.17 ± 13.22	39.13 ± 13.48	40.63 ± 9.09	0.09	2,51	.911
burden	13.06 ± 5.14	11.88 ± 4.68	13.11 ± 3.48	0.42	2,51	.659
vigilance	13.83 ± 4.67	15.18 ± 5.02	15.53 ± 4.15	0.69	2,51	.509
ability to act	12.28 ± 4.64	11.88 ± 5.01	12.0 ± 3.50	0.04	2,50	.963
STAI trait	34.35 ± 6.86	33.82 ± 6.70	30.79 ± 5.68	1.65	2,50	.203

Note. ASI = Anxiety Sensitivity Index, IUS = Intolerance of Uncertainty Scale, STAI = State-Trait-Anxiety Inventory.

4.4.1.2 Setting and ratings of the electric shock

The adjusted strength of the electric shock significantly differed between the two study days, $F_{1,49} = 9.87$, $p = .003$, $\eta_p^2 = .17$, with overall higher values on day 5 (1.23, 95%-CI[.44,

2.02]). However, participants of the three groups did neither choose different settings in general, $F_{2,49} = 2.78$, $p = .072$, $\eta_p^2 = .10$, nor depending on the study day, $F_{2,49} = 0.46$, $p = .634$, $\eta_p^2 = .02$. As can be seen in table 13, the values of the etifoxine group tended to be the highest on both days but there was neither a significant difference in comparison to alprazolam (2.19, 95%-CI[-.96, 5.34], $p = .274$) nor to placebo (2.78, 95%-CI[-.32, 5.88], $p = .092$).

Table 13

Means and Standard Deviations of the Applied Shock Strength (in mA) during the NPU at the Two Study Days

Study day	Placebo	Alprazolam	Etifoxine
	M ± *SD*	*M* ± *SD*	*M* ± *SD*
Day 1	5.61 ± 3.55	6.43 ± 2.20	8.16 ± 4.0
Day 5	6.84 ± 4.20	7.21 ± 2.46	9.85 ± 5.93

Importantly, the expectancy to receive a shock did not differ between the groups, $F_{2,51} = 0.14$, $p = .870$, $\eta_p^2 = .005$. The probability ratings changed significantly between the days, $F_{1,51} = 17.69$, $p < .001$, $\eta_p^2 = .26$, and differed between the conditions, $F_{1.44,73.34} = 310.43$, $p < .001$, $\eta_p^2 = .86$. They were further dependent on the cue, $F_{1,51} = 108.62$, $p < .001$, $\eta_p^2 = .68$ (see also table 14).

Table 14

Means and Standard Deviations of the Probability Ratings (%) for the Shock during the Different Conditions for the Two Days

Study day	Neutral		Predictable		Unpredictable	
	NoCue	Cue	NoCue	Cue	NoCue	Cue
	M ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*
Day 1	1.30 ± 3.61	1.02 ± 3.37	5.82 ± 12.62	58.41 ± 20.84	59.38 ± 21.71	51.45 ± 23.84
Day 5	.17 ± .80	.02 ± .14	.85 ± 3.36	53.51 ± 17.36	59.70 ± 22.37	37.48 ± 28.77

The mean strength of the shock (Day 1: $M = 6.52$, $SD = 1.18$; Day 5: $M = 6.64$, $SD = 1.18$) was similarly evaluated by the groups, $F_{2,51} = 1.99$, $p = .147$, $\eta_p^2 = .07$, on both testing days, $F_{1,51} = 0.56$, $p = .456$, $\eta_p^2 = .01$. Also the anxiety arising to the electric stimulation (Day 1: $M = 4.82$, $SD = 1.84$; Day 5: $M = 5.03$, $SD = 1.87$) did not differ between the days, $F_{1,51} = 1.06$, $p = .308$, $\eta_p^2 = .02$, and the three groups, $F_{2,51} = 1.48$, $p = .238$, $\eta_p^2 = .06$. The shock was

equally rated as painful (Day 1: M = 5.78, SD = 1.40; Day 5: M = 5.88, SD = 1.43) by the different groups, $F_{2,51}$ = 0.92, p = .405, η_p^2 = .04, on both days, $F_{1,51}$ = 0.36, p = .551, η_p^2 = .007.

4.4.1.3 Ratings of the white noise

Overall, participants experienced the white noise to be louder, $F_{1,51}$ = 22.99, p < .001, η_p^2 = .31, more unpleasant, $F_{1,51}$ = 18.52, p < .001, η_p^2 = .27, and more anxiogenic, $F_{1,51}$ = 13.02, p = .001, η_p^2 = .20, on day 1 of treatment in comparison to day 5. However, all parameters related to the tone were rated similarly by the three treatment groups: loudness, $F_{2,51}$ = 0.61, p = .549, η_p^2 = .02, unpleasantness, $F_{2,51}$ = 0.40, p = .672, η_p^2 = .02, and anxiety, $F_{2,51}$ = 1.83, p = .170, η_p^2 = .07.

4.4.2 Startle reactivity

4.4.2.1 Overall induction of fear and anxiety

Overall startle reactivity was shown to differ between the two treatment days, $F_{1,45}$ = 6.52, p = .014, η_p^2 = .13, with higher mean startle responses on day 1 of treatment (.12, 95%-CI[.03, -2.10]). Startle reactivity further differed according to the three conditions, $F_{2,4}$ = 123.53, p < .001, η_p^2 = .73, with the greatest responses during the U condition in comparison to the N condition (7.04, 95%-CI[5.77, 8.30], p < .001) and to the P condition (2.04, 95%-CI[.96, 3.12], p < .001). Additionally, overall responses during the P condition were higher than in the N condition (5.0, 95%-CI[3.92, 6.08], p < .001). Startle reactivity also differed according to the presentation of the cue, $F_{1,2}$ = 164.16, p < .001, η_p^2 = .79, with higher values during intervals when it was present (3.08, 95%-CI[2.60, 3.57], p < .001). There was no significant effect of treatment on overall startle reactivity, $F_{2,45}$ = 2.33, p = .109, η_p^2 = .09 (for an overview see table 15).

Table 15

Means and Standard Deviations (t scores) of the Startle Magnitude for the Different Conditions on Both Testing Days

	Neutral		Predictable		Unpredictable	
	NoCue	Cue	NoCue	Cue	NoCue	Cue
	M ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*
Placebo						
Day 1	45.66 ± 1.40	45.94 ± 1.90	47.28 ± 1.83	56.95 ± 4.03	52.69 ± 3.09	56.0 ± 2.87
Day 5	46.86 ± 1.85	46.75 ± 2.22	47.89 ± 2.04	55.50 ± 3.77	52.38 ± 2.78	53.68 ± 3.37
Alprazolam						
Day 1	46.63 ± 2.10	46.85 ± 2.73	47.93 ± 2.54	54.99 ± 4.35	52.34 ± 3.08	53.94 ± 3.72
Day 5	46.73 ± 1.71	46.97 ± 2.73	48.46 ± 2.66	54.14 ± 3.07	51.40 ± 3.10	54.41 ± 3.36
Etifoxine						
Day 1	46.07 ± 2.34	46.43 ± 2.02	48.43 ± 3.31	53.73 ± 2.12	52.99 ± 2.97	55.54 ± 3.90
Day 5	46.74 ± 2.08	46.48 ± 1.84	48.91 ± 2.04	53.84 ± 2.96	52.21 ± 2.14	54.95 ± 3.24

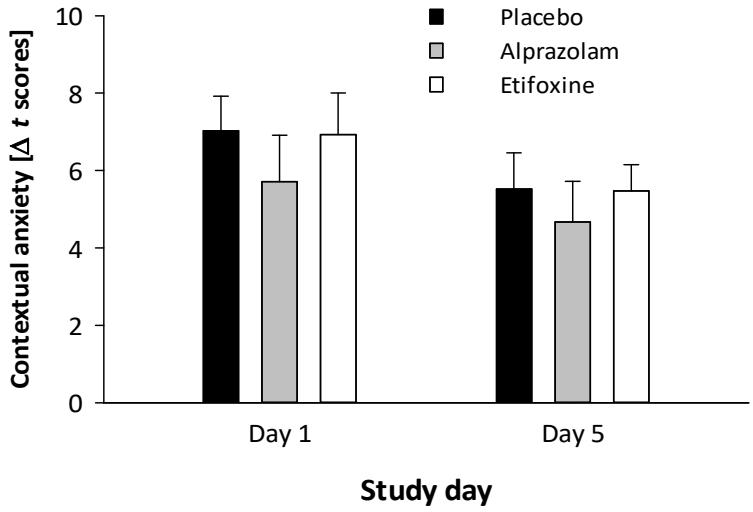
4.4.2.2 Contextual anxiety

Computations revealed a significant main effect of context, $F_{1,45} = 142.30$, $p < .001$, $\eta_p^2 = .76$, reflecting overall greater startle response during the U condition compared to the N condition for intervals when the cue was absent (see table 15 & figure 21). While overall startle reactivity did not differ between the two days, $F_{1,45} = 0.002$, $p = .962$, $\eta_p^2 = .00$, contextual anxiety changed across the days revealed by a significant interaction between context and day, $F_{1,45} = 5.49$, $p = .024$, $\eta_p^2 = .11$. Follow-up tests including the difference scores between the U and the N condition showed that contextual anxiety was greater on the first day of treatment (1.34, 95%-CI[.19, 2.48]).

However, as for cued fear there was no significant effect of treatment on overall startle reactivity, $F_{2,45} = 0.17$, $p = .848$, $\eta_p^2 = .007$, and further no effect on contextual anxiety as there was no significant interaction between context and treatment, $F_{2,45} = 0.50$, $p = .607$, $\eta_p^2 = .022$.

Figure 21

Anxiety Related Startle Response in the NPU Threat Test



Note. Startle response related to contextual anxiety is based on the difference of the t-scores (U – N) for the intervals when no cue was present. Error bars show standard errors.

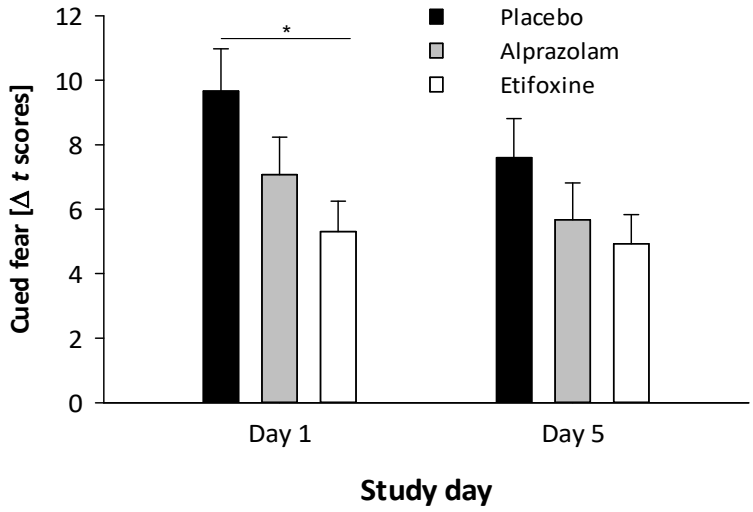
4.4.2.3 Cued fear

The startle magnitude during the predictable condition was significantly increased during the presence of the cue in comparison to periods when the respective cue was absent (see table 15 & figure 22; significant main effect of cue, $F_{1,45} = 160.79$, $p < .001$, $\eta_p^2 = .78$). Neither overall startle reactivity for the predictable condition independent of cue presentation differed significantly between the two testing days, $F_{1,45} = 0.12$, $p = .73$, $\eta_p^2 = .003$, nor fear potentiated startle reactivity, as there was no significant interaction between days and cue, $F_{1,45} = 2.92$, $p = .094$, $\eta_p^2 = .06$.

Overall startle reactivity in the predictable condition did further not differ between the three treatment groups, $F_{2,45} = 0.65$, $p = .529$, $\eta_p^2 = .03$. However, there was a significant effect of treatment on fear-potentiated startle revealed by a significant interaction between cue and treatment, $F_{2,45} = 3.80$, $p = .030$, $\eta_p^2 = .14$. Follow-up analyses including the difference scores for startles during presence / absence of the cue (Cue – NoCue) revealed a significant reduction of startle response by etifoxine (-3.52, 95%-CI[-6.74, -.30], $p = .028$) but not by alprazolam (-2.27, 95%-CI[-5.49, .96], $p = .262$) in comparison to placebo. However, the significant reduction of the fear potentiated startle in the etifoxine group was only found for day 1 of treatment (-4.37, 95%-CI[-8.41, -.32], $p = .031$) and not for day 5 of treatment (-2.68, 95%-CI[-6.51, 1.16], $p = .268$).

Figure 22

Fear Related Startle Response in the NPU Threat Test



Note. Startle response related to cued fear is based on the difference of the t-scores (Cue – NoCue) for the predictable condition. Error bars show standard errors. *$p < .05$.

4.4.3 Subjective ratings

4.4.3.1 Overall induction of fear and anxiety

Overall anxiety ratings were shown to differ between the two treatment days as there was a significant main effect of day, $F_{1,51} = 16.16$, $p < .001$, $\eta_p^2 = .22$, with higher values on day 1 of treatment (.42, 95%-CI[.19, .64]). Further, subjective anxiety differed between the three different conditions, $F_{1.42,72.22} = 263.66$, $p < .001$, $\eta_p^2 = .84$, reflecting the greatest anxiety during the U condition in comparison to the N condition (4.22, 95%-CI[3.64, 4.81], $p < .001$) and to the P condition (1.79, 95%-CI[1.41, 2.18], $p < .001$). Participants further stated higher subjective anxiety during the P condition in comparison to the N condition (2.43, 95%-CI[2.06, 2.80], $p < .001$). In accordance with the literature subjective anxiety also differed in response to the presence of the cue, $F_{1,51} = 133.27$, $p < .001$, $\eta_p^2 = .73$, with higher values during its presence (.91, 95%-CI[.75, 1.06]).

Overall subjective anxiety ratings did not differ with respect to the treatment, $F_{2,51} = 2.47$, $p = .095$, $\eta_p^2 = .09$ (for an overview see table 16).

Table 16

Means and Standard Deviations of the Anxiety Ratings for the Different Conditions on Both Testing Days

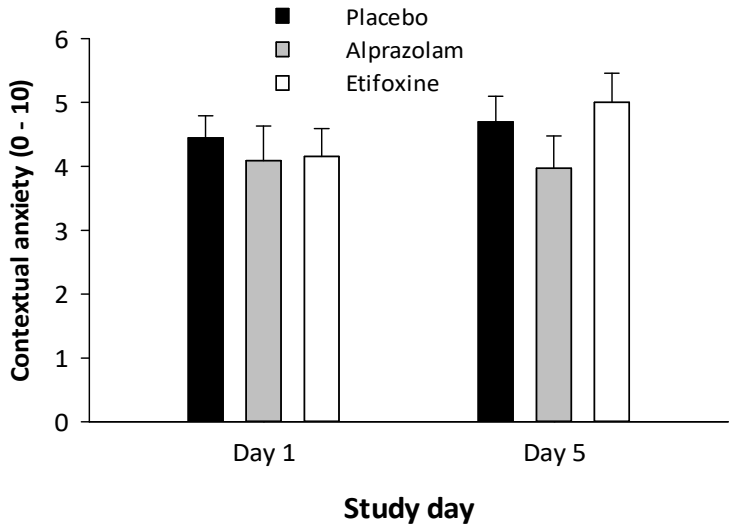
	Neutral		Predictable		Unpredictable	
	NoCue	Cue	NoCue	Cue	NoCue	Cue
	M ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*
Placebo						
Day 1	1.92 ± 1.42	1.97 ± 1.33	3.22 ± 1.65	5.56 ± 1.71	6.36 ± 1.62	6.75 ± 1.85
Day 5	1.28 ± .46	1.25 ± .46	2.03 ± .99	5.47 ± 1.50	5.97 ± 1.78	5.44 ± 2.09
Alprazolam						
Day 1	1.32 ± .39	1.38 ± .42	2.32 ± 1.38	5.09 ± 1.81	5.41 ± 2.41	4.85 ± 2.21
Day 5	1.12 ± .38	1.18 ± .43	1.47 ± .78	4.68 ± 1.95	5.09 ± 2.12	4.24 ± 2.49
Etifoxine						
Day 1	1.55 ± .85	1.55 ± .78	3.0 ± 1.36	5.55 ± 1.96	5.71 ± 1,71	5.84 ± 1.94
Day 5	1.29 ± .73	1.34 ± .71	2.11 ± 1.21	5.82 ± 1.98	6.29 ± 2.02	5.84 ± 2.13

4.4.3.2 Contextual anxiety

The experimental paradigm elicited contextual anxiety indicated by a significant effect of context with higher ratings for the U condition in comparison to the N condition, $F_{1,51}$ = 345.54, $p < .001$, $\eta_p^2 = .87$ (see figure 23). However, neither overall ratings differed significantly between the two testing days, $F_{1,51} = 2.69$, $p = .107$, $\eta_p^2 = .05$, nor those specifically reflecting contextual anxiety as there was no significant interaction between day and context, $F_{1,51} = 2.25$, $p = .140$, $\eta_p^2 = .04$. The applied anxiolytic substances did neither affect overall anxiety ratings for the neutral and the unpredictable condition, $F_{2,51} = 1.78$, $p = .180$, $\eta_p^2 = .07$, nor specifically contextual anxiety as there was no interaction between context and treatment, $F_{2,51} = 0.58$, $p = .566$, $\eta_p^2 = .02$.

Figure 23

Subjective Anxiety in the NPU Threat Test



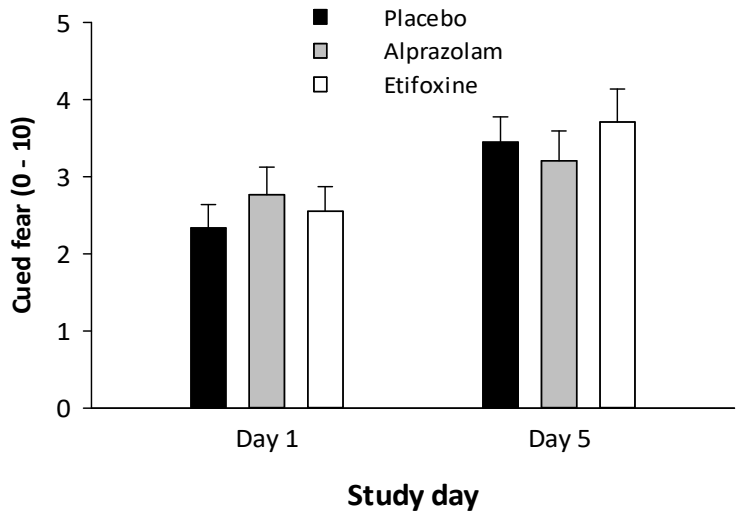
Note. Subjective anxiety is based on the difference scores (U – N) for the intervals when no cue was present. Error bars show standard errors.

4.4.3.3 Cued fear

Self-reported anxiety for the predictable condition was significantly increased by the presence of the cue reflecting specific fear (see figure 24; significant main effect of cue, $F_{1,51}$ = 257.97, $p < .001$, $\eta_p^2 = .84$). Overall ratings differed significantly between the two testing days, $F_{1,51} = 14.11$, $p < .001$, $\eta_p^2 = .22$, as well as specifically related to fear as there was a significant interaction between day and cue, $F_{1,51} = 26.62$, $p < .001$, $\eta_p^2 = .34$. Follow-up tests based on the difference scores (Cue – NoCue) revealed an increase of subjective fear from day 1 to day 5 of treatment (.90, 95%-CI[.55, 1.26]). The anxiolytic treatment did neither affect overall subjective ratings for the predictable condition, $F_{2,51} = 1.79$, $p = .178$, $\eta_p^2 = .07$, nor specific fear as there was no interaction between cue and treatment, $F_{2,51} = 0.15$, $p = .864$, $\eta_p^2 = .006$.

Figure 24

Subjective Fear in the NPU Threat Test



Note. Subjective fear is based on the difference scores (Cue – NoCue) for the predictable condition. Error bars show standard errors.

4.4.4 Further analyses

4.4.4.1 Baseline startle reactivity

In addition to the hypotheses on cued fear and contextual anxiety, we wanted to check for differences in baseline startle reactivity amongst the three treatment groups including the startle responses of the habituation phase before the start of the experiment. Analyses on t-transformed data showed that the baseline reactivity significantly differs between the two days, $F_{1,43} = 5.84$, $p = .020$, $\eta_p^2 = .12$, and according to the applied substances, $F_{1,43} = 3.88$, $p = .028$, $\eta_p^2 = .15$. Follow-up tests revealed significantly reduced baseline reactivity for the etifoxine group in comparison to placebo (-5.57, 95%-CI[-10.57, -.56], $p = .025$), while there was no significant effect of alprazolam (-3.23, 95%-CI[-8.23, 1.78], $p = .347$). There was no significant interaction between day and treatment, $F_{2,43} = 0.07$, $p = .93$, $\eta_p^2 = .003$.

We repeated those analyses for the raw data and confirmed the significant difference between the two days, $F_{1,43} = 7.39$, $p = .009$, $\eta_p^2 = .15$. As the effect of treatment reached significance on a level of 10 %, $F_{2,43} = 2.96$, $p = .062$, $\eta_p^2 = .12$, we checked which of the three treatment groups were responsible for that. Follow-up tests showed that baseline reactivity was slightly reduced in the alprazolam group compared to placebo although not significantly (-37.52, 95%-CI[-76.15, 1.11], $p = .060$). The other comparisons did not reach significance. There was no significant interaction between day and treatment, $F_{2,43} = 0.22$, $p = .802$, $\eta_p^2 = .01$.

4.4.4.2 TSPO polymorphism

We repeated the analyses for the startle response determined as cued fear or contextual anxiety, respectively, after exclusion of three participants of the etifoxine group that were homozygous concerning the TSPO related polymorphism rs6971 (see 2.2.3). For none of the two emotional states any main effect or interaction yielded deviant results after the exclusion of those participants. For the statistical results see table 17.

Table 17

Results of the Repeated Measures ANOVAs for Cued Fear and Contextual Anxiety after Correction for the TSPO Polymorphism

Parameter	Source	*F ratio*	*df*	*p*	η_p^2
Cued fear	Cue	147.57	1,43	**< .001**	.77
	Day	0.32	1,43	.576	.01
	Cue x day	2.40	1,43	.129	.05
	Treatment	0.43	2,43	.654	.02
	Cue x treatment	3.36	2,43	.044	.14
Contextual anxiety	Context	135.57	1,43	**< .001**	.76
	Day	0.01	1,43	.942	.00
	Context x day	6.46	1,43	**.015**	.13
	Treatment	0.12	2,43	.884	.01
	Context x treatment	0.52	2,43	.596	.02

4.5 Discussion

4.5.1 Overview

Within this part of the work, we investigated the acute and short-term effects of alprazolam and etifoxine on sustained anxiety and phasic fear in a standardized laboratory paradigm. We applied the NPU Threat Test on day 1 and 5 of treatment and assessed startle reactivity via an electrooculogram as well as subjective ratings in the two experimental groups and a placebo group. Importantly, our results show that the paradigm was suitable in eliciting differentiable responses of fear and anxiety. The highest startle amplitudes were noted in the predictable condition during presence of the fear-related cue, which indicated the application of an electric shock. This was followed by responses in the unpredictable condition independent of the cue resembling contextual or sustained anxiety, respectively. Overall, startle reactions in those two conditions exaggerated those of the neutral condition in which subjects were safe of any electric shocks. The same pattern as found in the startle data was also shown for the subjective ratings.

Against our hypothesis, none of the two compounds affected physiological and subjective measurements of contextual anxiety on any of the two testing days. However, etifoxine attenuated fear-related startle reactivity on day 1 of treatment but as alprazolam had no effects on the subjective experience of fear. For etifoxine none of the results changed after exclusion of participants that were homozygous for the gene polymorphism rs6971.

4.5.2 No effects of alprazolam and etifoxine on anxiety-related startle

Neither alprazolam nor etifoxine reduced the amplitude of the startle response in the unpredictable condition which has been identified to represent contextual anxiety (Schmitz & Grillon, 2012). Thus, we could not replicate the findings of previous studies that reported attenuating effects of alprazolam (Grillon et al., 2006; Riba et al., 2001) or other benzodiazepines (Graham et al., 2005; Scaife et al., 2005) within the context of the NPU Threat Test or akin paradigms. Thereby, the present study expands the inconsistent evidence commenced by other studies, which could not confirm those results as well (Acheson et al., 2012; Baas et al., 2009). Besides missing validation of the effects found after administration of a single dose of alprazolam, we could not confirm them for the anxiolytic TSPO ligand etifoxine. Interestingly, even extension of medication intake up to five days resembling a more chronic use did not engender inhibitory effects of the anxiolytic compounds.

One of the most striking differences between the present study and listed research on the attenuating effects of benzodiazepines in that context is constituted by the design. The present work is based on a between-subjects design whereas prior studies mostly applied a crossover design and compared the effects within subjects (Grillon et al., 2006; Riba et al., 2001). We tempted to account for the known interindividual variability of eyeblink measurements by performing transformation into standard values (Blumenthal et al., 2005; D. E. Bradford et al., 2015). However, possible differences on general startle reactivity between the three groups prior to administration of the substances, which might have biased our results, cannot be ruled out. Computations on the baseline activity during the habituation phase revealed significant differences between etifoxine and placebo for the t-standardized values. In contrast, a slight reduction of overall startle amplitude by alprazolam in comparison to placebo was shown for the raw data. This analysis must be interpreted with caution, though, as it is based on a very small number of evaluable trials and subjects were already aware of the upcoming paradigm and its inconveniences. The inclusion of a concrete baseline measurement as part of the screening before inclusion to the study would have been important to wipe out such uncertainties (Grillon & Baas, 2002). Indeed, the present work did include comparisons within subjects, as we administered the task for a second time after five days of treatment. In line with previous research, the paradigm has proven suitable for repeated measures, as startle reactivity during the unpredictable condition still exaggerated that of the safe context at the second session (Klumpers et al., 2010). Yet, the difference between those two conditions was lower in comparison to the first application indicating some habituation effect. Overall lowered reactivity on day 5 of treatment might have caused a floor effect and at least partly explain why it was not even attenuated by the anxiolytic substances after prolonged treatment.

We applied the NPU Threat Test once after a single dose of either placebo, 0.5 mg alprazolam or 50 mg etifoxine as well as after five days taking that dose three times a day. Election of the respective dosages was based on initial values prescribed within the clinical setting as well as on studies that reported anxiolytic effects of both substances in patients (Stein, 2015). However, with respect to startle reactivity in healthy subjects, they might have been too small. Although one study revealed a single dose of 0.5 mg alprazolam to be effective for the reduction of the fear-related startle stronger effects were yielded by a dose of 1 mg (Riba et al., 2001). Another study even reported attenuating effects of alprazolam only for 1 mg but not for 0.5 mg (Grillon et al., 2006). The assumption that the effects of the higher dose might have solely risen due to increased sedation was rebutted in that study by showing that the sedative non-anxiolytic diphenhydramine did not show such specific effects. Nevertheless, it would still

be possible that, for example, increased muscle relaxation could be responsible for those findings. Indeed, dose-dependency was also shown in an animal study on etifoxine which revealed effects on physiological responses to an anxiety-related context, which was conditioned by foregoing administration of electric shocks, only for higher doses of the TSPO ligand (Verleye & Gillardin, 2004).

Besides methodological reasons, effects of the applied medication might be restricted to highly anxious subjects. Indeed, questionnaires showed that the subjects of the present study were in a lower or medium range of anxiety sensitivity, uncertainty intolerance and trait anxiety. However, for example, trait anxiety of our sample with a mean of 32.6 (*SD* = 6.4) was similar to that reported from other studies which averaged at values of 28.2 (*SD* = 4.8) (Grillon et al., 2006) or 31.8 (*SD* = 7.3) (Baas et al., 2009). Nevertheless, it would for sure be interesting to transfer those investigations to samples of patients suffering from anxiety and other stress-related disorders, as those show deviant reactivity, most of all, elevated startle responses to contextual anxiety (Grillon et al., 2008; Pole, Neylan, Best, Orr, & Marmar, 2003).

Another important point concerns possible gender effects. While only male subjects were included in the present study, previous work was based on gender mixed samples with an even higher rate of females in some studies (Acheson et al., 2012; Grillon et al., 2006; Riba et al., 2001). It cannot be ruled out that the mentioned sample characteristics might at least partly explain the deviant results.

4.5.3 Reduction of fear-related startle after acute treatment with etifoxine

Concerning cued fear, the fact that the we did not find effects of alprazolam on startle reactivity during presence of the cue in the predictable condition is consistent with prior research (Baas et al., 2009; Grillon et al., 2006). This finding has mainly been contributed to the assumption of sustained anxiety and cued fear being distinguishable states that involve different mechanisms and are therefore differentially modulated by anxiolytic substances. Approval stems from animal studies that revealed stronger involvement of the central nucleus of the amygdala in phasic responses to explicit threat cues, whereas the bed nucleus of the stria terminalis seems more related to sustained responses to contextual stimuli (Walker et al., 2003).

In contrast to alprazolam, etifoxine did reduce the startle response to the fear related cue in the predictable condition on day 1 of treatment. This is somewhat surprising as the indications of both substances are more targeted on general states of anxiety with etifoxine being prescribed for general psychosomatic manifestations of anxiety (Biocodex, 2015) and alprazolam for the symptomatic treatment of acute and chronic states of tension, agitation and anxiety (ratiopharm

GmbH, 2015). However, it must be noted that the actual field of application and research confirming efficacy of etifoxine mostly concerns adjustment disorders with anxiety (Nguyen et al., 2006; Servant et al., 1998; Stein, 2015). This classification is related to the experience of specific stressors, mostly of psychological nature, and the appearance of pathological symptoms within a period of six months after the incident (Vanin & Helsley, 2008). Besides the stated research, to date, there exist few controlled trials on this disorder and there is little knowledge on characteristic disruptions within the central nervous system or on biological markers (Semprini, Fava, & Sonino, 2010; Stein, 2018). The specific effects of etifoxine on fear-related startle response and the close connection to that disorder might serve as a hint on possible markers, which recommends further laboratory research.

The twofold action mechanism of etifoxine with directly binding to the $GABA_A$ receptor and additionally modulating TSPO (Hamon et al., 2003) might serve as an explanation for its specific effect. Expression of the latter was significantly increased on day 1 of treatment in comparison to alprazolam and placebo while the difference decreased over the course of the study (see 3.5.6). An explicit connection between TSPO, modulating ligands like etifoxine and the regulation of specific structures related to fear remains to be further clarified. However, an animal study of neuropathic pain reported alleviating effects of etifoxine after local infusion into the basolateral amygdala suggesting a prominent role of this network (Zeitler et al., 2016). This part of the amygdala is strongly involved in positive and negative affect and further interacts with the central nucleus of the amygdala, which has been identified to play a major role for specific fear (Davis & Whalen, 2001; Walker et al., 2003). A relation between TSPO and specific fear can further be drawn from a study in healthy subjects who received XBD-173, another ligand that exclusively binds to the peripheral benzodiazepine receptor. After seven days of treatment, subjective experience of symptoms related to panic induced by injection of CCK-4 was significantly attenuated in comparison to placebo (Rupprecht et al., 2009).

Finally, the positive results found for etifoxine in the present work might have risen due to chance. However, because of their specificity this seems quite unlikely. Nevertheless, the question remains why the effect was only detectable on day 1 of treatment. Although the two testing days did not significantly differ with respect to the startle responses related to specific fear, they had apparently decreased, especially for the placebo and the alprazolam group, in comparison to the first implementation of the paradigm. Those slight changes, although not significant, might have contributed to the fact that the group differences indicating an effect of etifoxine were not evident on day 5 of treatment anymore. However, further research is needed to examine those guesses.

Repetition of the performed analyses after exclusion of three participants of the etifoxine group that there were homozygous for the TSPO related gene polymorphism rs6971 yielded no changes of any of the reported effects. For further discussion of that point see 3.5.8.

4.5.4 No effects of alprazolam and etifoxine on subjective fear and anxiety

Both, subjective ratings of contextual anxiety and specific fear were not affected by the anxiolytic treatment with alprazolam and etifoxine. Thus, although fear-related startle was shown sensitive to etifoxine on day 1 of treatment, those effects were not accessible to the subjective experience of fear. This is in line with previous studies that showed attenuating effects of anxiolytic or antidepressant compounds on fear- or anxiety-potentiated startle reactivity but not concerning respective self-reports (Grillon et al., 2006; Grillon, Levenson, & Pine, 2007). The present study showed that even longer administration of medication does not ensure subjective effects in a healthy sample.

Pharmacological compounds exert effects on a molecular level that are often reflected in changes of physiological parameters without being expressed in subjective measurements in healthy subjects. Since verbal reports of emotions involve a certain amount of cognitive activity, they might just be unable to uncover early drug effects. With respect to that, it must be noted that the ratings on the fearful cue and the anxiogenic context, which were stated on a scale from 1 to 10, did on average not exceed values of 7. Hence, although the three conditions were clearly separable with respect to the different emotional states, the subjects in our sample might not have experienced a notable level of anxiety and fear, which could have been attenuated by the treatment. Furthermore, in contrast to physiological measures, self-reports are more under conscious control and may be even more strongly susceptible to placebo effects.

Since the subjective ratings were acquired retrospectively after completion of the single blocks, subtle differences in responding to the six different conditions may have been concealed by the time elapsed. Therefore, further research should consider online assessment of the subjective state.

Lastly, other studies reported an association between ratings on fear and anxiety and reports on mental and physical sedation (Baas et al., 2002). However, the present study revealed only negligible differences with respect to sedation between the three groups (see also chapter 6) what could partly explain the missing findings on the other self-reports.

4.5.5 Conclusion

Using the NPU Threat Test, we revealed different effects of the benzodiazepine alprazolam and the TSPO ligand etifoxine on fear and anxiety in healthy subjects. While none of the substances had an impact on anxiety-related startle on any of the two testing days, etifoxine yielded attenuating effects on fear-potentiated startle on day 1 of treatment. Future research should target the question if the different modulation of the GABAergic system by the two anxiolytics might explain the observed differences. This might, for example, be realized by application of the fear-potentiated startle paradigm in animal studies or within the scope of neuroimaging studies that use versions of the NPU Threat Test, which are compatible with MRI scanning. Furthermore, it should be examined why the effects of etifoxine were restricted to the measurement on the first day of intake. Although a slight habituation effect was shown with respect to overall startle response on day 5 of treatment, in general, the paradigm was still capable of generating measurable differences of startle reactivity between the three conditions. Hence, we could confirm repeatability of the NPU Threat Test, which offers a good opportunity to investigate anxiolytic effects of pharmacological compounds, thereby differentiating between specific fear and contextual anxiety.

Overall, the present work joins the line of inconsistent findings reported from studies that investigated the effects of benzodiazepines on fear- and anxiety-related startle. With respect to methodology, future studies on the effects of alprazolam and etifoxine should include within-subjects comparisons or at least adequate baseline measurements as part of the screening procedure. Thereby, possible group differences before administration of the treatment could be ruled out and it could further be ensured that subjects display expected fear and anxiety reactions within the scope of the paradigm. To further disentangle the specific contribution of sedation and anxiolysis, future studies might profit from dose dependent comparisons as well online assessment of subjective experience.

CHAPTER 5: Effects of etifoxine & alprazolam on resting state connectivity

5.1 Theoretical background

In addition to the investigation of subjective, physiological and endocrine changes due to administration of psychoactive substances, the identification of neural networks underlying their therapeutic and sometimes sedative effects has increasingly gained interest. Studies assessing the neural activity of the brain rely on the quantification of changes of the blood oxygenation level-dependent (BOLD) signal due to higher oxygen consumption in activated regions, which was initially observed in response to external stimuli (Weishaupt, Koechli, & Marincek, 2009). However, research revealed that also during rest, fluctuations of low-frequency (< 0.1 Hz) in regions of the motor cortex were highly temporally correlated with each other and with other regions (Biswal, Yetkin, Haughton, & Hyde, 1995). Emerging research in this field has shown that so-called resting state networks (RSNs) are specific and follow replicable temporal and spatial patterns. Neuroimaging studies that considered comprehensive data sets of healthy subjects during rest revealed several networks representing functional systems, including areas that are related to visual or auditory processing, memory, motor and executive functions, and the so-called default-mode network. (Damoiseaux et al., 2006; Laird et al., 2011; Smith et al., 2009)

Meanwhile, resting state functional magnetic resonance imaging (rs-fMRI) has become a commonly used technique and was shown to be sensitive to different mental disease states (Fornito & Bullmore, 2010) and pharmacological manipulation (Honey & Bullmore, 2004). Thereby, it enables an analysis of drug effects that is not biased by the presence of any task.

There are several options to determine changes of brain activity during rest. One is so-called seed-based analysis, which allows the investigation of functional connectivity changes (Biswal et al., 1995). For this kind of analysis certain regions of interest are defined beforehand based on findings of previous research and changes of connectivity within these predefined areas or in relation to other areas are then analyzed. Using this technique, connectivity between the amygdala and the ventral medial prefrontal cortex as well as the orbitofrontal cortex was shown to be reduced in healthy subjects after administration of antidepressant medication for seven days (McCabe & Mishor, 2011). Since this study did not reveal any differences in mood ratings, the authors suggested that the dampened connectivity could resemble an earlier stage of rebalancing cortical distortions that are associated with depressive symptoms.

However, although commonly applied in the research on functional connectivity, the a priori selection of seed regions comes along with the risk of biased results or oversight of effects in other brain areas (Margulies et al., 2010). An alternative technique that does not rely on the specification of certain regions and constitutes a more data-driven approach is independent component analysis (ICA) (Beckmann, DeLuca, Devlin, & Smith, 2005). ICA aims at decomposing a multivariate signal into independent signals that are maximally non-Gaussian resulting in maps of voxels, which are spatially independent but show the same time courses of the BOLD signal (Bijsterbosch, Smith, & Beckmann, 2017). At group-level whole brain RSNs can be identified that share the mentioned characteristics across the group.

Using this approach, several studies showed effects of GABAergic compounds on functional connectivity within and between networks. It was shown that, for example, sedation induced by benzodiazepines like midazolam or zolpidem is associated with increased functional connectivity in sensory, motor and limbic brain regions (Greicius et al., 2008; Licata et al., 2013). Oral administration of ethanol, which is also known to modulate the GABAergic system, caused an increased connectivity within the visual network, especially in primary visual areas (Esposito et al., 2010). After 7-day administration of the anxiolytic agent diazepam, an increased connectivity within the medial visual network and the middle and inferior temporal network was reported by Pflanz and colleagues (2015). While the latter network is connected to emotional processing, the stronger activation of the medial visual system was attributed to the high occurrence of $GABA_A$ receptors within this area leading to high binding of GABAergic substances.

As far as we know, there is no other study that investigated the effects of alprazolam, etifoxine or any other TSPO ligand on resting state functional connectivity without further stimulation. However, one study investigated the effects of 1 mg alprazolam on pharmacologically induced panic by injection of the neuropeptide CCK-4 (Leicht et al., 2013). In line with previous literature, they found an increase of activity in the rostral anterior cingulate cortex, a region that is related to anxiety, which was attenuated by alprazolam. This is in accordance with another study that investigated the effects of alprazolam on an emotional face matching task in patients suffering from generalized anxiety disorder (G. G. Brown et al., 2015). They reported reduced activity of anxiety induced activation in the amygdala and the insula after acute administration of the benzodiazepine. Changes in brain activity after acute administration of alprazolam were further shown in a study that observed decreases in whole-brain cerebral blood flow of up to 30 % in comparison to placebo (Roy-Byrne et al., 1993). This was specified by another study that investigated the effects of 1 mg alprazolam in subjects with

or without a family history of alcoholism (Streeter et al., 1998). They reported a decrease of cerebral blood flow in the right inferior prefrontal cortex and the anterior cingulate cortex for the healthy subgroup.

With regard to etifoxine, there was just one study that applied another imaging technique - positron emission tomography - in rats (Bouillot et al., 2016). They compared binding affinity of flumazenil to the $GABA_A$ receptor, which is known to bind with comparable affinity as benzodiazepines, after injection of diazepam and etifoxine. While diazepam decreased binding of flumazenil, etifoxine led to an increase of flumazenil binding. The authors concluded that the allosteric effect of etifoxine might be associated to a disturbance of the structure of the GABAergic complex, thereby causing changes in other binding sites. Overall, they provided further evidence for the pharmacological differences that have been postulated for etifoxine and benzodiazepines in prior research.

5.2 Research questions and hypotheses

Given that resting state fMRI has proven to be sensitive for detecting drug-related effects, we aimed to identify alterations in resting-state connectivity patterns following the administration of etifoxine and alprazolam in comparison to placebo. Therefore, we conducted resting-state measurements on day 4 of treatment. We performed ICA and tested for alterations in commonly known RSNs (Smith et al., 2009) as well as further components that were chosen based on classification criteria for spatial distribution and time courses (Griffanti et al., 2017).

Based on previous research, we hypothesized that both substances would change resting-state functional connectivity within and between a variety of RSNs related to emotion processing and GABA activity in comparison to placebo. However, because of the different sedative properties and action mechanisms on the GABAergic system, we also expected to find differences for the activation patterns of the two anxiolytic compounds.

5.3 Methods and procedure

5.3.1 Resting state measurement

To address our questions concerning the effects of etifoxine and alprazolam on functional connectivity within and between brain networks, we performed resting state fMRI scans. The absence of any task throughout the complete session allowed the assessment of the medication effects without possible biases.

Functional resting state data were acquired using echo planar imaging (EPI) that was first introduced by Mansfield (1977) and allows fast acquisition of MRI data. Within the EPI multiple echoes are elicited by repeated switching of the frequency gradients after a single high frequency-pulse of 90°. Within one repetition time (TR = interval between two consecutive excitations of the same layer) up to 128 echoes can be acquired which yields an excellent temporal resolution (Weishaupt et al., 2009).

To control for the low spatial resolution of the functional data and to gain deeper information about the underlying anatomy, further structural images for the later registration of the functional data were gathered using a Magnetization Prepared Rapid Gradient Echo (MP-RAGE) scan. MP-RAGE is a 3D sequence used for the rapid acquisition of structural scans with high spatial resolution which is reached by a double phase coding (y- and z- direction). An initial 180° HF-pulse first aligns the magnetization antiparallel to the magnetic field. Dependent on the respective tissue this leads to a specific T1-relaxation, which describes the back flipping of the transversal magnetization into the z-direction whereby energy is released. After the inversion time, transversal magnetization and thereby the measurable signal is elicited. (Stöcker & Shah, 2013)

5.3.2 Scan parameters

For the resting state analysis, we acquired a whole-brain EPI sequence consisting of 450 volumes with axial slices of 3 mm thickness (voxel size = 3.0 × 3.0 × 3.0 mm^3, field of view (FOV) = 192 × 192 mm^2, echo time (TE) = 30 ms, TR = 2.0 s, flip angle = 75 °). We used a multi-band acceleration factor of 2 in a descending order.

The T1-weighted structural images consisted of 160 axial slices with a thickness of 1 mm using an MP-RAGE sequence (voxel size = 1.0 × 1.0 × 1.0 mm^3, FOV = 250 × 250 mm^2, TR = 1910 ms, TE = 3.67 ms, flip angle = 9 °).

We further acquired diffusion tensor imaging as well as magnetic resonance spectroscopy data. However, the analysis of these data was not part of the present thesis and will not be discussed here.

5.3.3 Procedure

Functional imaging took place on the premises of the Neuroradiology Department at the Bezirksklinikum Regensburg with a 3 Tesla scanner (Magnetom Prisma Siemens, Erlangen, Germany) and a 20-channel head-coil. Scanning was scheduled to start at 1 PM and participants were instructed to take their medication one hour prior to the MRI session. Due to technical problems and routine maintenance work, respectively, scanning had to be shifted to the evening for two participants.

After arrival at the scanner facility, one of the two MRI operators repeated the scanner specific screening for possible exclusion criteria. We ensured that all points had been understood by the subjects and that no changes had occurred since the first completion of the form. Then, participants were asked to complete VAS on their present mood, calmness, wakefulness, anxiety and concentration (see 3.3.2.2), both directly before as well as after the measurement. Before entering the scanner room, all participants were carefully checked to be completely free of any ferromagnetic object.

Inside the scanner room, we first attached the built-in photoplethysmograph to the index finger as well as the respiration belt to monitor pulse and respiration, respectively, throughout scanning. Participants were equipped with in-ear earplugs (3M E-A-R Classic, 3M, Neuss, Germany) and headphones to reduce the noise exposure. They further received an alarm bell, which they should ring in case of emergency. After positioning the subjects at the scanner table as comfortably as possible, cushions were put between the head and the coil to avoid movement. After information about the duration of the different scanning sequences, the operator left the room and further communication in between the scans took place via a speaker system. Participants were asked to close their eyes during the measurements, to think of nothing in particular and to try not to fall asleep.

The MRI scanning lasted about 54 minutes in total, including a head scout (00:14 minutes), resting state (15:08 minutes), structural data (04:27 minutes), diffusion tensor imaging (18:15 minutes), and magnetic resonance spectroscopy (16:05 minutes) measurements.

5.3.4 Data and statistical analysis

Data from n = 20 of the etifoxine group, n = 20 of the alprazolam group and n = 18 of the placebo group were included into the analysis. For two subjects there were no imaging data available, because of technical issues with the scanner for one subject of the placebo group and exclusion of another subject because of MRI contraindication that was just stated at the day of the scan.

The subjective data were analyzed using SPSS (version 25, IBM Statistics). FMRI data were analyzed using the FMRIB software library (FSL version 6.0.2, FMRIB Analysis Group, Oxford University, UK; http://fsl.fmrib.ox.ac.uk) and MATLAB (version R2017b, MathWorks, Natick, Massachusetts, US). Analysis of the imaging data was performed in collaboration with the Biomedical Imaging Group (Chair of Psychiatry and Psychotherapy, University of Regensburg) under supervision of Professor Jens Schwarzbach.

First, neuroimaging data were preprocessed. After converting the DICOM-files into NIfTI format that is required for FSL analysis, we applied bias correction and brain extraction (Smith, 2002) to the structural data and normalized them into standard space using the Montreal Neurological Institute (MNI)-152 template of 2 mm. The functional data were corrected for motion (Jenkinson, Bannister, Brady, & Smith, 2002), high-pass filtered with a cut-off of 100 seconds, spatially smoothed using a Gaussian kernel of full width at half maximum of 5 mm and slice time corrected because of the multiband acquisition. Afterwards the functional data were co-registered to the subject's own structural data. ICA-AROMA (Automatic Removal of Motion Artifacts) (Pruim et al., 2015) was used to identify components that represent motion artifacts. This procedure is based on a classification algorithm that decides upon the following four discriminative features: maximum correlation to realignment parameters, edge fraction, cerebrospinal fluid fraction, and high-frequency content. Using linear regression, the components labelled as noise were removed from the fMRI time-series and the denoised data were co-registered to standard space.

Determination of functional connectivity networks was based on ICA. The independent components (ICs) were extracted by Multivariate Exploratory Linear Optimized Decomposition into Independent Components (MELODIC) as implemented in FSL (Beckmann et al., 2005). Data were temporally concatenated across subjects and principal component analysis was used to estimate Bayesian dimensionality (Beckmann & Smith, 2004). To optimize component loading and avoid excessive splitting of known RSNs, the number of components was restricted to 40 beforehand. Furthermore, they were thresholded at values of $z > 3$. The obtained ICs were checked for meaningfulness based on spatial distribution and time courses

and components that resembled noise, for example, if comprising mainly white matter or much high-frequency signal, were excluded (Griffanti et al., 2017). Using correlation analyses we tested how much the remaining components resembled RSNs known from the literature (Smith et al., 2009).

To obtain subject-specific components from the extracted group spatial maps, we applied dual regression (Beckmann, Mackay, Filippini, & Smith, 2009). Testing for differences between the three groups was accomplished using voxel-wise, non-parametric permutation *t*-tests with 5000 permutations including the following contrasts: Etifoxine > Alprazolam, Alprazolam > Etifoxine, Etifoxine > Placebo, Placebo > Etifoxine, Alprazolam > Placebo, Placebo > Alprazolam. This included threshold-free cluster enhancement (Smith & Nichols, 2009) with a significance level of $p = .05$ and correction for multiple testing. Visualization of the results is based on computations which examined the voxels with the highest *z*-values for each contrast and component. Based on that we checked for each component to which other ICs these changed voxels were best associated.

5.4 Results

5.4.1 Self-reports before and after the MRI scan

Subjects reported significantly worse mood, $F_{1,55} = 12.76$, $p = .001$, $\eta_p^2 = .19$, less arousal, $F_{1,55} = 5.43$, $p = .023$, $\eta_p^2 = .09$, more fatigue, $F_{1,55} = 44.66$, $p < .001$, $\eta_p^2 = .45$, and decreased concentration, $F_{1,55} = 30.08$, $p < .001$, $\eta_p^2 = .35$, after the MRI scan in comparison to the assessment right before the scan (see also table 18). Subjective anxiety did not change significantly in response to the scanning session, $F_{1,55} = 3.62$, $p = .063$, $\eta_p^2 = .06$.

The three groups did not differ on any of the subjective parameters assessed with the VAS: mood, $F_{2,55} = 0.98$, $p = .381$, $\eta_p^2 = .03$, calmness, $F_{2,55} = 0.38$, $p = .686$, $\eta_p^2 = .01$, wakefulness, $F_{2,55} = 1.42$, $p = .251$, $\eta_p^2 = .05$, anxiety, $F_{2,55} = 0.47$, $p = .627$, $\eta_p^2 = .02$, and concentration, $F_{2,55} = 0.83$, $p = .441$, $\eta_p^2 = .03$. Further, there were no significant interactions between treatment and any of the subjective ratings: mood, $F_{2,55} = 0.82$, $p = .448$, $\eta_p^2 = .03$, calmness, $F_{2,55} = 0.14$, $p = .873$, $\eta_p^2 = .005$, wakefulness, $F_{2,55} = 0.74$, $p = .484$, $\eta_p^2 = .03$, anxiety, $F_{2,55} = 1.61$, $p = .209$, $\eta_p^2 = .06$, and concentration, $F_{2,55} = 0.48$, $p = .621$, $\eta_p^2 = .02$.

Table 18

Means and Standard Deviations of the Visual Analogue Scales before and after the MRI Scan

Parameter	Placebo		Alprazolam		Etifoxine	
	Pre scan	Post scan	Pre scan	Post scan	Pre scan	Post scan
	M ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*
Mood (0 – 10)	7.89 ± 1.08	7.17 ± 1.89	8.55 ± 0.83	7.38 ± 1.56	8.10 ± 0.97	7.60 ± 1.23
Calmness (0 – 10)	6.67 ± 1.65	7.56 ± 1.89	6.35 ± 2.08	6.95 ± 2.61	6.40 ± 2.56	7.45 ± 1.85
Wakefulness (0 – 10)	6.94 ± 1.59	5.17 ± 2.43	6.90 ± 1.71	4.60 ± 1.73	7.25 ± 1.74	5.75 ± 1.25
Concentration (0 – 10)	7.33 ± 1.14	6.0 ± 2.06	6.88 ± 1.15	5.73 ± 1.55	7.20 ± 1.36	6.35 ± 1.23
Anxiety (0 – 10)	2.89 ± 1.13	3.0 ± 2.11	2.80 ± 1.24	2.30 ± 1.17	3.20 ± 1.96	2.40 ± 1.05

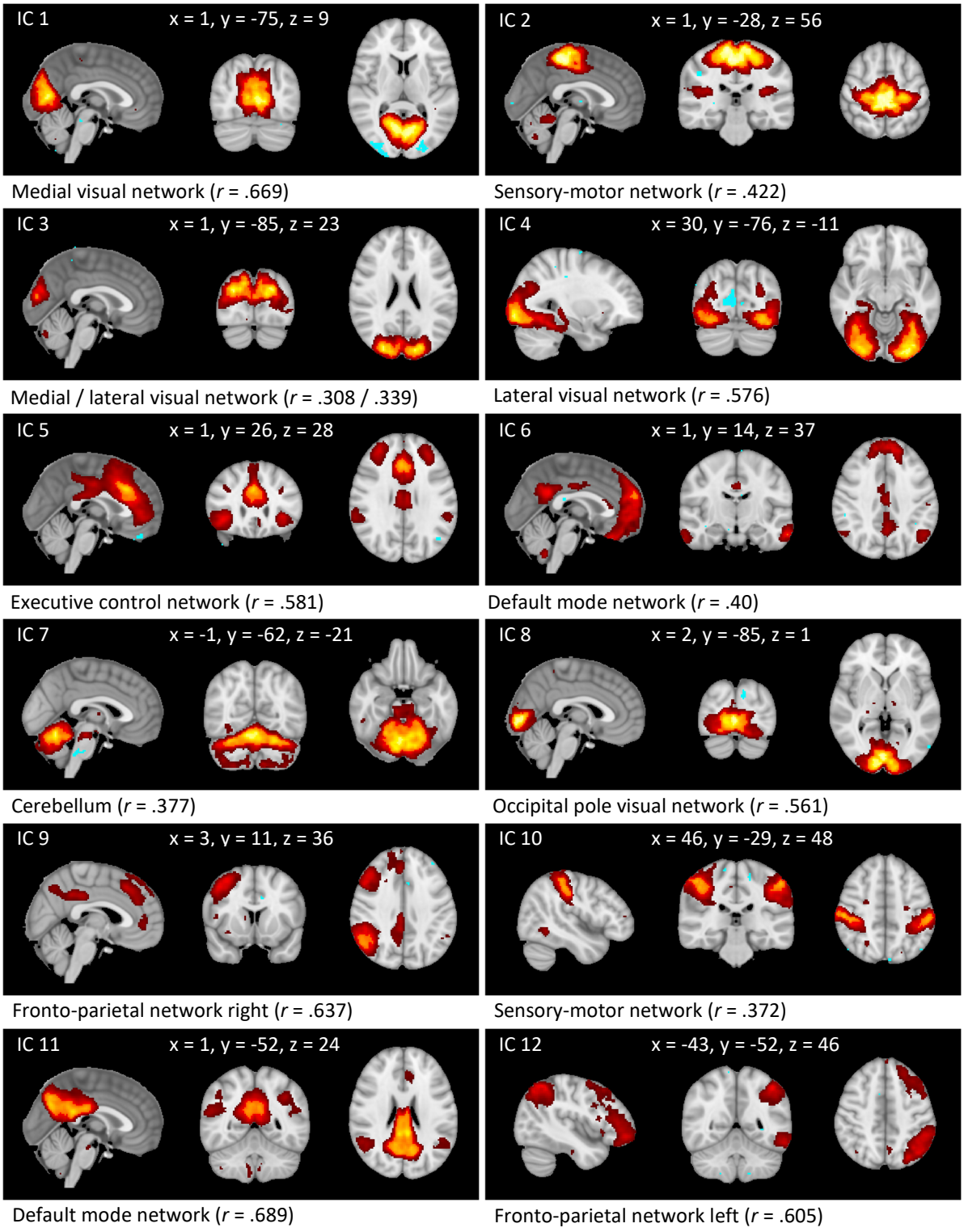
5.4.2 Extracted independent components

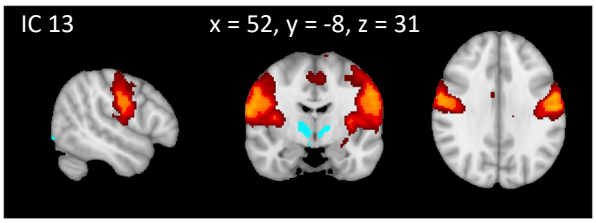
With respect to the detected 40 ICs, 30 were identified as networks of interest as they either resembled RSNs described by previous research (Smith et al., 2009) or otherwise fulfilled the criteria concerning spatial distribution and time courses (for the excluded components see appendix K). The RSNs as described by Smith et al. (2009) comprised the following networks: medial visual, sensory-motor, lateral visual, executive control, default mode, cerebellum,

occipital pole visual, left / right fronto-parietal, and auditory. Furthermore, we identified networks including putamen, thalamus, pallidum, middle temporal gyrus, orbitofrontal cortex, inferior frontal / paracingulate gyrus, pre- / postcentral gyrus, precuneus, angular gyrus, amygdala, parahippocampal gyrus, lateral occipital cortex and the medial frontal cortex (for an overview see figure 25).

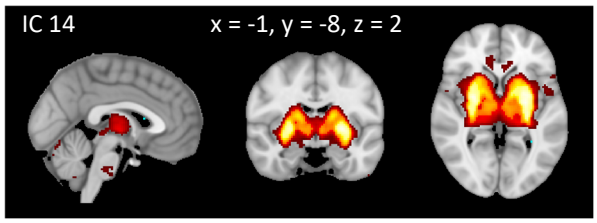
Figure 25

Overview of Independent Components of the Resting-State fMRI Data

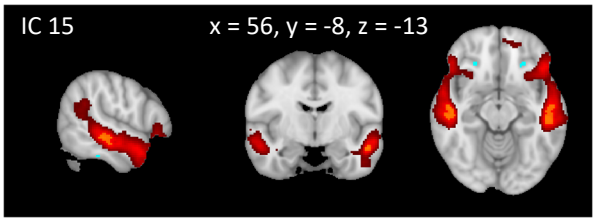




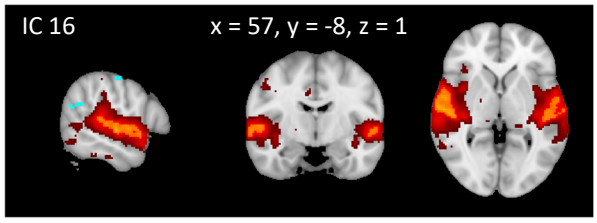
Sensory-motor network (*r* = .418)



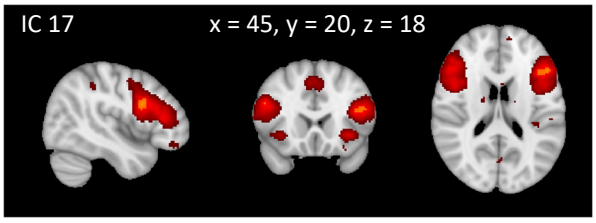
Putamen / thalamus / pallidum (no corr.)



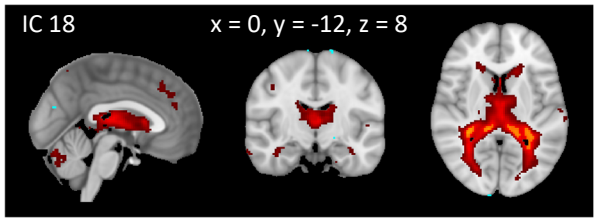
Middle temporal gyrus / orbitofrontal cortex (no corr.)



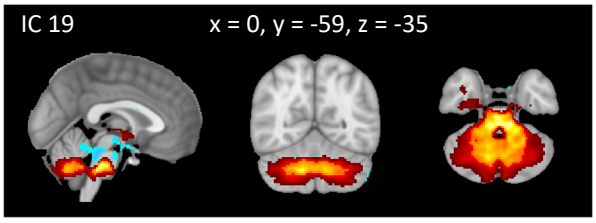
Auditory network (*r* = .549)



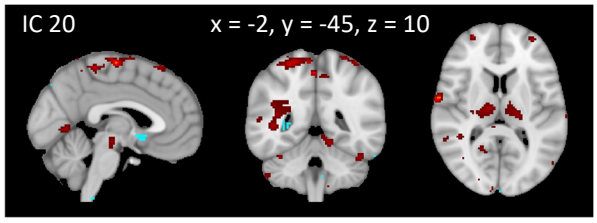
Inferior frontal / paracingulate gyrus (no corr.)



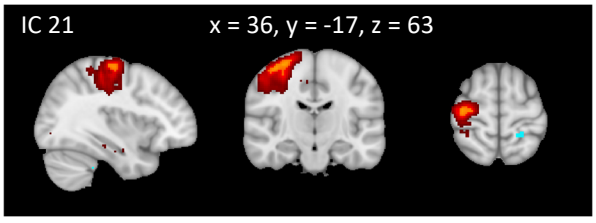
Thalamus (no corr.)



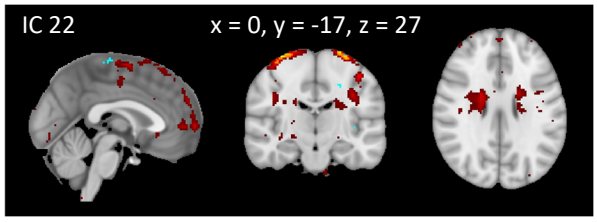
Cerebellum (*r* = .350)



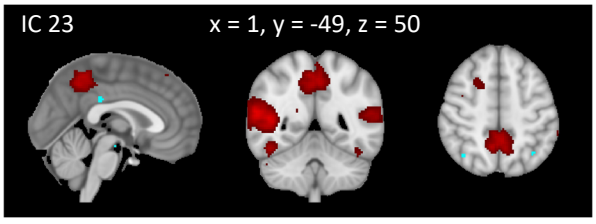
Thalamus / precentral gyrus (no corr.)



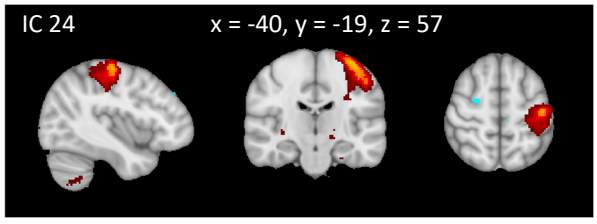
Motor cortex right (no corr.)



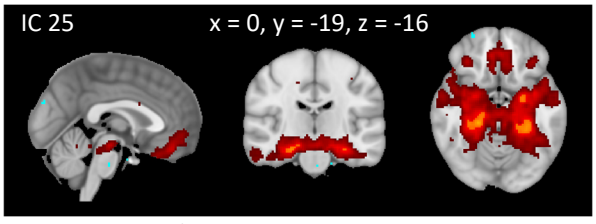
Precentral gyrus (no corr.)



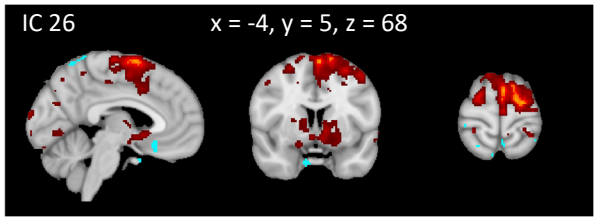
Precuneus / angular gyrus / lat. occ. cortex (no corr.)



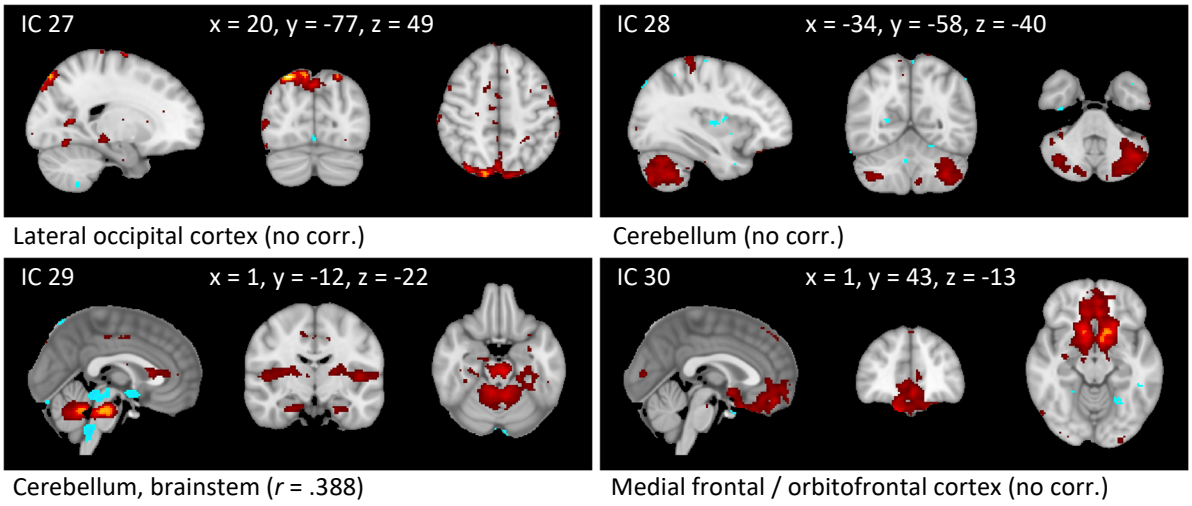
Motor cortex left (no corr.)



Paracingulate / parahippocampal gyrus, amygdala, medial frontal cortex (no corr.)



Superior frontal gyrus / putamen (no corr.)



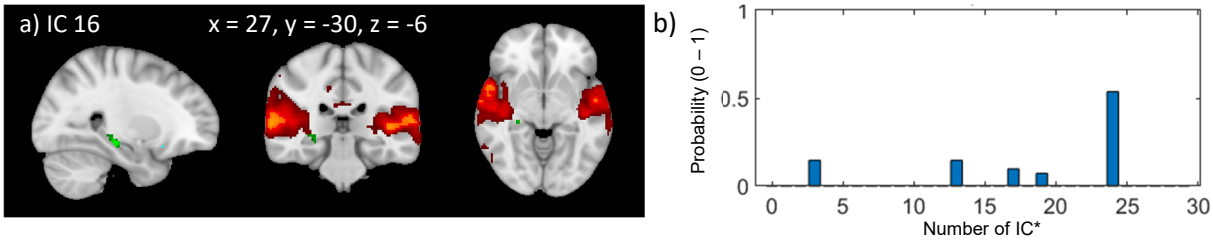
Note. Components of interest, as identified by ICA of all subjects' data (N = 58), overlaid onto the MNI 1-mm standard brain ($z > 3$); hot colors: regions with shared signal time-course, cold colors: anti-correlations with signal time-course; *r* indicates the correlation to a RSN from Smith et al. (2009) (only values $r > .3$ are reported).

5.4.3 Differences in functional connectivity between etifoxine and placebo

We revealed significant differences between etifoxine and placebo for the auditory network ($p = .003$). Significantly higher temporal coherence was observed in the placebo group between the auditory network and paracingulate / parahippocampal gyrus, amygdala and medial frontal cortex and further lateral visual areas, putamen, thalamus, pallidum, and precentral gyrus (see figure 26 a + b). There was no statistically significant increase of coherence in any network due to etifoxine in comparison to placebo.

Figure 26

Changes of Functional Connectivity for the Contrast Placebo > Etifoxine



Note. a) Statistical parametric maps for the contrast PLC > ETI for the auditory network overlaid onto the MNI 1-mm standard brain (hot colors: regions with shared signal time-course, cold colors: anti-correlations with signal time-course, green: regions with significantly increased functional connectivity in the placebo group compared to the etifoxine group). b) Map depicting the probabilities for IC 16 to which components changed voxels associate most (*scale starts from 0 = IC 1).

5.4.4 Differences in functional connectivity between alprazolam and placebo

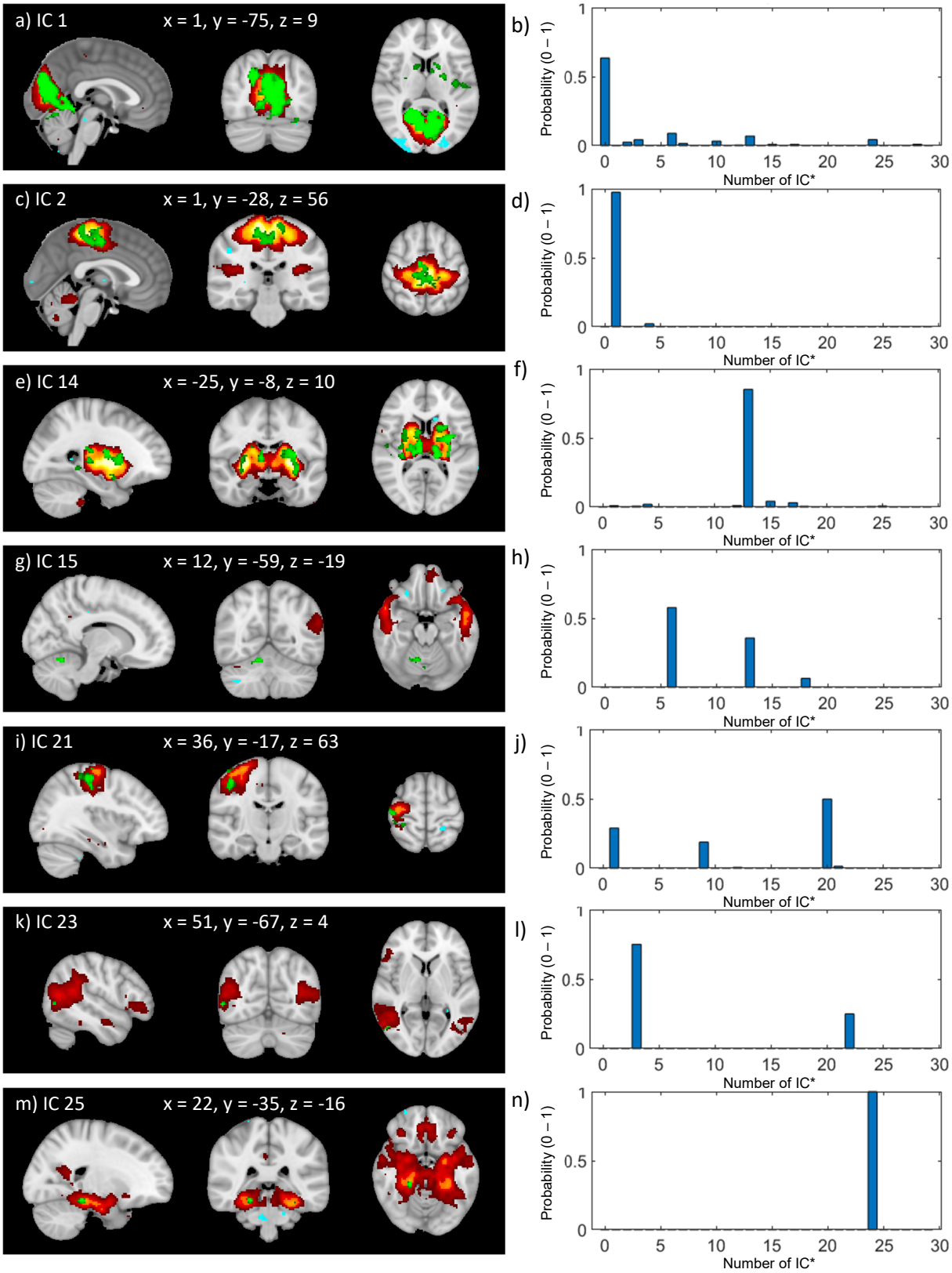
Significant differences between alprazolam and placebo were revealed for the medial visual network ($p = .0002$), sensory motor network ($p = .002$), putamen, thalamus, pallidum ($p = .002$), middle temporal gyrus, orbitofrontal cortex ($p = .001$), auditory network ($p = .007$), right motor cortex ($p = .001$), precuneus, angular gyrus, lateral occipital cortex ($p = .006$), paracingulate / parahippocampal gyrus, amygdala, medial frontal cortex ($p = .002$), and medial frontal / orbitofrontal cortex ($p = .001$).

Temporal coherence was significantly increased in the alprazolam group relative to the placebo group for the following ICs:

- within the medial visual network (see figure 27 a + b)
- within the sensory motor network (see figure 27 c + d)
- within a network including putamen, thalamus and pallidum (see figure 27 e + f)
- between a network spanning from middle temporal gyrus to the orbitofrontal cortex and the cerebellum, putamen, thalamus, and pallidum (see figure 27 g + h)
- within the right motor cortex and between this brain area and the sensory motor cortex (see figure 27 i + j)
- within a network including precuneus, angular gyrus and the lateral occipital cortex (see figure 27 k + l)
- within a network including the paracingulate / parahippocampal gyrus, amygdala and the medial frontal cortex (see figure 27 m + n)

Figure 27

Changes of Functional Connectivity for the Contrast Alprazolam > Placebo



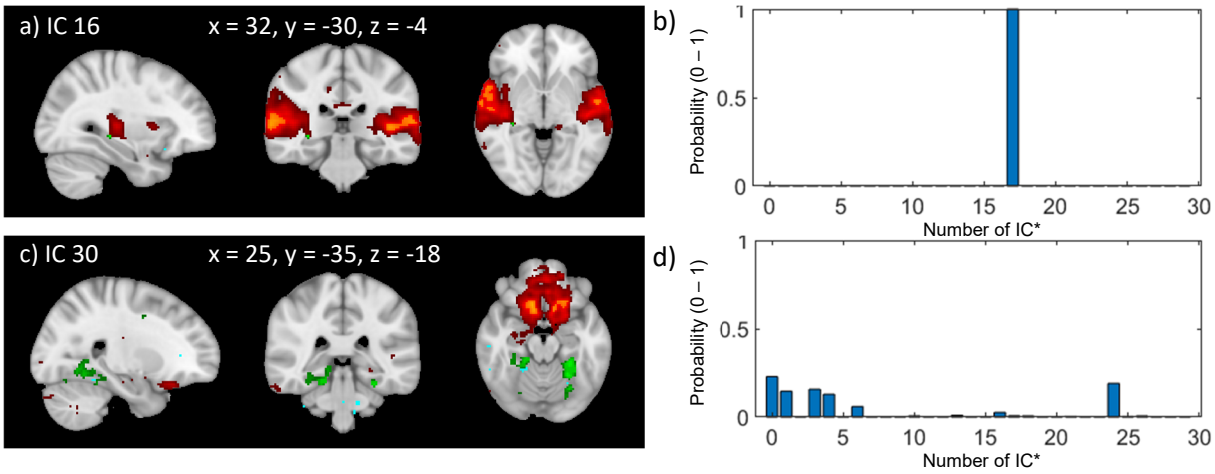
Note. Statistical parametric maps for the contrast ALP > PLC for a) medial visual network, c) sensory motor network, e) putamen, thalamus, pallidum, g) middle temporal gyrus, orbitofrontal cortex, i) right motor cortex, k) precuneus, angular gyrus, lateral occipital cortex and m) paracingulate / parahippocampal gyrus, amygdala, medial frontal cortex overlaid onto the MNI 1-mm standard brain (hot colors: regions with shared signal time-course, cold colors: anti-correlations with signal time-course, green: regions with significantly increased functional connectivity in the alprazolam group compared to the

placebo group). Maps depicting the probabilities for b) IC 1, d) IC 2, f) IC 14, h) IC 15, j) IC 21, l) IC 23 and n) IC 25 to which components changed voxels associate most (*scale starts from 0 = IC 1).

In contrast, temporal coherence was significantly decreased in the alprazolam group relative to the placebo group between the auditory network and the thalamus (see figure 28 a + b) as well as between a network including the medial frontal / orbitofrontal cortex and the medial visual network, the sensory motor network, lateral visual network, executive control network, cerebellum, and a network including the paracingulate / parahippocampal gyrus, amygdala and the medial frontal cortex (see figure 28 c + d).

Figure 28

Changes of Functional Connectivity for the Contrast Placebo > Alprazolam



Note. Statistical parametric maps for the contrast PLC > ALP for a) auditory network and c) medial frontal / orbitofrontal cortex overlaid onto the MNI 1-mm standard brain (hot colors: regions with shared signal time-course, cold colors: anti-correlations with signal time-course, green: regions with significantly increased functional connectivity in the placebo group compared to the alprazolam group). Maps depicting the probabilities for b) IC 16 and d) IC 30 to which components changed voxels associate most (*scale starts from 0 = IC 1).

5.4.5 Differences in functional connectivity between etifoxine and alprazolam

Significant differences between etifoxine and alprazolam were revealed in the medial visual network (p = .0004), putamen, thalamus, pallidum (p = .007), precentral gyrus (p = .001), precuneus, angular gyrus, lateral occipital cortex (p = .005), paracingulate / parahippocampal gyrus, amygdala, medial frontal cortex (p = .005), and medial frontal / orbitofrontal cortex (p = .0002).

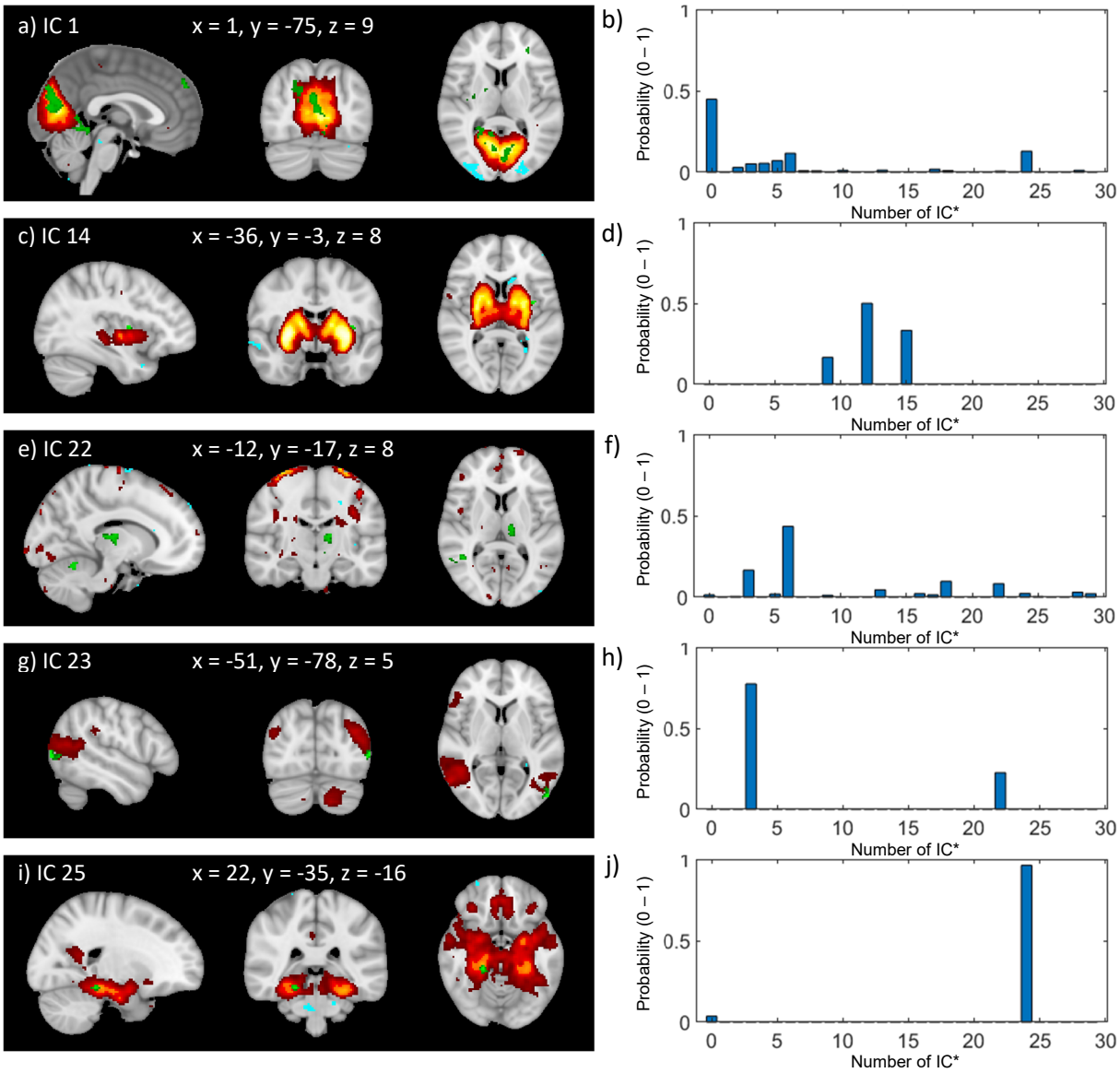
Significantly increased temporal coherence was observed in the alprazolam group relative to the etifoxine group for the following ICs:

- within the medial visual network (see figure 29 a + b)

- between a network including putamen, thalamus and putamen to the sensory motor network and the auditory network (see figure 29 c + d)
- between the precentral gyrus and the lateral visual network and the cerebellum (see figure 29 e + f)
- within a network including the precuneus, angular gyrus and the lateral occipital cortex and further between those areas and the lateral visual network (see figure 29 g + h)
- within a network including the paracingulate / parahippocampal gyrus, amygdala and the medial frontal cortex (see figure 29 i + j).

Figure 29

Changes of Functional Connectivity for the Contrast Alprazolam > Etifoxine



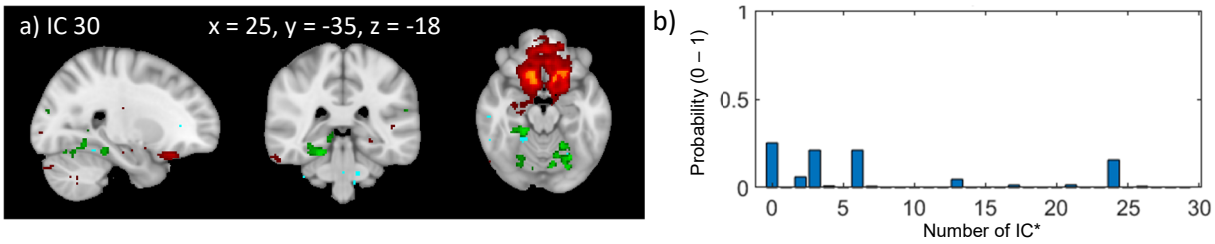
Note. Statistical parametric maps for the contrast ALP > ETI for a) medial visual network, c) putamen, thalamus, pallidum, e) precentral gyrus, g) precuneus, angular gyrus, the lateral occipital cortex and i) paracingulate / parahippocampal gyrus, amygdala and the medial frontal cortex overlaid onto the MNI 1-mm standard brain (hot colors: regions with shared signal

time-course, cold colors: anti-correlations with signal time-course, green: regions with significantly increased functional connectivity in the alprazolam group compared to the etifoxine group). Maps depicting the probabilities for b) IC 1, d) IC 14, f) IC 22, h) IC 23 and j) IC 25 to which components changed voxels associate most (*scale starts from 0 = IC 1).

Temporal coherence was significantly decreased in the alprazolam group relative to the etifoxine group between the medial frontal / orbitofrontal cortex and the medial / lateral visual network, cerebellum, and a network including the paracingulate / parahippocampal gyrus, amygdala, and the medial frontal cortex (see figure 30 a + b).

Figure 30

Changes of Functional Connectivity for the Contrast Etifoxine > Alprazolam



Note. Statistical parametric maps for the contrast ETI > ALP for a) the medial frontal / orbitofrontal cortex overlaid onto the MNI 1-mm standard brain (hot colors: regions with shared signal time-course, cold colors: anti-correlations with signal time-course, green: regions with significantly increased functional connectivity in the etifoxine group compared to the alprazolam group). b) Map depicting the probabilities for IC 30 to which components changed voxels associate most. (*scale starts from 0 = IC 1).

5.5 Discussion

5.5.1 Overview

In this section of the work, we investigated changes of functional connectivity within and between brain areas on day 4 of treatment with either a daily dose of 1.5 mg alprazolam, 150 mg etifoxine or placebo. Therefore, we performed resting-state fMRI and applied ICA to extract group components that are spatially independent while sharing the same time courses of BOLD signal. We identified 30 independent components including RSNs known from literature (Smith et al., 2009) and additional networks including areas like putamen, thalamus, pallidum, angular, and parahippocampal gyrus as well as regions of the frontal cortex.

Although there were no effects of the treatment on self-reported mood before or after the MRI scan, analyses revealed several changes of functional connectivity within and between the identified networks due to the anxiolytic medication while. Contrast analyses showed decreased functional connectivity between the auditory network and networks including the paracingulate / parahippocampal gyrus, amygdala, medial frontal cortex, and the thalamus due to etifoxine and alprazolam in comparison to placebo. Temporal coherence was further decreased by alprazolam in comparison to placebo and etifoxine between frontal areas and several brain regions related to vision, motor function, emotion and executive control. However, the most prominent findings were related to increases of coherence due to alprazolam in comparison to the two other groups for networks including widespread regions of the brain.

5.5.2 Decreased functional connectivity between the auditory network and other brain areas due to etifoxine and alprazolam

Similar results were revealed for alprazolam and etifoxine with respect to the auditory network as both compounds decreased functional connectivity between that component and other brain regions in comparison to placebo. While for etifoxine regions of the limbic system, basal ganglia and frontal areas were concerned, alprazolam specifically decreased temporal coherence between the auditory network and the thalamus.

Research on the effects of different CNS depressants on connectivity changes related to the auditory network has so far been contradictory. Similar to the results that were observed for etifoxine, one study found decreases in connectivity between the amygdala and the auditory cortex due to the benzodiazepine oxazepam (Flodin, Gospic, Petrovic, & Fransson, 2012). While the amygdala is known to be involved in the regulation of negative emotion (Nuss, 2015)

and salience attribution of stimuli (Costafreda, Brammer, David, & Fu, 2008), the middle temporal gyrus including the auditory cortex is related to processing of emotional audiovisual information (Park et al., 2010). Therefore, it was hypothesized that the functional uncoupling between brain areas related to perception and the amygdala might play a vital role in the calming effects of anxiolytic substances (Flodin et al., 2012). Within the present work, the anxiolytic treatment decreased synchrony between the auditory cortex and further structures of the limbic system including hippocampus, thalamus and prefrontal areas which are equally involved in the regulation of aversive states and the generation of responses to emotional stimuli (Nuss, 2015). Since subjects of this clinical trial were at rest and were not presented any emotional stimuli, it can only be speculated if the functional uncoupling of those areas was related to specific perceptions. For instance, it would be conceivable that the loud monotonous noise of the MRI scanner was not perceived as unpleasant due to the anxiolytic agents.

In addition to emotional aspects, the amygdala, the medial prefrontal cortex as well as parts of the thalamus are known to be involved in higher order processes including decision making and associative learning (Euston, Gruber, & McNaughton, 2012; Mitchell, 2015; Seymour & Dolan, 2008). Hence, decreased functional coupling between the auditory cortex and those areas might also indicate lowered higher order processing of perceived stimuli. However, these specific results do not speak for strongly sedative effects of the administered medication, since past research on the effects of CNS depressants mostly showed increased correlation of BOLD signal in auditory cortices, for example after administration of the anesthetic agent midazolam (Kiviniemi et al., 2005) or the hypnotic zolpidem (Licata et al., 2013). Those studies explicitly used dosages which were shown to elicit reliable subjective drug effects in healthy subjects (Licata, Mashhoon, Maclean, & Lukas, 2011). Combined with the fact that we did not find any group differences concerning self-reported sedation in the course of the MRI measurements, it might be concluded that the administered doses of alprazolam and etifoxine did not induce sedation, or that any possible sedation was not indicated by connectivity changes of the auditory network. Interestingly, other studies found no effects with respect to the auditory cortex after administration of diazepam (Pflanz et al., 2015) or alcohol (Esposito et al., 2010). However, it must be noted that those studies only reported changes of functional connectivity within networks, thereby possibly missing effects as those reported in the present work. To clarify the role of decreased or increased functional connectivity with respect to the auditory network in anxiolysis and sedation after administration of benzodiazepines and TSPO ligands, further research is clearly needed.

5.5.3 Decreased functional connectivity between the frontal cortex and other brain areas due to alprazolam

Alprazolam decreased functional connectivity between the medial frontal / orbitofrontal cortex and the medial / lateral visual network, cerebellum and a network including the paracingulate / parahippocampal gyrus, amygdala and the medial frontal cortex in comparison to both, placebo and etifoxine. In comparison to the placebo group further changes between that component and the sensory motor network as well as the executive control network were revealed.

As has been pointed out in the previous subchapter, the decreased connectivity between frontal areas, which are related to executive function (Tamminga, 2004) and regions which are involved in sensory or emotional processing, might indicate some functional uncoupling of the concerned processes. Furthermore, it might be relevant that alprazolam is a benzodiazepine which is not only indicated for the treatment of anxiety disorders, but also possesses antidepressant properties (Arvat et al., 2002). In a study that used seed-based analysis of resting-state data reduced connectivity between frontal areas and the amygdala was found after the intake of SSRIs and SNRIs, respectively, over a period of seven days (McCabe & Mishor, 2011). Thereby, our findings for alprazolam fit the results shown for antidepressant medication with decreasing resting-state functional connectivity in areas that are involved in reward and emotional processing. The lack of overlap with etifoxine might be due to the fact that – in the context of mental disorders – efficacy of the TSPO ligand has so far mostly been shown for anxiety disorders with a focus on adjustment disorder with comorbid anxiety (Alexandrowsky et al., 2010; Nguyen et al., 2006; Stein, 2015).

Since studies on other benzodiazepines mostly did not report decreased functional connectivity (Kiviniemi et al., 2005; Licata et al., 2013; Pflanz et al., 2015) but studies on stimulants did (S.-J. Li et al., 2000; Wong, Olafsson, Tal, & Liu, 2012), it has been concluded that such changes might indicate stimulation, alertness and increased excitability (Pflanz et al., 2015). Although we would not suppose that alprazolam exerted stimulating effects within the present sample, this might still show that this benzodiazepine exerted fewer sedative effects than the previously investigated GABAergic compounds. Furthermore, it must be noted that analyses within the mentioned studies on benzodiazepines mostly relied on RSNs known from literature (Smith et al., 2009). Although those comprise the left and right fronto-parietal network, effects specific for frontal regions might have been missed out using this approach.

5.5.4 Increased functional connectivity of several widespread resting state networks due to alprazolam

Most prominently, we observed increased functional connectivity within and between a series of the extracted independent components including the medial visual network, sensory motor network, limbic structures, basal ganglia as well as temporal and frontal regions after intake of alprazolam in comparison to etifoxine and placebo. Similar changes with respect to placebo and etifoxine were revealed within the medial visual network and with respect to a network including structures of the basal ganglia and the limbic system, and a network including precuneus, angular gyrus and the lateral occipital cortex. Altered functional connectivity that was specific for the comparison to placebo was observed within the sensory motor network, especially the right motor cortex, and between a network spanning from middle temporal gyrus and the orbitofrontal cortex. In contrast, specific changes with respect to etifoxine were shown for the precentral gyrus.

The changes of connectivity due to alprazolam concerned various components. However, especially with respect to visual, sensory motor, limbic, and temporal cortices the finding of increased temporal coherence due to alprazolam lines up with previous research. Similar effects were observed after administration of benzodiazepines like diazepam (Pflanz et al., 2015) or midazolam (Kiviniemi et al., 2005) as well as other $GABA_A$ receptor ligands like alcohol (Esposito et al., 2010), zolpidem (Licata et al., 2013) or bicuculline (Nasrallah, Singh, Yeow, & Chuang, 2017). This underlines the pharmacological similarity of alprazolam to the mentioned compounds, which was not confirmed for the TSPO ligand etifoxine in the present work. It has been concluded that increases in functional connectivity of RSNs might serve as an indicator for anxiolysis, sedation and a decreased level of consciousness, respectively (Pflanz et al., 2015). Still, it needs to be further disentangled which of those properties are most strongly related to the neural changes. Overall, the increased temporal coherence between networks due to pharmacological agents reflecting higher synchronicity of activity between the respective brain areas might indicate inhibition of differentiated neural processing. The present findings of alprazolam apparently affecting multiple RSNs in a seemingly non-specific manner fit previous research that identified decreases in whole-brain cerebral blood flow of up to 30 % after acute administration of alprazolam in comparison to placebo (Roy-Byrne et al., 1993).

Besides the question of the respective state that is reflected by the connectivity changes, there remains the question of the processes underlying the effects that were observed for alprazolam and other GABA modulating substances. A possible explanation lies in the density of GABAergic receptors within the affected regions. Studies using cytoarchitectonical brain

mapping revealed a high density of $GABA_A$ receptors in the primary visual cortex, the primary somatosensory cortex and the primary auditory cortex (Zilles & Amunts, 2009). In a study that applied positron emission tomography highest levels of central benzodiazepine receptors were found in the medial occipital and medial frontal areas (Abadie et al., 1992). Those brain regions might be particularly susceptible to benzodiazepines like alprazolam and respond with simultaneous changes of the BOLD signal. With respect to that, the differences in connectivity between alprazolam and etifoxine might be explained by the fact that they modulate distinct subunits of the $GABA_A$ receptor (Bouillot et al., 2016; Hamon et al., 2003), which are differentially distributed in the brain (Sieghart & Sperk, 2002). Furthermore, the twofold action mechanism of etifoxine and overall lower binding to the central benzodiazepine receptor in comparison to alprazolam might contribute to the distinct patterns of functional connectivity (Bouillot et al., 2016). Modulation of the GABAergic system by etifoxine furthermore seems to be strongly mediated by neurosteroids like allopregnanolone (Verleye et al., 2005), which was not found to be affected by the TSPO ligand in our sample of healthy male subjects.

Interestingly, in contrast to the present work, prior research on the effects of GABA modulating substances mostly reported no or only small changes of functional connectivity within subcortical areas like the thalamus although these areas show a high density of $GABA_A$ receptors (Nasrallah et al., 2017; Pflanz et al., 2015). Therefore, it was suggested that GABAergic neurons in subcortical regions might play a minor role in the increases of cortical connectivity (Nasrallah et al., 2017). However, studies in rats showed that thalamic connectivity to other areas is suppressed due to anesthesia (Liang, King, & Zhang, 2012). Hence it has been suggested that a lack of connectivity changes with respect to subcortical structures might be distinctive for pharmacological substances that induce high levels of sedation.

Further explanation of changed coherence within and between brain networks targets activity of the HPA axis and the related release of corticosteroids. Research has shown that undergoing an MRI session and prior expectations, respectively, increase subjective nervousness with heightened cortisol levels in some subjects (Keulers, Stiers, Nicolson, & Jolles, 2015; Muehlhan, Lueken, Wittchen, & Kirschbaum, 2011). Those increases of cortisol were shown to be associated with decreases in brain activity, for example, in regions of the cingulate cortex as well as frontal and parietal areas (Keulers et al., 2015). Furthermore, patterns of functional resting-state connectivity between regions of the visual cortex and other brain areas were shown to be associated with cortisol secretion in a dose-dependent manner (Muehlhan et al., 2020). Thus, changes of functional brain connectivity might indicate effects of cortisol on sensory processes. With respect to that, the probability of correct classification

between rest and stress based on connectivity changes of brain regions including the cingulate cortex, amygdala, and precuneus has been associated with individual increases of cortisol (Zhang et al., 2020). Within the present work, alprazolam but not etifoxine was shown to dampen stress-induced activity of the HPA axis (see 3.5.4), thereby confirming prior research on benzodiazepines. Although subjects reported low anxiety before and after the scan, they were more relaxed while stating worse mood at the end of the MRI session. Altered cortisol release related to subjective experiences during the MRI scan and concomitant changes of functional brain connectivity might have been selectively prevented in the group that was pretreated with alprazolam causing the differences found with respect to placebo and etifoxine.

While most of the differences were comparable with respect to placebo and etifoxine, alterations of connectivity within areas related to sensorimotor functions were restricted to the comparison with the group that did not receive an active substance. It might therefore be speculated that both anxiolytic compounds were to some extent related to changes of movement during the scan, possibly indicating muscle relaxing effects. Overall, it cannot be ruled out that some of the subjects fallen asleep during acquisition of the resting state data what might have contributed to our findings. We only occasionally asked our subjects about this after the scan and some could not answer the question clearly. Although no group differences were identified with respect to self-reported sedation, it seems possible that subjects who were pretreated with alprazolam became more sleepy while lying in the scanner and being exposed to the monotonous noise. If this was the case, the lowered activation – at least in visual, auditory and motor related regions – in this group might have contributed to the observed group differences concerning functional connectivity.

5.5.5 Conclusion

Using resting-state fMRI data, we revealed different effects for the benzodiazepine alprazolam and the TSPO ligand etifoxine on functional connectivity within and between various networks after four days of intake, while lacking effects on self-reports. Besides some results indicating decreased temporal coherence due to both compounds, most prominently, increased connectivity within and between networks was observed in response to the benzodiazepine. It seems likely that the revealed differences are related to increased sedation in comparison to placebo and etifoxine, at least to a certain amount. Furthermore, stronger activation of the GABAergic system as well as inhibition of stress-induced activity of the HPA axis might contribute to effects that were revealed explicitly for alprazolam. Overall, the results

of the present work further underline the divergent pathways of action for the two anxiolytic substances.

As far as we know, the present work was the first to investigate the effects of alprazolam and etifoxine on resting-state functional connectivity in healthy subjects. Therefore, the observed changes require further clarification and replication within future studies. Those studies should include within-subjects measurements and target different doses of the medication as well as several time points of assessment. Furthermore, it would be interesting to investigate the effects of the anxiolytic agents in response to experimental paradigms which induce fear and anxiety. For instance, this might be accomplished with a version of the NPU Threat Test that is compatible with the environment of the MRI scanner. Since decreased or at least altered density of the central benzodiazepine receptor was shown in patients suffering from various forms of anxiety disorders (Hasler et al., 2008; Malizia et al., 1998; Tiihonen et al., 1997), it would moreover be interesting to compare the effects of alprazolam and etifoxine in respective samples.

Overall, the differences between the three groups in the present work concerned more ICs than reported in other studies. On the one hand, this might be due to the relatively long scan duration and multiband acquisition yielding a high amount of analyzable data. On the other hand and in contrast to previous research, we did not restrict our analysis to RSNs known from the literature. While our approach might thus better represent the structured components which are specific to the present dataset, it may also have split up some of the known networks, making the interpretation more difficult and less comparable to the existing literature (Bijsterbosch et al., 2017). However, restricting the networks to predefined template maps might more strongly bias the analysis and thereby lead to overlooking effects related to other networks. Hence, future research might follow-up by comparing different kinds of approaches for the investigation of the effects of alprazolam and etifoxine on functional connectivity in the brain.

CHAPTER 6: Effects of etifoxine & alprazolam on attention, alertness and general state

6.1 Theoretical background

Although not recommended by guidelines as treatment of first choice, benzodiazepines like alprazolam still belong to the most prescribed drugs for the therapy of anxiety disorders (Bandelow, Michaelis et al., 2017). However, besides their benefits comprising rapid onset and well-validated efficacy, common criticism affects the risk of dependency, especially if taken over longer periods, rebound effects after withdrawal and most of all the sedative properties that might restrict an individual in activities of daily life (Koelega, 1989; Lader, 2011; Verster & Volkerts, 2004). In the search for alternatives, anxiolytics targeting the translocator protein were shown to act as promising candidates, as they possess a similar efficacy without most of the adverse effects including sedation and addiction potential (Nothdurfter, Rammes et al., 2012; Nuss, Ferreri, & Bourin, 2019). Especially in situations that require full performance and attention, this could yield a decisive factor to opt targeted application of those substances. In general, besides ascertaining efficacy related parameters, the improvement of pharmacological treatment relies on the standardized assessment of side effects and adverse reactions due to drug administration.

Within patient studies, information on adverse effects after application of substances is often gathered using self-reports. Following this scheme, studies directly comparing self-reported side effects of various benzodiazepines and etifoxine yielded contradictory results. One study reported significantly more adverse events mostly related to somnolence, sedation and fatigue after 28 days intake of 1.5 mg alprazolam in comparison to 150 mg etifoxine in a sample of patients suffering from adjustment disorder with anxiety (Stein, 2015). However, within another study addressing the same disorder condition no such differences after intake of etifoxine or lorazepam for 28 days were reported (Nguyen et al., 2006).

In contrast, studies that involved healthy subjects and assessed detrimental effects of the substances after a single dose using experimental tasks yielded more consistent results. In one study, the effects of single doses of either 50 mg or 100 mg etifoxine, 2 mg lorazepam or placebo on psychomotor performance, free recall and vigilance were compared in healthy subjects (Micallef et al., 2001). While detrimental effects on all measurements were reported for the group that received the benzodiazepine, no such effects were found for either dosage of the TSPO ligand. Similar results favoring etifoxine were shown in a study that administered the

same medication to a sample of healthy elderly including measurements of alertness and cognitive functions (Deplanque et al., 2018). A favorable profile against alprazolam was also shown for another TSPO ligand (XBD-173) with respect to self-reported side effects after intake of seven days (Rupprecht et al., 2009).

A common target of studies assessing cognitive changes due to drug administration is constituted by vigilance or sustained attention. Although often used interchangeably, sustained attention more describes the continuous maintenance of alertness over a longer period, while vigilance more in detail depicts a certain readiness to detect and respond to changes of stimuli that are hardly to detect or occur at irregular intervals (Ballard, 1996). One experimental paradigm that was initially developed to investigate the aftermath of brain damage and integrates both concepts is the Continuous Performance Test (CPT) (Rosvold, Mirsky, Sarason, Bransome, & Beck, 1956). In this test, subjects are required to monitor series of letters over a certain time and to respond differently to target and non-target letters by button-press or retention of an answer, respectively. Within the original version, the target was constituted by an X, while all the other presented letters served as non-targets. The ability to sustain attention is amongst others quantified by measures of reaction time, correct responses, omissions, and false alarms over the total experiment or more specifically in relation to performance decrements over time (Ballard, 2001). Further parameters that were shown suitable for performance monitoring include, for example, the sensitivity index *d'*, which was derived from signal detection theory and describes the ability to discriminate targets from non-targets (Grier, 1971; Robinson et al., 2013).

Modifications of the CPT aimed for the improvement of ecological validity and appeal by integrating the task into virtual reality based scenarios (Mühlberger et al., 2020) or increased the complexity as, for example, in the AX-CPT in which targets are constituted by a sequence of letters (Marcora, Staiano, & Manning, 2009). The CPT and its variations have mainly been applied in studies that investigated the compensating effects of stimulants like methylphenidate with regard to hyperactivity and attention deficits (Mühlberger et al., 2020; Riccio, Waldrop, Reynolds, & Lowe, 2001). However, they were also shown suitable for the examination of cognitive changes due to the presence of mental disorders (Robinson et al., 2013) or concerning the effects of substances like ketamine (Umbricht et al., 2000) or alcohol (Dougherty et al., 1999). Although those conditions or substances incorporate deficits that slightly differ from those elicited by benzodiazepines, they all share the constrain of certain capabilities. Together with studies that revealed disrupted vigilance after administration of 0.5 mg or 0.8 mg alprazolam within similar tasks lasting 60 minutes (Kozena, Frantik, & Horvath, 1995) or 20

minutes, respectively (Suzuki, Uchiumi, & Murasaki, 1995), the CPT seems to yield an appropriate paradigm for the objective assessment of side effects due to drug administration.

6.2 Research questions and hypotheses

The stated literature showed almost concordantly impairment of measures related to attention after intake of benzodiazepines and a favorable profile for etifoxine. Therefore, within this part of the work, we aimed to compare the effects of those two substances on vigilance and sustained attention after acute and short-term treatment. For that, we administered a vigilance task – the AX-CPT - at the screening day as well as on day 1 and 5 of treatment with the medication.

We expected the subjects of the alprazolam group to exhibit slower reaction to the target variables in comparison to the participants of the etifoxine and the placebo group on both treatment days. Furthermore, we expected this group to show a higher rate of omission and commission errors emphasized by a lower ability to discriminate between targets and non-targets indicated by the sensitivity index *d'*.

To underline the findings of the standardized vigilance task, we administered several questionnaires to further assess self-reports on side effects. Analogous to the objective measurements, we anticipated more reports of side effects, especially in relation to sedation effects in the alprazolam group in comparison to etifoxine and placebo. This was expected to be accompanied by other phenomena affecting sleep quality duration and withdrawal symptoms.

6.3 Methods and procedure

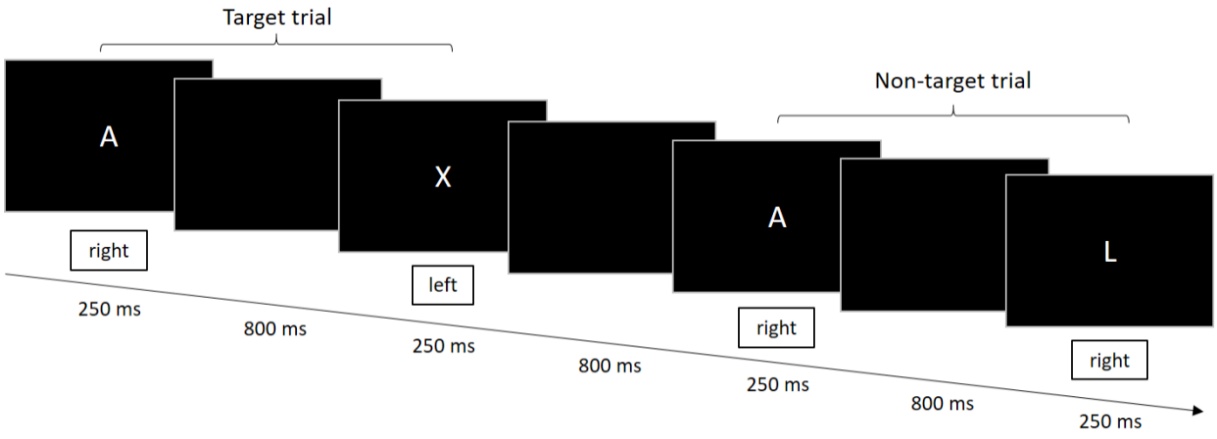
6.3.1 Continuous Performance Test (AX-CPT)

The Continuous Performance Test (CPT, AX-version) used in this study was set up using the software Presentation (version 19.0, Neurobehavioral Systems Inc., Albany, California, US) and presented at a desktop PC. In this task series of letters were presented one at a time and participants were instructed to respond differently to letters of target and non-target trials (see figure 31). Target trials (AX) were constituted by the combination of an X (probe) following an A (cue) and participants were prompted to press the left mouse button only for the probe letter (X). For the cue (A) and letters of non-target trials, they should press the right mouse button. Non-target trials consisted of either a sequence with any other letter following the cue (AY), any other letter preceding the probe (BX) or a combination of two letters including neither the probe nor the cue (BY).

The complete task lasted about 25 minutes and was divided into four blocks each lasting 378 seconds (~ six minutes). Within the blocks, the different sequences were shown in a pseudo-randomized order with the following probabilities: 70 % target AX trials (n = 126 per block), 10 % AY trials (valid cue with non-target probe, n = 18 per block), 10 % BX trials (invalid cue with target probe, n = 18 per block), 10 % BY trials (invalid cue with non-target probe, n = 18 per block). The letters were presented centrally on a black screen (white font, Helvetica, 24-point) for a duration of 250 ms followed by an interstimulus interval of 800 ms.

Figure 31

Experimental Procedure of the AX-CPT



6.3.2 Outcome measures AX-CPT

The following performance measures of the AX-CPT were included into our analysis: mean reaction time for correct answers to the probe in AX trials (RT_AX_hits in ms), commission errors (integration of false button presses in AY, BX and BY trials in %) and omission errors (integration of all missed responses in %). Further, we included the sensitivity index *d'* that was computed using the following formula:
(|*z*-value(proportion of all hits) - *z*-value(proportion of all false alarms)|) (Robinson et al., 2013).

The values were exported separately for each of the four blocks from the text file that was generated automatically by the Presentation software using a script that was created with MATLAB (version R2017b, MathWorks, Natick, Massachusetts, US). For the analyses of the screening day, we computed the means of the four outcome parameters for the blocks two, three and four. We excluded the results of the first block to account for the time needed to adapt to the test procedure during the first administration. For day 1 and 5 of treatment, we included the results of all four blocks into our analysis.

6.3.3 Procedure AX-CPT

The AX-CPT was conducted at the screening day to obtain an unmedicated baseline measurement of all participants as well as on day 1 and 5 of treatment about one and a half hours after intake of the morning medication. After arrival of the participants at the laboratory of the Chair of Clinical Psychology and Psychotherapy of the University of Regensburg, they were seated in front of the PC and provided a document containing information and instructions on the task (see appendix L). At the end of the document, subjects had to complete a paper-pencil, which was equivalent to the computer task, to check if the instructions were understood. Possible questions were clarified by the examiner and participants were reminded to respond as fast but accurately as possible and to stay attentive throughout the complete task.

The actual experiment was preceded by a training phase containing 20 trials that adapted individually to the performance of the subjects. It restarted if less than 50 % of the trials were answered correctly.

6.3.4 Subjective measurements

Questionnaire on side effects

A self-designed questionnaire was administered to monitor the appearance of physical and mental symptoms that may have arisen due to the study medication. It was based on the most frequent side effects stated in the SmPCs of alprazolam and etifoxine and comprised 18 items (e.g. dizziness, confusion, fatigue or nervousness) as well as two further fields for sensations not pre-formulated (see appendix E). Participants were asked to rate daily if they experienced the respective symptom within the last 24 hours and in case they did, to further state the severity as either mild, moderate or strong.

Diary on mental state

To track the general state of the subjects throughout the study, we administered a self-designed questionnaire on each of the five treatment days (see appendix F). Within that, we asked for the sleep duration of the preceding night as well as a respective rating on sleep quality on a scale from 1 = not relaxing (*nicht erholsam*) to 10 = very relaxing (*sehr erholsam*). Further, subjects should state if they were exercising or drinking alcohol the day before and in case they did, state the extent. Finally, we requested if there were any stressful events or other kind of special incidents.

Follow-up questionnaire

The phone post-inquiry seven days after the end of the study was conducted using a standardized guideline. As during the study, participants were asked for the duration and quality of the sleep during the preceding night. Further, the desire to continue intake of the study medication as well as the stress level at the evening of the last study day were assessed on a scale from 1 = very low (*sehr gering*) to 10 = very high (*sehr groß*). Any physical or mental anomalies after termination of participation in the study should be indicated. At the end, every subject was asked for an estimation on which medication they thought to have received.

6.3.5 Statistical analysis

Statistical analysis for the AX-CPT was performed for $n = 20$ of the etifoxine group, $n = 19$ of the alprazolam group and $n = 20$ of the placebo group. One subject was excluded

because of issues with the experiment. We used the software SPSS (version 25, IBM Statistics) to compute the statistical analysis and set the significance level for all analyses at $\alpha = .05$.

Using univariate ANOVAs, we first checked if there were significant group differences concerning performance during the AX-CPT at the screening day. We performed this analysis for each mean score of the four parameters (RT_AX_hits, commission errors, omission errors, sensitivity index *d'*) computed over the blocks 2, 3 and 4 and defined treatment as between subjects factor.

To compare effects of the medication on overall performance during the AX-CPT, we computed repeated measures ANOVAs including the within subjects factor day (screening, day 1, day 5) and the between subjects factor treatment separately for the four performance parameters.

To assess the effects of the medication more specifically on sustained attention, we used repeated measures ANOVAS including the within subjects factor block (block 1, block 4) and the between subjects factor treatment separately for each performance parameter and for each of the two treatment days (day 1, day 5). Before computing the repeated measures ANOVAs, we tested for homogeneity of variances using Levene's tests and sphericity with Mauchly-tests. In case of violation of sphericity, we report Greenhouse-Geisser (GG) corrected values. Significant main effects or interactions were followed up by post-hoc t-tests or univariate ANOVAs to determine differences between the groups or time points. In these cases, alpha error accumulation because of multiple testing was accounted for by Bonferroni correction.

Reporting on the occurrence of side effects during the study is mainly based on descriptive statistics. For a comparison of the overall occurrence of adverse events we further computed a univariate ANOVA for the total number of AEs with treatment as between subjects factor. To investigate effects of the medication on sleep duration, we computed a repeated measures ANOVA including the within subjects factor study day (day 1, day 5) and the between subjects factor treatment.

6.4 Results

First, analyses of possible group differences concerning the outcome parameters of the AX-CPT at the screening day are reported. This is followed by comparisons of the overall performance between the three groups on the screening day and day 1 and 5 of treatment. Afterwards, separate analyses of block 1 and 4 of the task for each the two treatment days are shown. Following, the results of the self-report questionnaires including adverse events, sleep duration, general mental state, and observations after the end of the study will be presented.

6.4.1 Continuous Performance Test (AX-CPT)

6.4.1.1 Data of the screening day

Analysis on the data of the screening day yielded no significant group differences in overall performance indicated by the reaction time for AX hits, commission errors, omission errors, and the sensitivity index *d'* (see table 19).

Table 19

Means, Standard Deviations and Statistics for the Outcome Parameters of the AX-CPT at the Screening Day

Parameter	Placebo	Alprazolam	Etifoxine	Statistics		
	M ± *SD*	*M* ± *SD*	*M* ± *SD*	*F* ratio	*df*	*p*
RT_AX_hits [ms]	262.63 ± 73.32	257.17 ± 67.14	235.38 ± 46.25	1.04	2,56	.361
Commission errors [%]	34.89 ± 13.12	33.28 ± 17.75	34.35 ± 17.41	0.05	2,55	.953
Omission errors [%]	3.46 ± 4.48	3.08 ± 5.32	3.24 ± 3.69	0.03	2,56	.967
Sensitivity index *d'*	.18 ± 1.98	.09 ± 1.99	-.26 ± 1.89	0.27	2,56	.762

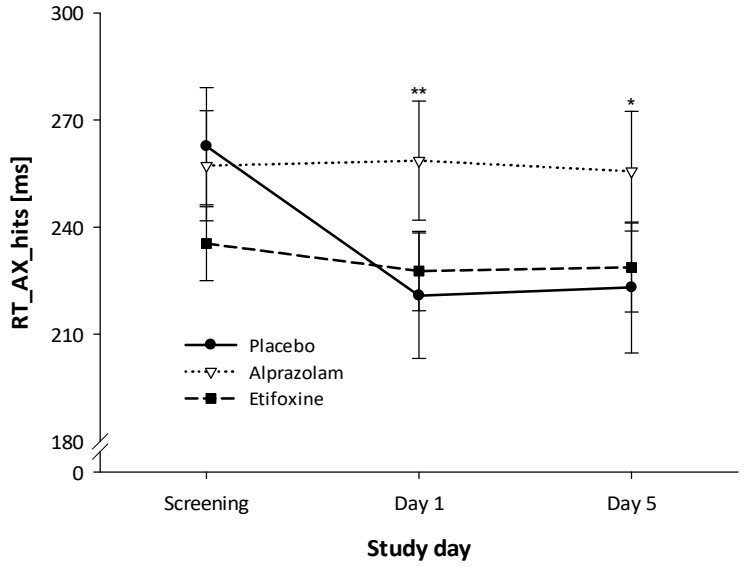
6.4.1.2 Overall test performance throughout the study

For the reaction time on correct responses to targets (RT_AX_hits), there was a significant difference between the three study days, $F_{1.39,77.86} = 8.59$, $p = .002$, $\eta_p^2 = .13$. Follow-up tests showed that the subjects reacted faster to the targets on day 1 of treatment (16.04, 95%-CI[5.82, 26.27], $p = .001$) as well as on day 5 of treatment (15.93, 95%-CI[1.90, 29.96], $p = .021$) in comparison to the screening day, respectively.

There was no overall difference of reaction time to targets between the groups, $F_{2,56}$ = 0.93, p = .402, η_p^2 = .03. However, there was a significant interaction between day and treatment for RT_AX_hits, $F_{2.78,77.86}$ = 5.34, p = .003, η_p^2 = .16 (see figure 32). Follow-up analyses to compare RT_AX_hits between the groups separately for the three days showed that they did not differ significantly on any of the days: screening, $F_{2,56}$ = 1.04, p = .361, day 1, $F_{2,56}$ = 1.69, p = .193, and day 5, $F_{2,56}$ = 1.16, p = .321. To check how RT_AX_hits changed within each of the groups we further computed repeated measures ANOVAs including the within subject factor day separately for the three treatment groups. It showed that this parameter significantly changed above the three days in the placebo group, $F_{1.30,24.67}$ = 18.94, p < .001, η_p^2 = .50, but neither in the etifoxine group, $F_{1.36,25.76}$ = 0.55, p = .515, η_p^2 = .03, nor in the alprazolam group, $F_{1.21,21.74}$ = 0.08, p = .828, η_p^2 = .004. Follow-up post hoc t-tests for placebo showed that subjects of this group reacted significantly faster on day 1 (41.82, 95%-CI[22.73, 60.91], p < .001) as well as on day 5 of treatment (39.54, 95%-CI[13.55, 65.53], p = .002) in comparison to the screening day.

Figure 32

Reaction Time to Targets in the AX-CPT by Group and Study Day



Note. Overview of group differences concerning the reaction time to targets (RT_AX_hits in ms) at the screening and on day 1 and 5 of treatment. Error bars show standard errors. Asterisks mark significant difference to the screening, *p < .05, **p < .01.

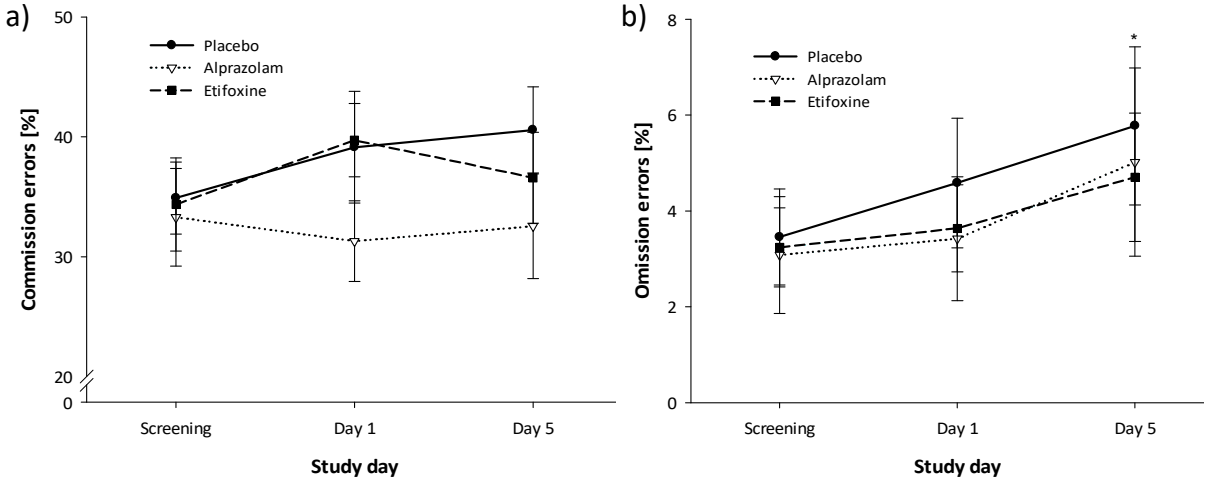
For commission errors, computations yielded neither a significant main effect of days, $F_{2,110}$ = 1.27, p = .285, η_p^2 = .02, nor due to the treatment, $F_{2,55}$ = 1.07, p = .351, η_p^2 = .04. Further, there was no significant interaction between days and treatment, $F_{4,110}$ = 0.92, p = .458,

η_p^2 = .03, indicating that the groups did not significantly differ concerning the commission errors over the three days.

For omission errors, there was a significant main effect of days, $F_{1.27,70.97}$ = 7.92, p = .004, η_p^2 = .12. Follow-up tests revealed a significantly higher rate of omission errors on day 5 compared to the screening day (-1.91, 95%-CI[-3.51, -.31], p = .014) as well as in comparison to day 1 (-1.29, 95%-CI[-2.21, -.36], p = .004) (see figure 33). However, there was no general effect of treatment, $F_{2,56}$ = 0.13, p = .880, η_p^2 = .005, and no significant interaction between days and treatment, $F_{2.54,70.97}$ = 0.21, p = .857, η_p^2 = .008.

Figure 33

Commission and Omission Errors in the AX-CPT by Group and Study Day

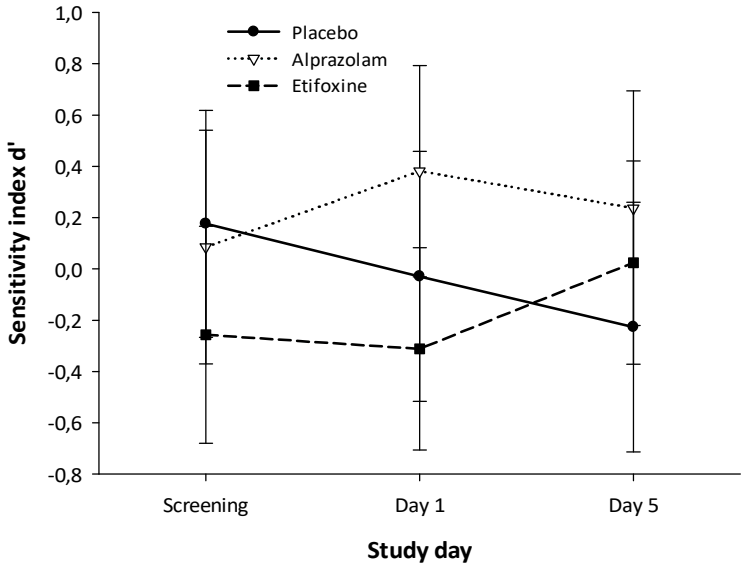


Note. Overview of group differences concerning commission (a) and omission errors (b) (both in %) at the screening and on day 1 and 5 of treatment. Error bars show standard errors. Asterisks mark significant difference to the screening, *p < .05.

For the sensitivity index *d'*, we found no main effect of day, $F_{1.68,94.28}$ = 0.00, p = .996, η_p^2 = .0, and neither a general effect of treatment, $F_{2,56}$ = 0.26, p = .775, η_p^2 = .009, nor a significant interaction between days and treatment, $F_{3.37,94.28}$ = 1.34, p = .265, η_p^2 = .05 (for an overview see figure 34).

Figure 34

Sensitivity Index d' in the AX-CPT by Group and Study Day



Note. Overview of group differences concerning the sensitivity index *d'* at the screening and on day 1 and 5 of treatment. Error bars show standard errors.

6.4.1.3 Comparison of the blocks 1 and 4 separately for day 1 and 5 of treatment

Day 1 of treatment

Comparisons of block 1 and 4 on day 1 of treatment indicated no significant changes across the blocks, $F_{1,57} = 2.28$, $p = .137$, $\eta_p^2 = .04$, or between the groups, $F_{2,57} = 2.17$, $p = .124$, $\eta_p^2 = .07$, for RT_AX_hits. There was further no significant interaction between blocks and treatment, $F_{2,57} = 2.11$, $p = .130$, $\eta_p^2 = .07$.

The same was shown for commission errors as there was neither a significant main effect of block, $F_{1,56} = 0.76$, $p = .388$, $\eta_p^2 = .01$, nor for treatment, $F_{2,56} = 0.55$, $p = .581$, $\eta_p^2 = .02$, for RT_AX_hits. The interaction between blocks and treatment did also not reach significance, $F_{2,56} = 1.30$, $p = .282$, $\eta_p^2 = .04$.

Subjects made significantly more omission errors during block 4 in comparison to block 1, $F_{1,57} = 21.78$, $p < .001$, $\eta_p^2 = .28$ (see also table 20). However, the anxiolytic did neither affect omission errors in general, $F_{2,57} = 0.48$, $p = .624$, $\eta_p^2 = .02$, nor the change across blocks, $F_{2,57} = 0.70$, $p = .499$, $\eta_p^2 = .02$.

The sensitivity index *d'* did neither change between blocks, $F_{1,57} = 0.00$, $p = 1.0$, $\eta_p^2 = .00$, nor between the three groups, $F_{2,57} = 0.19$, $p = .825$, $\eta_p^2 = .007$. There was further no significant interaction between blocks and treatment, $F_{2,57} = 0.00$, $p = .999$, $\eta_p^2 = .00$.

Table 20

Means, Standard Deviations and Statistics for the Parameters of the AX-CPT for Block 1 and 4 on Day 1 of Treatment

Parameter	Placebo	Alprazolam	Etifoxine
	M ± *SD*	*M* ± *SD*	*M* ± *SD*
Block 1			
RT_AX_hits [ms]	237.06 ± 70.0	259.83 ± 79.31	233.26 ± 58.91
Commission errors [%]	43.47 ± 27.31	31.87 ± 19.55	42.22 ± 24.92
Omission errors [%]	2.75 ± 3.89	2.15 ± 4.35	2.14 ± 2.50
Sensitivity index *d'*	.01 ± 1.96	.20 ± 1.84	-.18 ± 1.92
Block 4			
RT_AX_hits [ms]	210.26 ± 87.88	265.78 ± 74.92	223.21 ± 56.63
Commission errors [%]	42.59 ± 24.86	42.59 ± 29.32	43.33 ± 21.48
Omission errors [%]	5.80 ± 7.77	3.77 ± 5.51	4.54 ±4.83
Sensitivity index *d'*	.01 ± 2.01	.20 ± 1.67	-.17 ± 1.66

Day 5 of treatment

Comparisons of block 1 and 4 on day 5 of treatment indicated a significant difference between the blocks for RT_AX_hits, $F_{1,57} = 8.98$, $p = .004$, $\eta_p^2 = .14$. There was no effect of the treatment on the overall reaction time, $F_{2,57} = 1.59$, $p = .212$, $\eta_p^2 = .05$, and no significant interaction between blocks and treatment, $F_{2,57} = 1.36$, $p = .264$, $\eta_p^2 = .05$.

The emergence of commission errors did not change between the first and the last block of the task, $F_{1,48} = 0.34$, $p = .562$, $\eta_p^2 = .007$. It was further not affected by the treatment in general, $F_{2,48} = 0.95$, $p = .393$, $\eta_p^2 = .04$, or across the blocks, $F_{2,48} = 0.10$, $p = .909$, $\eta_p^2 = .004$. Like on the first day of treatment, participants made significantly more omission errors during the last block in comparison to the first one, $F_{1,57} = 13.53$, $p = .001$, $\eta_p^2 = .19$ (see also table 21). There was no effect of the anxiolytic substances on omission errors in general, $F_{2,57} = 0.10$, $p = .904$, $\eta_p^2 = .004$, or on the change across blocks, $F_{2,57} = 0.04$, $p = .965$, $\eta_p^2 = .001$.

The sensitivity index *d'* did not significantly change across the two blocks, $F_{1,57} = 0.00$, $p = 1.0$, $\eta_p^2 = .00$, or between the groups, $F_{2,57} = 0.24$, $p = .790$, $\eta_p^2 = .008$. There was no significant interaction between blocks and treatment, $F_{2,57} = 0.03$, $p = .973$, $\eta_p^2 = .001$.

Table 21

Means, Standard Deviations and Statistics for the Parameters of the AX-CPT for Block 1 and 4 on Day 5 of Treatment

	Placebo	Alprazolam	Etifoxine
	M ± *SD*	*M* ± *SD*	*M* ± *SD*
Block 1			
RT_AX_hits [ms]	230.88 ± 74.91	269.28 ± 81.85	230.40 ± 57.02
Commission errors [%]	44.72 ± 23.34	36.28 ± 29.30	36.63 ± 23.46
Omission errors [%]	4.81 ± 7.86	4.18 ± 8.71	3.78 ± 5.34
Sensitivity index *d'*	-.23 ± 2.19	.16 ± 1.93	.08 ± 1.83
Block 4			
RT_AX_hits [ms]	213.02 ± 93.67	250.57 ± 74.49	227.65 ± 60.91
Commission errors [%]	44.29 ± 23.56	36.27 ± 22.27	36.53 ± 20.70
Omission errors [%]	6.49 ± 7.66	5.48 ± 9.06	5.63 ± 7.47
Sensitivity index *d'*	-.21 ± 2.12	.23 ± 1.88	.03 ± 1.67

6.4.2 Reported side effects / adverse events

6.4.2.1 Observations during the study

Most importantly, no serious adverse event occurred in any of the treatment groups and no subject withdraw participation in the study. During the treatment period, 17 subjects in the etifoxine group experienced in total 90 adverse events (AEs) compared to 19 participants experiencing 109 AEs in the alprazolam group. Interestingly, also in the placebo group 15 participants reported in total 82 AEs. Of those $n = 44$ were related to sedation effects in the etifoxine group, while this was the case for $n = 69$ for the alprazolam group and $n = 36$ for the placebo group.

The total occurrence of AEs did not differ significantly between the three groups, $F_{2,57} = 0.68$, $p = .510$. However, there was a significant group difference for adverse events related to sedation, $F_{2,57} = 4.99$, $p = .010$. Follow-up tests revealed that there was a significantly higher occurrence of AEs related to sedation in the alprazolam group compared to the placebo group (1.65, 95%-CI[.31, 2.99], $p = .011$). However, there were no differences between alprazolam and etifoxine (1.25, 95%-CI[-.09, 2.59], $p = .077$) nor for etifoxine and placebo (.40, 95%-CI[-.94, 1.74], $p = 1.0$). Table 22 lists the most reported adverse reactions separately for the three groups.

Table 22

Overview of the Most Frequent Adverse Events in the Three Treatment Groups

Symptom	Placebo	Alprazolam	Etifoxine
		n	
Drowsiness	4	12	6
Increase of appetite	3	7	7
Sleeplessness	5	4	5
Nervousness	8	6	7
Headache	7	3	8
Dizziness	4	9	3
Inner unrest	7	4	5
Sleepiness	9	13	10
Concentration difficulties	3	12	6
Fatigue	13	18	12

In addition to the self-reports of adverse events, we registered changes of blood parameters on the last day of treatment in comparison to the screening. In case of relevant deviations, we tracked the respective parameters until they had subsided to an acceptable level. For an overview of changes of blood parameters see table 23.

Table 23

Overview of Changes of Blood Parameters throughout the Study

Group	Parameter (reference range)	Value		
		Screening	Day 5	Follow-up
Etifoxine (*n* = 1)	GPT (10-50 U/l)	51	57	+ 2 weeks: 65 + 3 weeks: 66 + 7 weeks: 48
	Triglycerides (10-200 mg/dl)	199	398	+ 2 weeks: 287 + 3 weeks: 319 + 7 weeks: 273
Etifoxine (*n* = 1)	GOT (10-50 U/l)	27	55	+ 2 weeks: 37
Placebo (*n* = 1)	Lipase (13-60 U/l)	49	97	+ 1 week: 35
Placebo (*n* = 1)	Creatinine (0.1-1-17 (mg/dl)	1.17	1.21	+ 1 week: 1.20

Note. GPT = Glutamic pyruvate transaminase, GOT = Glutamic oxaloacetate transaminase, TSH = Thyroid-stimulating hormone.

6.4.2.2 Observations after the end of the study

When asked for the estimation of the respective treatment group the subjects thought they had been allocated to, only 7 subjects of the placebo group, 8 from the alprazolam group and 1 of the etifoxine chose the correct group with reasonable certainty. The others either guessed another group or could not decide between two options (see also table 24).

Table 24

Estimations of the Group Allocation by the Subjects

Symptom	Placebo	Alprazolam	Etifoxine
		n	
Placebo	7	5	4
Alprazolam	7	8	7
Etifoxine	-	3	1
Placebo or etifoxine	3	3	6
Placebo or alprazolam	1	-	-
Alprazolam or etifoxine	2	1	1
No statement possible	-	-	1

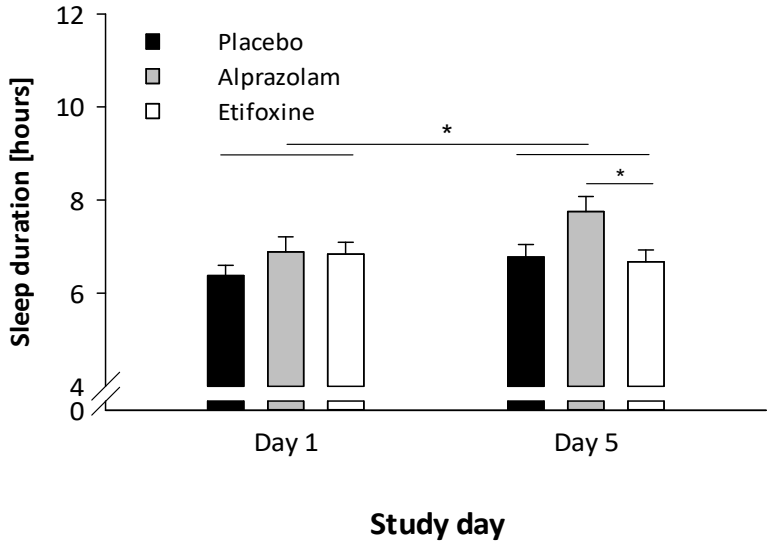
Two subjects of the alprazolam group reported a low to medium desire to take the medication after the end of the study, while this was the case for none of etifoxine group. Interestingly, also two subjects of the placebo reported a low desire to continue medication.

6.4.2.3 Effects on sleep duration

We further checked if there were differences between the three groups concerning the sleep duration the nights before the testing on day 1 and 5 of treatment. Our analysis yielded a significant main effect of study day, $F_{1,57} = 4.19$, $p = .045$, $\eta_p^2 = .07$ (see figure 35). There was no significant difference between the treatment groups, $F_{2,57} = 2.88$, $p = .064$, $\eta_p^2 = .09$, and no significant interaction between study day and treatment, $F_{2,57} = 2.74$, $p = .073$, $\eta_p^2 = .09$. As we expected differences according to the medication on day 5 of treatment, we further computed a univariate ANOVA for this time point to compare the sleep duration between the groups and found a significant difference, $F_{2,57} = 4.34$, $p = .018$, $\eta_p^2 = .13$. Post hoc t-tests for the factor group yielded significant differences between alprazolam and etifoxine (1.08, 95%-CI[.08, 2.07], $p = .030$) but not between alprazolam and placebo (.98, 95%-CI[-.02, 1.97], p = .056) or etifoxine and placebo (-.10, 95%-CI[-1.09, .89], $p = 1.0$).

Figure 35

Sleep Duration during the Nights before Day 1 and 5 of Treatment



Note. Group differences concerning the sleep duration (in hours) the nights before day 1 and 5 of treatment. Error bars show standard errors. *$p < .05$.

6.5 Discussion

6.5.1 Overview

Within this part of the work, we investigated side effects after acute and short-term administration of alprazolam and etifoxine with a focus on vigilance and sustained attention. Therefore, we assessed four parameters of the Continuous Performance Test (AX-CPT) at the screening as well as on day 1 and 5 of treatment complemented by self-reports and measurement of mental and physical phenomena.

There was neither severe impairment of vigilance or sustained attention measured with the AX-CPT after a single dose of 0.5 mg alprazolam or 50 mg etifoxine nor after five days intake of three times that dose. Only for reaction time on target letters, we found improvement over the three testing days for the placebo group, which was absent in the alprazolam and the etifoxine group. Overall, we registered a high number of adverse events, surprisingly, for all three groups. Importantly, there were no serious cases. Intake of alprazolam caused a higher frequency of adverse events related to sedation accompanied by increased sleep duration at the end of the treatment. For two subjects of the placebo group as well as for two of the etifoxine group, blood parameters had to be followed-up, as they had changed with respect to the baseline values.

6.5.2 No considerable performance deficits due to alprazolam and etifoxine

Since alprazolam but not etifoxine binds to subunits of the $GABA_A$ receptor that are related to sedative effects (Poisbeau et al., 2018; Rudolph & Knoflach, 2011), we would have expected stronger impairment of performance in the AX-CPT by the benzodiazepine. More precisely, we thought to replicate findings from studies that showed increased error rates as well as reduced response rate by benzodiazepines with simultaneously no such impairment by etifoxine (Deplanque et al., 2018; Micallef et al., 2001). However, in the present work there were no group differences for any of the parameters analyzed for the AX-CPT on any of the testing days. Those comprised reaction time for correct responses to target letters, commission and omission errors as well as the sensitivity marker *d'*, which puts both, hit rate and error rate, into context. The only striking finding was that subjects of the placebo group became faster on day 1 and 5 of treatment in comparison to the screening day, while no such improvement was shown for subjects that had received etifoxine or alprazolam. Hence, there might have been some habituation or learning effect due to repetition of the paradigm, which was hampered by

the active treatment. Overall, reaction times were already quite low at the screening day for all subjects in comparison to those yielded for healthy controls of other studies that applied the AX-CPT (Brambilla et al., 2007; Robinson et al., 2013). Although there were no significant differences, the fastest responses were shown for the etifoxine group, which might have impeded further improvement at the subsequent testing days. Subjects of the alprazolam group, however, were more similar to the placebo group with respect to the time needed for correct responding. The fact that they did not improve equally over the days might hint at least little impairment effect of the benzodiazepine, although this can only be guessed.

While the fact that we did not find detrimental effects on alertness for etifoxine matches existing literature, the results for alprazolam might seem surprising. The missing overlap to past studies, which reported sedating effects of benzodiazepines in comparison to etifoxine, might be constituted by the fact that they used a dose of 2 mg lorazepam as control substance (Deplanque et al., 2018; Micallef et al., 2001). A comparative study on lorazepam and alprazolam found detrimental effects of 2 mg lorazepam on reaction time and all other tests related to cognitive functioning that were applied in that study, while alprazolam impaired performance only for some of the parameters in a dose-dependent manner (Vermeeren et al., 1995). Other studies on alprazolam that reported performance deficits applied either a higher dose (Suzuki et al., 1995) or longer task duration (Kozena et al., 1995). Thus, the combination of dose and task characteristics in the present study might have been inadequate to measure detrimental effects of alprazolam.

For the Continuous Performance Test, especially worsening of performance across the single blocks has been emphasized to indicate reduced sustained attention (Ballard, 1996). However, we only found omission errors to be increased from the first to the last block on day 1 and 5 of treatment. With respect to reaction time, subjects got even faster at the end of the task, which lasted about 25 minutes, on day 5 of treatment. The fact that subjects showed only little or no exhaustion throughout the task raises the question if the applied version of the AX-CPT was suitable to emphasize impairment effects of the medication. Although there exist studies that examined the effects of sedative compounds using versions of the CPT (Dougherty et al., 1999; Umbricht et al., 2000), it has mainly been applied within the context of ADHD and testing of stimulants (Mühlberger et al., 2020; Riccio et al., 2001). Besides potential specificity of subjects' condition and applied substance, task characteristics like stimulus duration, length of the interstimulus interval and required response behavior might have influenced our findings, too. It could further be criticized that we repeated the same paradigm three times throughout the study such that learning effects might have arisen, which impeded the detection of true

medication effects. Although such an influence cannot be ruled out completely, it must be noted that the order of letter presentation was randomized for each acquisition in the present work such that subjects cannot have learned concrete patterns. Furthermore, repetition of testing paradigms was performed before in studies that either trained their subjects on the tests prior to inclusion (Micallef et al., 2001) or applied crossover designs (Deplanque et al., 2018; Umbricht et al., 2000).

Subjects performed the AX-CPT about one and a half hours after intake of the morning medication on each of the testing days. As both compounds are known to possess a rapid onset of action, we expected possible sedative effects to appear even at such an early time point of measurement. However, since peak plasma concentrations are reached after one to two hours for alprazolam (Verster & Volkerts, 2004) and within two to three hours for etifoxine (Choi & Kim, 2015), it would be interesting to include several time points for the assessment of side effects in future research. Especially, since some subjects verbally reported increased levels of tiredness in the evening, it might be interesting to consider testing at a later daytime.

6.5.3 More side effects related to sedation after intake of alprazolam

Almost all subjects, even those of the placebo group, experienced at least one adverse event during participation in the study, most of them related to drowsiness, fatigue, concentration difficulties, and sleepiness. Overall, the number of reported symptoms did not differ between the three groups, while there were more reports related to sedation by subjects that were allocated to the treatment with alprazolam in comparison to placebo. We could not replicate findings in patients that showed significantly more adverse events related to sedation and fatigue after several days intake of alprazolam in comparison to etifoxine (Stein, 2015). However, in that study side effects were asked only after intake of seven and 28 days, respectively, and the authors did not describe the exact way of assessment. Thus, it must be assumed that they did not use a specific questionnaire, but more relied on open questioning and spontaneous reports. In the present work, participants were asked to state the appearance of any symptoms on each of the five treatment days within the scope of a predefined questionnaire. Although there was further space for open comments, they reported almost no symptoms in addition to the pre-formulated ones. Hence, it is possible that the administered reporting form caused participants of all three groups to even pay attention to smallest changes of the listed body sensations over the single days what might explain the relatively high number and overall missing group differences. The high prevalence of events related to sedation in all groups might further be explained by the fact that subjects had to complete the questionnaire at 8:30 AM and

had to show up at the research station at 8 AM or take the medication at that time on days without an appointment at the clinics, respectively. Especially for students, which represent about two thirds of our sample this, time might have been far ahead of their usual wake-up time. Overall increased tiredness across the study was also reflected in prolonged sleep duration during the night before day 5 of treatment in comparison to the night before the start of substance intake, especially for subjects that received alprazolam.

Previous studies reported significant differences between benzodiazepines and etifoxine with respect to adverse events, especially after treatment discontinuation (Nguyen et al., 2006; Stein, 2015). Within the present trial, only two subjects of the alprazolam group reported a low to medium desire to take the medication after the end of the study while none of the etifoxine group, but two subjects of the placebo group reported a low desire to do so. The duration of intake has been chosen specifically to avoid any risk of addiction or withdrawal symptoms which were shown to exert at least slightly already after seven days for alprazolam (Rupprecht et al., 2009).

Interestingly, not even half of the participants per group named the correct substance when asked for an estimation of the group they thought they had been allocated to. The most votes were casted for alprazolam underlining the hypothesis that subjects were overall quite tired in the present study independent of the actual treatment. This was followed by ratings for placebo while only few subjects clearly expressed the estimation of having received etifoxine. This might be attributable to considering the TSPO ligand as the “new” substance with subjects having only little idea of common side effects of that compound, while benzodiazepines inclusive of their adverse effects are more commonly known.

It must be noted that two subjects of the etifoxine group had to reappear after the end of the study as they exhibited changes of blood parameters related to liver function in comparison to the baseline values. Those were followed-up as they exceeded the inclusion criterion of being > 10 % above the reference range. Although all parameters were still not of clinical relevance according to medical assessment and declined after several weeks, this must be kept in mind when considering the prescription of etifoxine over a longer period. Changes of triglycerides for one subject of the etifoxine group as well as lipase and creatinine, respectively, for two subjects of the placebo group, however, might be explained by general fluctuations in those subjects or a possible influence of eating prior to the blood taking. While subjects were instructed to be sober at the screening day that was not prescribed for day 5 of treatment.

6.5.4 Conclusion

Within the scope of the AX-CPT, that was administered in the previous work we did neither observe serious impairment of vigilance and sustained attention after a single dose of 0.5 mg alprazolam or 50 mg etifoxine nor after five days of taking that dose three times a day. Together with reported mental and physical symptoms, we conclude an overall safe profile of the administered doses for both anxiolytic agents, since there were no serious adverse events and no study dropouts in any of the groups. Only when focusing on symptoms related to sedation, there were more reports from subjects that were allocated to receive alprazolam. During treatment with etifoxine, possible changes of parameters related to liver function must be considered and require regularly monitoring. Overall, we cannot conclude clear superiority of etifoxine compared to alprazolam with respect to self-reports and measurement of adverse events or regarding objective measurements of vigilance and sustained attention from the present work.

Our findings stand in contrast to patient studies that reported a favorable profile of side effects for etifoxine in comparison to benzodiazepines. However, those studies either used benzodiazepines, which are known to be more sedative than alprazolam, or applied higher doses. Further, adverse events were not assessed in such detail or even using a questionnaire as we did in the present work. To follow-up on the objective assessment of possible performance decrements due to the anxiolytic agents, future studies might apply more complex testing. For instance, the Cambridge Neuropsychological Test Automated Battery (CANTAB, Cambridge Cognition, Bottisham, UK), which addresses aspects like memory, executive function as well as attention and psychomotor speed within several subtests, could be concerned. Furthermore, more realistic situations like writing an exam might help in transferring observed effects into everyday life.

CHAPTER 7: General Discussion

The following chapter provides an overarching discussion of the results from the four main parts of the present work in which we aimed to investigate the role of two distinct benzodiazepine receptors, the $GABA_A$ receptor and the translocator protein, in stress and anxiety. Firstly, findings of the different paradigms and multimodal measurements that were applied in the clinical trial are summarized and their implications will be discussed. Subsequently, general methodological considerations and limitations of the clinical trial will be outlined and future prospects will be introduced.

7.1 Summary of findings

Within the present randomized clinical trial, healthy male subjects were either administered a daily dose of 1.5 mg alprazolam, 150 mg etifoxine or placebo for a period of five days. While the former selectively modulates the $GABA_A$ receptor, etifoxine acts twofold by directly binding to the $GABA_A$ receptor at sites distinct to those of benzodiazepines and further by modulation of the mitochondrial TSPO. Across the course of the study, different effects for the two anxiolytic compounds were revealed on a molecular, neuronal and physiological level.

Within **chapter 3**, we described the effects of alprazolam and etifoxine after five days of intake on **acute psychosocial stress** elicited by the Trier Social Stress Test in Virtual Reality. The most prominent finding was an inhibition of the stress-induced cortisol release by alprazolam but not by etifoxine. Surprisingly, etifoxine did not stimulate the synthesis of allopregnanolone although it increased expression of TSPO, especially at an early stage of intake. In general, within the scope of the VR-TSST, we only observed molecular changes due to the medication and there were no effects on any of the subjective or physiological parameters. Nevertheless, various relation of the individual stress markers within the three groups suggests that they might be differentially susceptible to the medication.

Chapter 4 compared the effects of the two IMPs on **fear and anxiety** using the NPU Threat Test on day 1 and 5 of treatment. We revealed different effects for the benzodiazepine alprazolam and the TSPO ligand etifoxine on fear and anxiety in healthy subjects. In contrast to past research, none of the anxiolytic substances had an impact on anxiety-related startle.

However, etifoxine yielded attenuating effects on fear-potentiated startle on day 1 of treatment. Overall, there were no effects of the medication on subjective measures of the emotional states.

Within **chapter 5**, we revealed different effects of the benzodiazepine alprazolam and the TSPO ligand etifoxine on **resting-state functional connectivity** within and between various brain networks after four days of intake. Some results showed decreased temporal coherence due to both anxiolytic compounds. However, most prominently, we observed increased connectivity within and between several resting-state networks in the alprazolam group in comparison to etifoxine and placebo.

Within **chapter 6**, we compared the effects of alprazolam and etifoxine on **attention, alertness and general mental state**. Within the scope of the AX-CPT, we did neither observe serious impairment of vigilance and sustained attention after a single dose of 0.5 mg alprazolam or 50 mg etifoxine, nor after five days of taking that dose three times a day. In neither group, there was any serious adverse event or study dropout. When focusing on symptoms related to sedation, there were more reports from subjects of the alprazolam group. For two subjects of the etifoxine group we registered slight changes of blood parameters related to liver function.

7.2 Implications

The comparison of the two anxiolytic substances alprazolam and etifoxine provided further insights into the role of the central and the peripheral benzodiazepine receptor in mechanisms that are related to stress and anxiety. Both substances modulate the release of the inhibitory neurotransmitter GABA with alprazolam binding to the $GABA_A$ receptor with higher affinity than etifoxine (Hamon et al., 2003; Schmoutz et al., 2014), which in turn additionally modulates the translocator protein (Costa et al., 2017). For alprazolam, there already existed experimental research on the effects on stress and anxiety related parameters in healthy subjects (Fries et al., 2006; Grillon et al., 2006; Rohrer et al., 1994). In contrast, for etifoxine, the present work was the first to experimentally investigate those aspects in a non-clinical sample. Thereby, we used multimodal techniques and assessed changes due to the pharmacological treatment not only after a single dose but further after intake over several days.

First, our findings suggest that the affinity with which a ligand binds to the $GABA_A$ receptor, also referred to as central benzodiazepine receptor, might play a crucial role for the

initiation of processes of the organism in response to psychosocial stress. For the alprazolam group, we found an inhibition of stress-induced cortisol release, which reflects activity of one of the two major stress systems – the HPA axis. In contrast, this was not shown after administration of etifoxine. Those striking results might serve as an indicator of the disparate strength with which the two compounds modulate the $GABA_A$ receptor. This might be explained or complemented by the fact that etifoxine modulates subunits of this receptor complex that are distinct from those of benzodiazepines (Bouillot et al., 2016; Poisbeau et al., 2018). Those subunits are differentially distributed in the central nervous system and are suggested to have unique functions leading to specific effects of respective ligands (Sieghart & Sperk, 2002). Just as the subunits, the density of GABA receptors in general was shown to vary between different brain areas (Abadie et al., 1992; Zilles & Amunts, 2009). Following this, the different effects of alprazolam and etifoxine on stress-induced cortisol release were likewise reflected in changes of functional brain connectivity during rest within the present work. Most of all, we found increased connectivity within and between various RSNs after administration of alprazolam in comparison to etifoxine and placebo. Since the concerned networks, inter alia, comprised brain regions that yield a high density of GABA receptors, this might indicate a stronger modulation of the GABAergic system by the benzodiazepine. In general, both the effects on cortisol and on neuronal connectivity changes underline the divergent pathways of action taken by the two applied substances. Those need to be kept in mind regarding their application in the clinical field, since patients suffering from different forms of anxiety disorder were shown to yield reduced cerebral benzodiazepine receptor binding in several brain areas including frontal, temporal and parietal regions (Hasler et al., 2008; Tiihonen et al., 1997). Hence, depending on the availability and functioning of central benzodiazepine receptors, anxiolytic agents might differentially evolve their therapeutic effects in various subtypes of anxiety disorders.

Besides possible explanations that involve direct binding to the central benzodiazepine receptor, effects of etifoxine might even more strongly depend on the synthesis of neurosteroids through binding to TSPO (Ugale et al., 2007; Verleye et al., 2005). Stimulating effects with respect to the genesis of neurosteroids were shown in cell culture and, for example, in rat plasma (Liere et al., 2017; Wolf et al., 2015). The present work, however, was the first to assess the effects of the TSPO ligand on allopregnanolone levels in humans and could not replicate the findings of preclinical research. However, since those neurosteroids themselves are strong modulators of the $GABA_A$ receptor (Majewska et al., 1986), they play an important role in anxiolysis (Rupprecht, 2003; Schüle et al., 2014) and probably in the effects of etifoxine. It is

unclear why there was no effect of etifoxine in the present work, especially since TSPO expression was found increased after administration of this compound. On the one hand, this might suggest that TSPO is less relevant for the synthesis if neurosteroids than assumed and requires further activity of other components that are related to the mitochondrial complex, as has already been pointed out in previous research (Banati et al., 2014; Selvaraj & Stocco, 2015). On the other hand, it seems possible that the specific effects of etifoxine just or more strongly evolve in patients, who were shown to yield altered expression of TSPO (Abelli et al., 2010; Chelli et al., 2008; Gavish et al., 1996; Johnson et al., 1998) as well as disrupted levels of neurosteroids (Ströhle et al., 2003). Such alterations in pathological states might lead the systems to become more susceptible to modulating effects of the TSPO ligand.

The striking effects of etifoxine on the fear-potentiated startle response in the NPU Threat Test suggest a specific indication of the TSPO ligand for the treatment of fear-related disorders. This conclusion seems reasonable, since etifoxine has mostly been shown effective within clinical studies that included patients suffering from one particular disorder – adjustment disorder with anxiety (Alexandrowsky et al., 2010; Nguyen et al., 2006; Stein, 2015). This might differentiate etifoxine from benzodiazepines that were most consistently shown to reduce the startle response related to contextual anxiety, which is more closely linked to general anxiety disorder (Grillon et al., 2006). However, since this was the first report of that specific finding, this possible association needs to be further clarified within future research.

In contrast to previous research (Deplanque et al., 2018; Micallef et al., 2001; Nguyen et al., 2006), the present work did not find clear superiority of etifoxine against the benzodiazepine with respect to self-reported adverse events and objective measurements of alertness and attention. While for etifoxine is consistent with our hypotheses that there was no severe impairment of those parameters, the results at first seem surprising for alprazolam. However, the missing overlap to previous studies might mostly be attributable to the lower dose and type of the benzodiazepine that was administered in the present work, since others often used compounds that are known to be more sedative than alprazolam. Importantly, based on the reported mental and physical symptoms, we may conclude an overall safe profile of the applied doses for both substances from the present work. Etifoxine might particularly be preferable for longer use since previous work reported no withdrawal effects. However, a possible impact on liver function must be considered and monitored in such cases.

7.3 Methodological considerations

The presented results were gathered within the scope of a randomized double-blind clinical trial, which aimed at best possible standardization as well as unbiased implementation and analysis. However, in addition to the methodological comments that were provided specifically for the different parts, there are some considerations concerning the complete clinical trial, which will be outlined in the following.

Since the subjective stress level in response to the VR-TSST constituted the primary endpoint of the clinical trial, it was designed to best fit the requirements needed for the application of that paradigm. Amongst other concerns, the knowledge that psychosocial stressor tasks are rather vulnerable to habituation effects (Klumpers et al., 2010; Wüst et al., 2005) led us to apply a between-subjects design in the present study. The chosen design, however, might have impeded the detection of effects in the other paradigms as, for example, the measurement of the startle response in the NPU Threat Test is more reliable within subjects (Blumenthal et al., 2005). Likewise, the analysis of functional MRI data has been shown to be preferably accomplished intra-individually (Cole, Smith, & Beckmann, 2010). Future studies might therefore profit from crossover designs, which include adequate washout phases and compare the effects of the anxiolytic substances within the individual subjects.

In addition to the design, the sample size of the present study with $n = 20$ for each group was chosen based on calculations for the primary endpoint of the trial, too. However, for some of the analyses more subjects might be required to be able to identify effects of the anxiolytic treatment. Future research should aim to repeat parts of the present work in larger samples and try to further clarify the findings for the different parameters. Importantly, since we found significant differences in molecular and physiological parameters as well as functional brain connectivity despite of the current sample size, it is more likely that the present work underestimates the effects of alprazolam and etifoxine instead of reporting false positive results.

To ensure homogeneity of the sample and to avoid an interfering influence of sex hormones on the endocrine measurements in the VR-TSST (Childs et al., 2010; Santl et al., 2019), only male subjects aged between 18 and 55 years were included in the present work. However, to allow generalization of the results and to address the entire target group for pharmacological agents, future studies might include females in different phases of the menstrual cycle as well as subjects of different age groups. Besides those demographic variables, it is obvious that the mere characteristics of a pharmacological trial particularly attract individuals that exhibit a low level of trait anxiety, thereby possibly facing a floor effect. Since the physiological and endocrine stress and anxiety related systems show altered reactivity in

highly anxious individuals and patients (Martin et al., 2009; G. E. Miller et al., 2007; Möhler, 2012), it would be important to replicate the presented findings, as possible, by directly comparing healthy control and patient samples.

Within the present study, a fixed dosage regimen was applied for all subjects. Some of our subjects yielded a BMI > 25, thereby being classified as overweight. Importantly, subjects of the three groups did not differ with respect to their body mass index so that a negative impact of differences concerning the body type on the reported results can be excluded. Indeed, as already pointed out in the specific parts, it might be possible that the dosage of both compounds was too little to exert effects that go over molecular and neuronal aspects. Similarly, the duration of intake might be reconsidered. The duration of five days was chosen to fulfill requirements of the ethics committee with a specific focus on the addiction potential of alprazolam, which evolves after longer intake. Furthermore, administration longer than five days might have resulted in analyzing habituation effects rather than original drug effects. Nevertheless, especially with respect to etifoxine, it would be worthy to conceptualize follow-up studies that target different dosage and time windows for the investigation of effects in healthy subjects.

It might be criticized that we did not monitor blood concentration of alprazolam and etifoxine throughout the study. Since subjects took the medication autonomously in the evenings as well as on day 2 and 3 of treatment, it cannot be guaranteed that every capsule was taken by each participant. However, commitment was created by close supervision of the subjects and by instructing them to record the respective time points of intake in a diary. Further, on testing days on which the experimental paradigms were applied medication was taken in front of the study personnel. Since we observed effects of the medication with etifoxine, for example, increasing TSPO expression or alprazolam blunting the stress-induced cortisol release, we can assume that most of the capsules were taken. Still, it is possible that some effects were masked by different levels of drug concentration between subjects if some of them did not take the capsules as prescribed.

7.4 Outlook

The current work presented the first clinical trial that compared the effects of alprazolam and etifoxine on stress and anxiety related parameters in healthy subjects and showed different effects on a molecular, physiological and neuronal level. In any case, more research is required, which aims at replicating and further clarifying the discussed results under consideration of the

mentioned methodological considerations. Thereby, especially the affinity with which a compound binds to the $GABA_A$ receptor seems to play a vital role for its impact on activity of the HPA axis and related neuronal and physiological processes. Besides further clarification of that association, future studies should address the question if pharmacological inhibition of stress-induced activity of the HPA axis is rather advantageous or unfavorable with respect to repeated stress exposure and long-term outcome. Likewise, it needs to be further investigated to which extent and under which conditions TSPO is involved in the emergence of anxiolytic effects of pharmacological ligands in humans.

A major advantage of the present work was the inclusion of multimodal measurements for the quantification of the effects. However, future research might even add by combining the different techniques that were administered in the clinical trial. For instance, the application of psychosocial stress tasks or anxiety evoking paradigms within the MRI scanner would allow more insight to the interdependencies between psychological and neuronal processes that evolve in response to the pharmacological treatment. Simultaneous and multiple assessment of molecular markers like cortisol or allopregnanolone but also their precursors ACTH or pregnenolone and parameters, which are related to activity of the SAM axis including catecholamines like adrenaline and noradrenaline, might further contribute to the explanation of observed effects.

In general, future experimental work might consider the application of a crossover design and the inclusion of other subpopulations like females, highly anxious individuals and clinical patients. With respect to subjective assessment of emotional states, the application of online measurements could provide a helpful tool to detect slight differences even in healthy subjects.

Scientific work, which aims at further clarification of the effects of anxiolytic substances like alprazolam and etifoxine and concerns the mentioned suggestions, might contribute to a deeper understanding of underlying systems and mechanisms and promote pharmacological prevention and treatment options for mental disorders.

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APPENDIX

A. RECRUITMENT ANNOUNCEMENT

Probanden gesucht

Das Klinikum für Psychiatrie und Psychotherapie der Universität Regensburg
sucht ab sofort:

Gesunde Männer
zwischen 18 und 55 Jahren

für eine klinische Studie zur Wirkung angstlösender Medikamente
bei akutem psychosozialem Stress.

Die Studie erstreckt sich über 7 Tage (5 Besuche am Bezirksklinikum).

Aufwandsentschädigung 500 €

Die Studie beinhaltet:
5-tägige Einnahme eines angstlösenden Medikaments oder Placebo, medizinische Untersuchungen, psychologische Stresstestungen, subjektive Ratings, physiologische Messungen, Messungen von Blutparametern, Kernspintomographie

Bei Interesse erhalten Sie weitere Informationen unter:
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Leitung der Klinischen Prüfung:
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E-Mail: Caroline.Nothdurfter@medbo.de

Aushang vom XX.XX.XXXX

B. QUESTIONNAIRE BLOOD TAKING

Die folgenden Fragen beziehen sich auf den **Zeitpunkt der Blutentnahme** und sind für die korrekte Auswertung der Blutwerte wichtig.
Kreuzen Sie daher bitte im Folgenden das jeweils für Sie Zutreffende an.

1)	Ich fühle mich erkältet.					ja O	nein O
2)	Ich habe erhöhte Temperatur / Fieber.					ja O	nein O
3)	Ich bin traurig.						
	O	O	O	O	O	O	O
	gar nicht	sehr schwach	schwach	etwas	ziemlich	stark	sehr stark
4)	Ich fühle mich ängstlich.						
	O	O	O	O	O	O	O
	gar nicht	sehr schwach	Schwach	etwas	ziemlich	stark	sehr stark
5)	Wie haben Sie in der vergangenen Nacht geschlafen?						
	O	O	O	O	O	O	O
	ausge-zeichnet	sehr gut	Gut	mittel	schlecht	sehr schlecht	mise-rabel
6)	Seit wie vielen Stunden sind Sie **heute** bereits wach? _____ Stunde(n)						
7)	Haben Sie **heute** bereits körperlich anstrengende Tätigkeiten ausgeführt (Gymnastik, Sport)?					ja O	nein O
8)	Haben Sie **heute** bereits Medikamente eingenommen?					ja O	nein O
	Falls ja: Welche? _____						
9)	Haben Sie **heute** bereits etwas gegessen?					ja O	nein O
10)	Haben Sie **heute** bereits Zigaretten / Zigarren / Pfeife geraucht?					ja O	nein O
	Falls ja, wie viele _____,						
	und vor wie vielen Minuten die letzte? _____ Minuten						
	und wie viele rauchen Sie derzeit pro Tag? _____						
11)	Haben Sie **heute** bereits Kaffee oder Schwarztee getrunken?					ja O	nein O
	Wie viele Tassen trinken Sie derzeit pro Tag? _____						

C. VISUAL ANALOGUE SCALES

Bitte setzen Sie im Folgenden auf einer Skala von 1 bis 10 ein Kreuz unter der Zahl, die Ihr momentanes Empfinden am besten ausdrückt.

1. Im Augenblick fühle ich mich

Sehr schlecht — Sehr gut

1 2 3 4 5 6 7 8 9 10

---

2. Im Augenblick bin ich

Sehr aufgeregt — Sehr ruhig

1 2 3 4 5 6 7 8 9 10

---

3. Im Augenblick bin ich

Sehr müde — Sehr wach

1 2 3 4 5 6 7 8 9 10

---

4. Im Augenblick bin ich

Sehr gelassen — Sehr ängstlich

1 2 3 4 5 6 7 8 9 10

---

5. Im Augenblick bin ich

Sehr zerfahren — Sehr konzentriert

1 2 3 4 5 6 7 8 9 10

D. SOCIODEMOGRAPHIC QUESTIONNAIRE

Schulabschluss:

□ noch Schüler
□ kein Schulabschluss
□Hauptschulabschluss oder gleichwertiger Abschluss
□ Real- (Mittel-) oder Handelsschule oder gleichwertiger Abschluss
□ (Fach-)Abitur
□ Sonstiges: _____

Berufsabschluss:

□ noch Schüler/ in Ausbildung/ im Studium
□ kein Ausbildungsabschluss
□ Lehre bzw. Berufsausbildung/ Fachschule/ Techniker
□ Universitäts- bzw. Fachhochschulabschluss
□ Sonstiges: _____

Familienstand:

□ verheiratet/in eingetragener Lebenspartnerschaft
□ geschieden/getrennt lebend
□ ledig, in einer Partnerschaft
□ ledig, nicht in einer Partnerschaft
□ verwitwet
□ sonstiges: _____

Wohnsituation:

□ allein
□ mit Ehepartner(in)/Lebenspartner(in)
□ in Wohngemeinschaft
□ bei den Eltern oder Verwandten

Wohnumgebung:

□ städtisch
□ Stadtrand
□ ländlich

Aktuelle Tätigkeit:

□ berufstätig
□ mithelfend im eigenen Betrieb
□ Hausfrau/Hausmann
□ Schüler
□ Student
□ in Berufsausbildung
□ arbeitslos
□ arbeitsunfähig (krankgeschrieben)
□ Sonstiges: _____

Monatlich zur Verfügung stehendes Geld:

□ < 200 Euro
□ 200 – 500 Euro
□ 500 – 800 Euro
□ 800 – 1100 Euro
□ > 1100 Euro

Beruf:

□ Leitender Angestellter
□ Nichtleitender Angestellter
□Beamter des höheren oder gehobenen Dienstes
□ Beamter des mittleren oder einfachen Dienstes
□ Facharbeiter mit abgelegter Prüfung
□ Sonstiger Arbeiter
□ Noch Schüler/Auszubildender/Student
□ Sonstiges: _____

Falls Sie studieren oder studiert haben, geben Sie bitte die Fachrichtung(en) an (unabhängig davon, ob Sie einen Abschluss erhalten haben):

_____

Falls Sie in Ausbildung sind oder eine Ausbildung abgeschlossen haben, geben Sie bitte die Bezeichnung der Ausbildung an:

_____

E. QUESTIONNAIRE SIDE EFFECTS

Im Folgenden finden Sie eine Reihe an möglichen körperlichen und psychischen Empfindungen. Bitte geben Sie für jede Empfindung an, ob Sie diese in den letzten 24 Stunden verspürt haben und wenn ja, wie stark diese war.

Empfindung / Symptom	**Zutreffendes bitte ankreuzen**	**Wie stark war die Empfindung?**		
		leicht	mittel	stark
1. Benommenheit	□ JA □ NEIN	□	□	□
2. Hautreaktion	□ JA □ NEIN	□	□	□
3. Appetitmangel	□ JA □ NEIN	□	□	□
4. Appetitzunahme	□ JA □ NEIN	□	□	□
5. Verwirrtheit	□ JA □ NEIN	□	□	□
6. Halluzinationen	□ JA □ NEIN	□	□	□
7. Wutanfälle	□ JA □ NEIN	□	□	□
8. Schlaflosigkeit	□ JA □ NEIN	□	□	□
9. Nervosität	□ JA □ NEIN	□	□	□
10. Kopfschmerzen	□ JA □ NEIN	□	□	□
11. Verstopfung	□ JA □ NEIN	□	□	□
12. Übelkeit	□ JA □ NEIN	□	□	□
13. Schwindel	□ JA □ NEIN	□	□	□
14. Innere Unruhe, Nervosität	□ JA □ NEIN	□	□	□
15. Verschlafenheit	□ JA □ NEIN	□	□	□
16. Konzentrationsschwierigkeiten	□ JA □ NEIN	□	□	□
17. Änderungen der Libido	□ JA □ NEIN	□	□	□
18. Müdigkeit	□ JA □ NEIN	□	□	□
21. Andere:		□	□	□
22. Andere:		□	□	□

F. DIARY GENERAL MENTAL STATE

1. Wie viele Stunden haben sie in der letzten Nacht geschlafen?

_____ Stunden

2. Wie bewerten Sie die Qualität Ihres Schlafes in der letzten Nacht? (bitte ankreuzen)

1	2	3	4	5	6	7	8	9	10
Nicht erholsam				mittelmäßig					sehr erholsam

3. Haben Sie sich gestern sportlich betätigt?

□ ja □ nein

Falls ja, wie lange: _____ Minuten Sportart: _____

4. Haben Sie gestern Alkohol getrunken?

□ ja □ nein

Falls ja, wie viel: _____

5. Hatten Sie gestern ein besonders stressiges Ereignis (z. B. Prüfung)?

□ ja □ nein

Falls ja, welches: _____

6. Besonderheiten

_____

_____

G. POST STUDY QUESTIONNAIRE

Diesen Fragebogen am Telefon mit dem Probanden durchgehen!

(Instruktion für Ratingskalen:

„Bitte geben Sie jeweils die Zahl an, die Ihrem Empfinden am meisten entspricht.“)

1. Wie viele Stunden haben sie in der letzten Nacht geschlafen?

_____ Stunden

2. Wie bewerten Sie die Qualität Ihres Schlafes in der letzten Nacht? (bitte ankreuzen)

1	2	3	4	5	6	7	8	9	10
Nicht erholsam				mittelmäßig					sehr erholsam

3. Wie groß war Ihr Verlangen im Lauf der letzten Woche, das Medikament weitereinzunehmen?

1	2	3	4	5	6	7	8	9	10
Sehr gering					mittelmäßig				sehr groß

4. Hatten Sie im Verlauf der letzten Woche körperliche Auffälligkeiten?

□ ja □ nein

Falls ja, welche:

_____

5. Hatten Sie im Verlauf der letzten Woche psychische Auffälligkeiten?

□ ja □ nein

Falls ja, welche:

_____

6. Wie hoch war Ihr Stresslevel am Abend des letzten Studientags?

1	2	3	4	5	6	7	8	9	10
Sehr gering				mittelmäßig					sehr groß

7. Bitte geben Sie im Folgenden Ihre Einschätzung darüber an, in welcher Gruppe Sie gewesen sind.

□ Placebo

□ Alprazolam

□ Etifoxin

□ eines der beiden Medikamente aber ich weiß nicht welches

H. PRE-RECORDED QUESTIONS VR-TSST

Speech

1 „Ihr Lebenslauf und Ihre Zeugnisse liegen uns bereits vor, bitte berichten Sie weiter über Ihre persönlichen Eigenschaften."

2 „Sie haben noch Zeit, bitte fahren Sie mit Ihren Eigenschaften fort."

3 „Das ist ja alles schön und gut, aber kommen Sie noch auf Ihre negativen Eigenschaften zu sprechen"

4 "Kommen Sie noch mal auf Ihre negativen Eigenschaften zurück."

5 „Warum halten Sie sich selbst für geeigneter als andere Bewerber?"

6 „Vervollständigen Sie bitte den Satz 'ich bin der/die beste in ... '"

7 „Was schätzen Ihre Familie und Ihre Freunde besonders an Ihnen?"

8 „Welche Führungsqualitäten besitzen Sie?"

9 „Was schätzen Sie an Kollegen?"

Math task

1 „Fehler. 2023 bitte."

2 „Fehler. Bitte noch einmal von vorne."

3 „Bitte bemühen Sie sich, etwas schneller zu rechnen."

4 „Bitte sprechen Sie die Zahl nach dem Schema 1992."

I. PROBAND INFORMATION NPU THREAT TEST

Sehr geehrter Versuchsteilnehmer,

bitte lesen Sie die nachfolgende Aufklärung aufmerksam durch. Im Folgenden werden Ihnen noch einmal Details zu den physiologischen Messungen und zum Ablauf dieser Untersuchung erklärt. Sollten Ihnen Teile der Aufklärung unklar sein oder Sie weitere Informationen über einzelne Aspekte der Untersuchung wünschen, gibt Ihnen Ihr/e Untersuchungsleiter/in gerne Auskunft.

Wie ist der Ablauf?

Zunächst werden Ihnen Fragebögen und Skalen zu Ihrem Befinden vorgelegt. Während der Untersuchung werden wir Ihre Herzrate (EKG), Hautleitfähigkeit/Schweißdrüsenaktivität (EDA) und Augenmuskelaktivität (EMG) messen. Dazu wird der Versuchsleiter mehrere Messelektroden auf Ihre Hand, auf Ihren Oberkörper und Ihr Gesicht ankleben.

Der NPU- Threat-Test besteht aus drei Bedingungen: 1) kein Elektroschock (**N**o event = N-Bedingung), 2) vorhersehbarer Elektroschock (**P**redictable = P-Bedingung), 3) unvorhersehbarer Elektroschock (**U**npredictable = **U**-Bedingung). In der N-Bedingung sind sie vor einem Elektroschock sicher. In der P-Bedingung ist es für Sie vorhersehbar, wann Sie einen Elektroschock erhalten, da dieser durch eine geometrische Form angekündigt wird. Im Gegensatz dazu ist ein Elektroschock in der U-Bedingung nicht vorhersehbar, da er nicht angekündigt wird. Der Test besteht aus zwei Blöcken mit einer 5-minütigen Pause dazwischen. Während der Pause und am Ende werden Ihnen noch kurze Fragebögen vorgelegt. Der Termin dauert ca. 1 Stunde.

Welche Risiken sind mit der Erhebung verbunden?

Vor dem Anbringen der Elektroden wird die Haut mit einer alkoholischen Lösung desinfiziert und mit einer Art Peeling behandelt. Das kann zu einer kurzfristigen Hautirritation führen. Anhaltende Folgen sind nicht zu erwarten. Manche der dargebotenen Stimuli sind unangenehm (elektrische Stimulation, laute Geräusche) aber nicht gefährlich oder schmerzvoll. Auch hier sind keine anhaltenden Folgen zu erwarten.

J. INSTRUCTION SHOCK WORK-UP

---

*Bitte alle nicht kursiven Absätze **vorlesen**!*

Zur Erforschung bestimmter Prozesse beim Menschen (wie Furcht, Angst und Vermeidung) ist es unumgänglich, unangenehme Reize zu präsentieren, die in der Lage sind, diese Prozesse anzustoßen.

In dieser Studie handelt es sich hierbei um elektrische Reize, deren Stärke man gut als unangenehm oder schmerzhaft einstellen kann, welche aber gesundheitlich vollkommen unbedenklich sind.

Anlegen der Elektrode oben mittig am rechten Unterarm.

Unser Ziel ist es, die Schwelle zu finden, an der die Reize für dich nicht mehr nur „unangenehm“ sind, sondern eine schmerzhafte Qualität entwickeln. Dies ist eine persönliche Einschätzung und fällt daher bei jedem Menschen anders aus. Deshalb wollen wir nun mit dem folgenden Verfahren deine individuelle Schwelle für die elektrischen Reize ermitteln, die später im Experiment verwendet werden. Um diese Schwelle möglichst effizient zu finden, setzen wir einen Algorithmus ein, der anhand deiner Antwort die Stärke des nächsten Reizes vorgibt.

Wenn ein Reiz für dich schmerzhaft ist, teile das sofort mit. Das Ziel ist nicht, Stärke zu demonstrieren. Andererseits möchten wir dich darauf hinweisen, dass die durch die Studie gewonnenen Erkenntnisse darauf basieren, dass der Reiz für dich wirklich unangenehm ist.

Bitte achte darauf, wie sich die Reize für dich anfühlen und versuche, nicht zu viel darüber nachzudenken, sondern möglichst spontan zu antworten, ob der Reiz für dich noch „unangenehm“ oder bereits „schmerzhaft“ ist.

Wenn du bereit bist, werde ich den Countdown für die elektrische Stimulation auslösen und von 3 an herunterzählen. Bist du bereit?

QUEST Prozedur durchführen:
VP soll dabei weder Trial-Zahl noch eingestellte Stromstärke auf Monitor oder Digitimer sehen

---

Nach QUEST Prozedur:
Finale Stromstärke = Endergebnis x 1.3 (Wert wird automatisch berechnet)

Gleich wird ein Reiz präsentiert, der etwas stärker ist als die eben identifizierte Schwelle. Wenn dieser Reiz für dich aushaltbar ist, werden wir ihn im Laufe des Experiments verwenden.

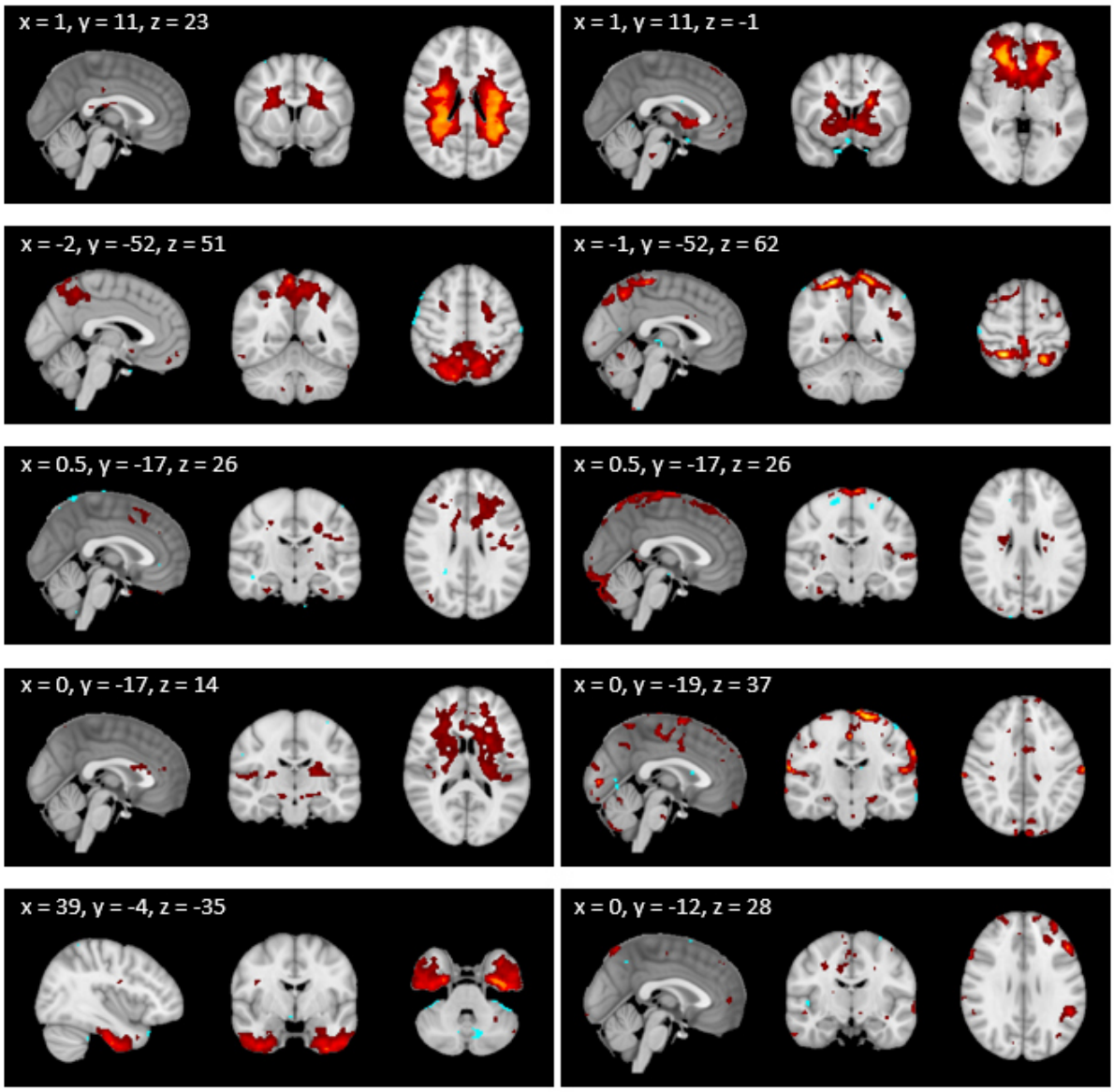
Wenn du bereit bist, werde ich den Countdown auslösen und von 3 an herunterzählen. Bist du bereit?

Falls „nicht aushaltbar“ (möglicherweise mehrfach); Werte werden automatisch berechnet

---

Wir werden den Abstand zur Schwelle nun etwas reduzieren. Bist du bereit?

K. EXCLUDED COMPONENTS RESTING STATE FMRI



L. PROBAND INFORMATION AX-CPT

Sehr geehrter Versuchsteilnehmer,

bitte lesen Sie die nachfolgende Information zur Testung aufmerksam durch.

Im Folgenden werden Ihnen noch einmal Details zum Ablauf erklärt. Sollte Ihnen noch etwas unklar sein oder Sie weitere Informationen über einzelne Aspekte wünschen, gibt Ihnen Ihr/e Untersuchungsleiter/in gerne Auskunft.

Wie ist der Ablauf?

Im folgenden Experiment werden Ihnen nacheinander Folgen von Buchstaben gezeigt.

Ihre Aufgabe ist es, für jeden Buchstaben, den Sie sehen, durch Drücken der entsprechenden Taste anzugeben, ob es sich um ein Target (= Zielbuchstabe) handelt.

Der **Target-Buchstabe** in diesem Experiment ist ein **X,** jedoch **NUR dann, wenn das X auf ein A folgt.**

Wenn ein X nach irgendeinem anderen Buchstaben kommt, sollten Sie es nicht als Target betrachten.

Wenn Sie beispielsweise die Sequenz A, X, B, X sehen, ist nur der zweite Buchstabe der Sequenz ein Target. Alle anderen Buchstaben sind keine Targets.

Wenn der Buchstabe, der erscheint, ein **TARGET** ist, drücken Sie die **LINKE Maus-Taste.**

Wenn der Buchstabe, der erscheint, **KEIN TARGET** ist, drücken Sie die **RECHTE Maus-Taste.**

Versuchen Sie, so schnell und genau wie möglich zu antworten.

Der Test besteht aus einer Übungsphase und insgesamt 4 Blöcken.

Er dauert ca. 25 Minuten.

Zwischen den Blöcken erscheint jeweils die Instruktion: „*Um fortzufahren, drücken Sie die linke Maustaste.*“ Bitte fahren Sie dann möglichst zügig fort.

Es ist sehr wichtig, dass Sie die gesamte Testung über aufmerksam bleiben.

Sollten noch Fragen bestehen, können Sie diese jetzt dem Versuchsleiter stellen.

Übung

Bitte kreuzen Sie im Folgenden an, welche Maustaste im Experiment für den jeweiligen Buchstaben gedrückt werden soll.

Buchstabe	**Maustaste**	
	Links	Rechts
A	☐	☐
X	☐	☐
C	☐	☐
B	☐	☐
A	☐	☐
Y	☐	☐
B	☐	☐
X	☐	☐
A	☐	☐
X	☐	☐
R	☐	☐
T	☐	☐
A	☐	☐
X	☐	☐