

Dare to Approach

Effects of Testosterone on
Avoidance in Social Anxiety

Dorien Enter

The logo for the Behavioural Science Institute is a circular emblem with a grey border. Inside the circle, the text "Behavioural Science Institute" is written in a clean, sans-serif font, arranged in three lines. A grey pointer-like shape extends from the top right of the circle towards the text.

Behavioural
Science
Institute

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General Introduction



More than a century ago the distinguished neurologist and physiologist Charles Edouard Brown-Séquard published an article in leading medical journal *The Lancet* titled “The effects on man by subcutaneous injections of a liquid obtained from the testicles of animals.” (Brown-Séquard, 1889). In this article he describes enthusiastically how he extracted a liquid from a mixture of dog and guinea pig blood, semen, and testicles, painfully injected it in his own thigh, and subsequently experienced huge invigorating effects. Despite the fact that the experiment was not received well by his colleagues, it gave an important boost to “the study of the internal secretion of glands, a branch of physiology which gives promise of leading to important advances in therapeutics.” (Bowditch, 1897, p. 96).

Nowadays we know that the effects reported by Brown-Séquard must be ascribed to a placebo response, but we also know that the substance he was after is the gonadal steroid hormone testosterone (Cussons, Bhagat, Fletcher, & Walsh, 2002). Besides having virilizing and anabolic effects, more recent psychophysiological research has shown that testosterone also bears anxiolytic properties and stimulates social approach motivation. It plays a crucial role in the regulation of social behavior, together with the steroid hormone cortisol, which is associated with stress and social avoidance. Imbalances in testosterone and cortisol levels are linked to psychopathology. This thesis will focus on social anxiety disorder (SAD), characterized by increased cortisol responses and persistent avoidance of social situations. Although the role of cortisol in social fear and avoidance in SAD has been explored, studies on the actions of testosterone thus far are lacking. The research presented in this thesis investigates whether testosterone levels are deviating in SAD, tests whether administering testosterone could counteract the persistent social avoidance tendencies in SAD, and explores some of the neuroendocrinological mechanisms. This not only advances our theoretical understanding of steroid involvement in social emotional behavior, but also may have implications for the treatment of SAD.

Psychoneuroendocrinology of social motivational behavior

Social environment, emotion, and motivation

Like many social species, humans organize themselves in social status hierarchies, which help stabilize the social organization of individuals (Gilboa-Schechtman & Shachar-Lavie, 2013; Sapolsky, 2005). Emotional facial expressions play an important role in social communication within this system and ensure a non-violent regulation of the status hierarchy. Smiling faces initiate social interaction, but also function as a signal of appeasement, whereas angry expressions typically convey social dominance and are being perceived as a threat (Öhman, 1986). For a socially anxious individual such a sign of a potentially aggressive encounter is highly distressing and therefore avoided. In contrast,

a more dominant approach-oriented individual would rather engage in the confrontation, because such an encounter may end up in a possibly rewarding outcome, such as increased social status and its benefits. Accordingly, SAD may be based on this ubiquitous social hierarchy system, with patients displaying exaggerated socially submissive behavior (Gilbert, 2001; Hermans & van Honk, 2006; Öhman, & Wiens, 2003; Weisman, Aderka, Marom, Hermesh, & Gilboa-Schechtman, 2011)

Adaptive regulation of social approach or avoidance responses is crucial for successful social functioning. In response to a social stimulus (e.g., an angry facial expression), the individual will show a quick and automatic sequence of response stages, the freeze-fight-flight response (Bradley, Codispoti, Cuthbert, & Lang, 2001). After the initial freeze response in which the individual ceases all ongoing activity, and quickly and subconsciously assesses the situation, it will select either to approach the situation (i.e., fight), or to avoid it (i.e., flight; Blanchard, Griebel, Pobbe, & Blanchard, 2011). Typically, the automatic evaluation directly results in a behavioral disposition towards the stimulus: appetitive stimuli will elicit approach responses, whereas aversive stimuli will elicit avoidance tendencies. These types of automatic tendencies are suggested to play a prominent role in the maintenance of psychopathology (Blanchard et al., 2011; Turk, Lerner, Heimberg, & Rapee, 2001; Wong & Moulds, 2011). When the system is impaired they may lead to aggression in case the fight response is triggered too easily, or in persistent avoidance when the flight-response is too prominent (Blanchard et al., 2011), as is the case with SAD. The hormones testosterone and cortisol are important in the regulation of such social motivational behavior, as will be described below.

Neurobiology underlying social motivational behavior

Approach-avoidance responses are mediated by complex interacting neural networks, which can be categorized in the so called Emotional Network, Reward Network, and the Cognitive Control Network (Cremers & Roelofs, 2016), which will be broadly described hereafter. The amygdala plays a central role in the Emotion Network: its subnuclei process salient information from the environment, such as emotional facial expressions, and trigger behavioral responses in response to these environmental stimuli. The Basolateral Amygdala (BLA) receives input from the thalamus and sensory cortices (such as fusiform gyrus, involved in face processing), whereas the Central Amygdala (CeA) orchestrates autonomic responses by projections to the Periaqueductal Grey (PAG) initiating freeze, to brainstem nuclei for release of neurotransmitters, and the hypothalamus for release of Corticotropin Releasing Hormone (CRH) and Gonadotropin Releasing Hormone (GnRH), which eventually leads to enhanced cortisol and testosterone levels respectively. The amygdala is also connected to the Reward Network, which comprises the ventral tegmental area (VTA), striatum (including the nucleus accumbens (NAcc), and medial prefrontal cortex (mPFC) (Haber & Knutson, 2010). Striatal dopamine transmission is es-

sential for the adaptive regulation of social behavior as it is involved in reward learning, (i.e., obtaining social reward but also avoiding punishment; see Delgado, Jou, LeDoux, & Phelps, 2009), behavioral activation, and motivational behavior (Cools, 2008; Yacubian & Büchel, 2009). The anterior prefrontal cortex plays a crucial role in the Cognitive Control Network as it is involved in the regulation of emotion (Damasio, 1994; Rolls, 1999), and in social motivational behavior as it inhibits the amygdala, making it possible to control and override automatic behavioral approach and avoidance tendencies (Roelofs, Minelli, Mars, van Peer, & Toni, 2009; Volman, Roelofs, Koch, Verhagen, & Toni, 2011), and modulates mesolimbic striatal activity (Grace, Floresco, Goto, & Lodge, 2007; Wager, Davidson, Hughes, Lindquist, & Ochsner, 2008). Naturally, this description is a highly simplified one, and many other brain regions partake in these networks.

Hormonal regulation of social motivational behavior

The Hypothalamus-Pituitary-Gonadal (HPG) axis with its end product testosterone plays a key role in the neuroendocrine regulation of social motivational behavior in both sexes. Testosterone levels follow a pulsatile, seasonal, and diurnal cycle in which levels are highest upon waking and typically decline with 50% during the day (Dabbs, 1990). Under influence of signals from mainly the amygdala Gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus, which stimulates the production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the pituitary gland, which in turn triggers production of testosterone and estradiol in the gonads (i.e., testes and ovaries). In addition, small amounts of testosterone are produced in the adrenal cortex, and synthesized in the brain from cholesterol and other steroid precursors. Testosterone is able to cross the blood-brain barrier, and besides having (epigenetic) organizational effects on brain structure during pre- and early postnatal development, testosterone also influences emotion, motivation, and behavior later in life (i.e., activational effects; Lombardo et al., 2012; McHenry, Carrier, Hull, & Kabbaj, 2014). Actions of testosterone are brought about directly via androgen receptors but also via metabolites such as estradiol, dihydrotestosterone, and 3 α -diol, which binds to the γ -aminobutyric acid (GABA-A) receptor (Balthazart & Ball, 2006; Wood, 2008). The effects can either be slow and long-lasting (i.e., hours-days) via a genomic pathway featuring intracellular steroid receptors, or rapid (i.e., seconds-minutes) via membrane-bound (steroid) receptors, which exert non-genomic actions in the cell. Importantly, testosterone acts through a steroid-responsive network which includes the amygdala, hypothalamus, hippocampus, and PAG, among other limbic areas (Wood, 1996), and hence influences the flight-fight response. Naturally, testosterone interacts with other neurotransmitters and -peptides, such as serotonin (probably via estradiol), vasopressin, oxytocin, and dopamine. With regard to the latter, testosterone enhances dopamine transmission in the mesolimbic system, which in turn can lead to increased reward sensitivity and augmented motivational behavior (de Souza

Silva, Mattern, Topic, Buddenberg, & Huston, 2009; Hermans et al., 2010; Welker, Gruber, & Mehta, 2015).

The HPG-axis works in antagonism with the Hypothalamus-Pituitary-Adrenal (HPA) axis, in such a way that the end product of the latter (i.e., cortisol, released in response to stress) disrupts production and inhibits actions of testosterone, which in turn inhibits the stress-induced activation of the HPA-axis at the hypothalamus (Viau, 2002). Both neuroendocrine axes are important in the regulation of social-motivational behavior: higher basal cortisol levels, and low testosterone, are associated with social subordination stress and avoidance behavior, whereas higher basal testosterone and low cortisol facilitates social dominance and approach behavior (Bedgood, Boggiano, & Turan, 2014; Mehta & Josephs, 2010; Sapolsky, 1990, 1991; Honk et al., 1999).

Baseline hormone levels are in general predictive of psychological traits and behavior (Welker, 2015), whereas social events are typically associated with a temporary surge or decline in hormone levels (Casto & Edwards, 2016; Maner, Miller, Schmidt, & Eckel, 2008; Sapolsky, 1991). The social challenge hypothesis states that testosterone levels rise in preparation to a challenging encounter in which social status might be threatened, thereby initiating approach motivation and simultaneously reducing fear (Archer, 2006; Mazur & Booth, 1998; Wingfield, Hegner, Dufty Jr., & Ball, 1990). Several studies featuring single dose testosterone administration, which leads to a transient increase in testosterone levels, to healthy female participants confirmed the causal relationship between testosterone and its effects on the social motivational system. The findings show that testosterone administration reduces fear and sensitivity to threat and punishment, enhances reward sensitivity, and promotes social approach motivation aimed at achieving social status (i.e., social reward; see for a review Bos, Panksepp, Bluthe, & van Honk, 2012). These actions are brought about by anxiolytic effects (GABA, androgen receptors; McHenry et al., 2014) and upregulation of the dopaminergic system (de Souza Silva et al., 2009), in addition to biasing the amygdala towards threat approach (Radke et al., 2015), and reducing prefrontal control over the amygdala (Schutter & van Honk, 2004; van Wingen, Mattern, Verkes, Buitelaar, & Fernández, 2010; Volman, Toni, Verhagen, & Roelofs, 2011). Although associated with aggression (Montoya, Terburg, Bos, & van Honk, 2012), the effects of testosterone on social motivational behavior depend on social context and individual differences, and thus do not entail aggressive behavior per se, but could also lead to prosocial behavior when this is more appropriate to ensure an increase in social status (Boksem et al., 2013; Carré et al., 2016; Eisenegger, Haushofer, & Fehr, 2011; Mehta & Josephs, 2010; Stanton & Schultheiss, 2009; van Honk, Terburg, & Bos, 2011).

Social Anxiety Disorder

Clinical description

Social Anxiety Disorder (SAD) is characterized by an intense fear of social situations in which the individual may be scrutinized by others (American Psychiatric Association, 2013). The affected individual fears that he/she will behave, or show anxiety symptoms, in a way that will be negatively evaluated and will lead to rejection by others. Social situations, such as social interactions, are therefore avoided or endured with intense fear or anxiety. Avoidance behavior plays a crucial role in the persistence of the disorder, and hinders extinction of fear in social situations as it reduces the opportunity for accommodation to and reevaluation of the situation (Clark & Wells, 1995). In addition, when engaging in social interaction, someone with SAD typically avoids eye contact (Stein & Stein, 2008). As eye contact is important in social communication, this characteristic hinders social interactions and influences how others respond to the person with SAD, reinforcing the social fear-avoidance cycle. Furthermore, there is evidence that SAD persists because of biased processing of social information, favoring disorder-relevant information, which leads to interpretation of the situation as more negative than it was in reality (Heeren, Lange, Philippot, & Wong, 2014; Stein & Stein, 2008). With a lifetime prevalence rate of 7-12% SAD is the most common anxiety disorder and among the most common psychiatric disorders (Kessler, Berglund, et al., 2005). Onset occurs in childhood or early adolescence, and SAD affects more women than men. The disorder typically leads to significant distress, and - when left untreated - tends to follow a chronic, unremitting course leading to substantial impairments in vocational and social functioning. Treatment of SAD consists of pharmacotherapy and/or psychotherapy, mainly cognitive behavioral therapy aiming at acquiring the behavioral and cognitive skills to function effectively. Exposure therapy is part of the latter, and aims at fear extinction by repeated or prolonged exposure to feared social situations, leading to a reduction of anxiety and avoidance behavior. Notwithstanding the efficacy of current evidence based psychological and pharmacological treatments in SAD, nonresponse rates in large clinical trials have been up to 50% (Hofmann & Bögels, 2006; Stein & Stein, 2008), leaving considerable room for improvement.

Neurobiology of SAD

SAD is associated with deviations in the neuroendocrine brain circuits underlying social motivational behavior. Several meta-analytic studies have consistently shown a hyperactive amygdala in response to social threat, probably reflecting enhanced processing of and attention to threat (Brühl, Delsignore, Komossa, & Weidt, 2014; Cremers & Roelofs, 2016; Fouché, van Der Wee, Roelofs, & Stein, 2013). In addition, prefrontal structures are also more active than in healthy controls, however prefrontal-amygdala connectivity seems to be reduced (Cremers & Roelofs, 2016; Fouché et al., 2013; although

evidence is inconsistent, see Brühl et al., 2014), indicating an inability to regulate sub-cortical regions. Interestingly, pharmaco- and psychotherapy seem to “normalize” these responses (Fouche et al., 2013; Freitas-Ferrari et al., 2010). Studies also show alterations in striatal functioning in SAD, but findings are mixed (Freitas-Ferrari et al., 2010). A recent fMRI study in patients with SAD compared to healthy controls reported reduced striatal activity in anticipation of social reward and relative increased striatal activity for avoiding social punishment (Cremers, Veer, Spinhoven, Rombouts, & Roelofs, 2014). These findings suggest that patients with SAD show a reduced motivation to obtain social reward and relative increased motivation to avoid social punishment compared to healthy controls. In addition, patients with SAD showed a reduced pattern of fronto-striatal connectivity during reward and punishment anticipation, relative to healthy controls.

Hormonal regulation in SAD

Patients with SAD show an increased cortisol response to social stress, compared to healthy participants and patients with Post-Traumatic Stress Disorder (PTSD), and this response was associated with social avoidance behavior (Roelofs, van Peer, Berretty, de Jong, et al., 2009). Studies combining cortisol administration with Electroencephalography (EEG) in patients with SAD confirmed a causal relationship between cortisol and increased early processing of emotional faces during social avoidance (Van Peer, Spinhoven, & Roelofs, 2009), and modulation of early threat processing depending on motivational context and symptom severity (van Peer, Spinhoven, & Roelofs, 2010). In addition, both higher baseline levels of cortisol and exogenous cortisol are associated with EEG wave activity patterns related to anxiety and behavioral inhibition (Schutter & van Honk, 2005; van Peer, Roelofs, & Spinhoven, 2008), whereas testosterone has an opposite effect (Schutter & van Honk, 2004). This thesis contains the first published studies on the role of the HPG-axis and testosterone in SAD. Studies on testosterone in social anxiety are scarce, and although testosterone levels in socially anxious men dropped in response to losing a competition, no differences in baseline levels of testosterone were reported (Gerra et al., 2000; Maner et al., 2008). Previous research mainly focused on the role of testosterone in depression, and found that reduced testosterone levels are associated with depressive moods and anxiety (Ebinger, Sievers, Ivan, Schneider, & Stalla, 2009; McHenry et al., 2014). Interestingly, testosterone administration for the treatment of depression has been applied since mid-twentieth century (Altschule & Tillotson, 1948), and positive effects on mood have been reported (Ebinger et al., 2009). Besides cortisol and testosterone, also other neuropeptides and hormones are involved in social anxiety and avoidance, such as oxytocin, vasopressin, and progesterone (Crespi, 2016; Maner, Miller, Schmidt, & Eckel, 2010; Meyer-Lindenberg, Domes, Kirsch, & Heinrichs, 2011).

Aim of this thesis

In sum, individuals with SAD show alterations in the regulation of social motivational behavior characterized by persistent social avoidance, increased cortisol responses, enhanced threat sensitivity, and probably reduced reward processing, a pattern that is associated with socially submissive behavior. Considering that testosterone is associated with social dominance, entailing enhanced approach motivation, and reduced fear and threat sensitivity, it is striking that so far no studies have investigated the role of testosterone in SAD. This thesis aims to elucidate the role of testosterone in the neuroendocrine mechanisms underlying social fear and avoidance in SAD. We hypothesize that 1) SAD is associated with reduced testosterone levels compared to healthy controls, and 2) that administering testosterone to people with SAD will alleviate social fear and avoidance, and will promote prosocial behavior. The first hypothesis was tested by assessing salivary testosterone levels in a large group of people including SAD patients and healthy controls. We tested the second hypothesis by investigating the effects of single dose testosterone administration in women with SAD as compared to healthy controls, on several indexes of motivational behavior and attention. The background of the used methods is briefly outlined in Box 1.1. In addition, based on animal and human evidence on the role of dopamine in social approach and avoidance behavior, and given the important interactions between testosterone and striatal dopamine signaling (de Souza Silva et al., 2009; Hermans et al., 2010), an initial study was performed to explore the interaction between striatal dopaminergic polymorphisms and social approach-avoidance tendencies.

Outline of this thesis

Social reward processing is of importance for the adaptive regulation of social behavior, but it has not yet been explored how it affects actual social approach-avoidance behavior. **Chapter 2** describes a first study on the effect of alterations in the mesolimbic reward system on social approach-avoidance tendencies. An implicit social AAT is performed by two groups of participants who carry different variants of a gene involved in striatal dopamine transmission, which affects their reward sensitivity.

Thus far no studies have systematically tested the effects of testosterone on actual social approach-avoidance action tendencies. **Chapter 3** presents a first study testing whether single dose testosterone administration diminishes threat avoidance and promotes behavioral approach actions that have an immediate effect on the relative distance of the social threat stimulus, by using an implicit social AAT performed by healthy individuals.

Box 1.1. General methodology

Exogenous testosterone Administering testosterone enables determination of a causal relationship between a rise in testosterone levels and its effects on the social motivational system. We applied sublingual administration of testosterone cyclodextrin, a method that has been used for over a decade in healthy and highly anxious individuals, and has convincingly shown consistent psychophysiological and behavioral effects approximately 4–6 hours after administration (see for a review Bos et al., 2012). Its pharmacodynamic profile has been established using a reliable automatic and non-habitual measure (Tuiten et al., 2000). The delay between administration and subsequent effects is related to how much of the substance crosses the blood brain barrier, which also depends on the free fraction of testosterone and serum SHBG saturation, and the fact that effects are due to slow genomic effects via steroid receptors, and other routes of action (van der Made et al., 2009; van Rooij et al., 2012).

Action tendencies The automatic and motivationally driven tendency to approach or avoid a certain stimulus translates to motor responses, and can be objectively measured by using a social approach–avoidance task (AAT; Chen & Bargh, 1999; Heuer et al., 2007; Roelofs et al., 2005; Rotteveel & Phaf, 2004). This reaction time task requires participants to approach and to avoid socially appetitive and aversive visually presented stimuli (happy and angry faces, respectively) by pulling (approach) or pushing away a joystick (avoidance). Pulling or pushing the joystick increases or decreases the size of the picture respectively, giving the impression of moving towards or moving away from the participant. Affect congruent responses (i.e., approaching happy or avoiding angry faces), lead to quicker responses than when these automatic tendencies need to be overridden as is the case with affect-incongruent responses (i.e., Approaching angry or avoiding happy faces). Highly socially anxious individuals have consistently been shown to push away angry faces faster compared to neutral ones (Heuer et al., 2007; Roelofs et al., 2010).

Gaze behavior Avoidance of eye contact is a characteristic and persistent social feature in people with SAD, and is typical socially submissive behavior aimed at reducing social threat (Horley et al., 2003, 2004; Öhman, 1986). Gaze behavior can be assessed by using eye-tracking, which is the process of measuring where the participant is looking on a screen by tracking eye movements. The eye tracker uses infrared light that is reflected from the eye and sensed by a video camera: moving the eye causes changes in reflections which are used to extract information on the position of the eye. Eye movements are typically categorized into fixations (i.e. when the gaze stops in a certain position for about 100ms) and saccades (i.e., when it moves from one to another position). Individuals with SAD tend to avoid looking at the eyes of emotional facial expressions presented on computer screens (e.g., Horley et al., 2004; Moukheiber et al., 2010).

Electroencephalography We used EEG to capture early attention processes, which lay at the basis of the flight–fight response. Cognitive processes in the brain cause waves of electric currents that can be measured at the scalp using EEG. In response to sensory events, certain patterns in voltage changes emerge at typical topographical locations on the scalp, called Event Related Potentials (ERPs). We performed a spatiotemporal clustering analysis (Brunet et al., 2011; Murray et al., 2008) on the ERPs, as it can tease apart the following two ERP effects: 1) topographic modulations, which reflect a change in neural sources, indicating the activation of different cognitive processes (a qualitative change in processing), and 2) amplitude modulations, which, in absence of a concurrent topographic modulation, reflect the increase or decrease in strength of a common cognitive process (a quantitative change in processing; Murray et al., 2008; Pourtois et al., 2008).

Low endogenous levels of testosterone have been associated with major depression, but data on testosterone levels in anxiety disorders are scarce. **Chapter 4** maps salivary testosterone levels in the Netherlands Study of Depression and Anxiety (NESDA; Penninx 2008)), a longitudinal cohort study comprising 2981 participants.

Chapter 5 presents a first study that tests the effects of testosterone on actual social approach–avoidance action tendencies in SAD. Using the same implicit social AAT as in chapter 3, we investigate whether administering a single dose of testosterone can counteract the automatic social avoidance tendencies in patients with SAD.

Previous eye tracking studies have confirmed that avoidance of eye contact is a characteristic and persistent social feature in SAD. **Chapter 6** tests the hypothesis that

administering a single dose of testosterone promotes socially dominant gaze behavior and thus will be able to alleviate submissive gaze avoidance in individuals with SAD.

It remains unknown whether testosterone affects early automatic or later, more controlled, processing-stages of social threat. In **Chapter 7** we therefore investigate the effects of testosterone on social threat processing in participants with SAD and healthy participants, using temporally fine-grained recordings of event-related brain potentials (ERPs) during an emotional Stroop task with subliminally presented angry, happy and neutral faces.

Finally, **chapter 8** presents a summary and integration of the empirical chapters, discusses the strengths and limitations, and considers future theoretical and clinical perspectives.



Dopamine transporter polymorphisms affect social approach–avoidance tendencies

Enter, D., Colzato, L. S., & Roelofs, K. (2012). *Genes, Brain and Behavior*, 11(6), 671–676.

Abstract

There is increasing interest in the role of striatal dopaminergic activity in social approach–avoidance motivation. The 9-repeat allele of the dopamine transporter (DAT) gene, associated with increased striatal dopamine levels, has been found to be related to increased sensitivity to reward. However, it remains unexplored whether this polymorphism influences automatic action tendencies in the social domain. We set out to test experimentally whether human carriers of the 9-repeat allele show increased approach–avoidance tendencies compared to non-9-repeat carriers. One hundred and one healthy adults, genotyped for the DAT gene, performed the social Approach–Avoidance Task, a reaction time task requiring participants to approach or avoid visually presented emotional (happy and angry) faces, by pulling a joystick towards them or pushing the joystick away from themselves, respectively. In accordance with expectations, 9-repeat carriers showed stronger approach–avoidance effects compared to non-9-repeat carriers. These results suggest a role for striatal dopaminergic polymorphisms in motivational responses to social-emotional cues. Our findings may be relevant in the selection of candidate genes in future studies involving social behavior.

Introduction

The dopamine transporter (DAT) is responsible for dopamine (DA) reuptake in the striatum (Sesack, Hawrylak, Matus, Guido, & Levey, 1998). Genetic variations in the dopamine transporter gene (DAT1, SLC6A3) relate to deviations in expression. The chromosome 5p15.3 (Giros et al., 1992; Vandenberghe et al., 1992) contains a 40 base pair variable number of tandem repeats in the 3'-untranslated region (3'-UTR-VNTR). Alleles with a number of repeats ranging from 3 to 13 have been described, but the alleles with 9 and 10 repeats are the most frequently reported (Kang, Palmatier, & Kidd, 1999; Mitchell et al., 2000). The 9-repeat allele is associated with a reduced expression of the transporter, resulting in higher DA concentrations in the striatum as compared to the 10-repeat allele (Heinz et al., 2000; VanNess, Owens, & Kiltz, 2005).

Typically, striatal dopaminergic activity is associated with motivational processes, and there is emerging evidence for a significant role of repeat polymorphisms in DAT genes. For example, DAT1 9-repeat carriers showed more striatal activity during processing of a monetary reward than 10-repeat carriers (Dreher, Kohn, Kolachana, Weinberger, & Berman, 2009; Forbes et al., 2009), suggesting increased reward sensitivity in 9-repeat carriers (see also Aarts et al., 2010). Recent work has also provided evidence for striatal involvement in the processing of social rewards. For instance, Spreckelmeyer and colleagues (2009) found increased striatal responding during the anticipation of social reward, signaled by emotional facial stimuli in healthy individuals. Interestingly, activity in overlapping brain regions was also related to the motivation to avoid social punishment (signaled by angry faces) in patients with social phobia (Cremers et al., 2014). These findings suggest that striatal functioning is not restricted to the sensitivity to obtaining social reward but also to avoiding punishment (see also Beninger, Mason, Phillips, & Fibiger, 1980; Darvas, Fadok, & Palmiter, 2011; Delgado et al., 2009). Indeed, also in healthy individuals changing a picture of an angry facial expression to one of a neutral look, elicits reward related activity in the ventral striatum (Mühlberger et al., 2011).

On the basis of these studies, it can be suggested that striatal functioning, and the specific role of DAT1 polymorphisms, is of crucial importance for the adaptive regulation of social behavior (e.g., Caldú & Dreher, 2007; Yacubian & Büchel, 2009). However, direct evidence for such relation is lacking. The aim of this study was therefore to test the relationship between DAT1 polymorphisms and alterations in social motivational behavior directly, by using an implicit social approach–avoidance task (AAT). The AAT is a valid and reliable measure of social approach–avoidance tendencies (Heuer, Rinck, & Becker, 2007; Roelofs et al., 2010; Roelofs, van Peer, et al., 2009; Roelofs, Minelli, et al., 2009). This reaction time task requires participants to approach and avoid socially appetitive and aversive visually presented stimuli (happy and angry faces, respectively) by pulling (approach) or pushing away a joystick (avoidance). Happy and angry faces elicit automatic approach

and avoidance tendencies, respectively (Chen & Bargh, 1999; Roelofs et al., 2010; Seidel, Habel, Kirschner, Gur, & Derntl, 2010).

On the basis of the rewarding nature of approaching happy faces and avoiding social threat, we hypothesized that healthy individuals carrying the DAT1 9-repeat polymorphism would show increased social approach–avoidance tendencies on the AAT.

Methods

Participants

One hundred and one young Caucasian healthy adults (26 male/75 female), with a mean age of 23 years ($SD = 2.4$, range 19–31), served as participants in exchange for partial fulfillment of course credits or a financial reward. The sample was drawn from adults in the Leiden and Rotterdam metropolitan area (The Netherlands) who volunteered to participate in studies of behavioral genetics. Exclusion criteria were any major medical illness that could affect brain function, current substance abuse, neurological conditions, a history of head injury, or a personal history of psychiatric treatment. Participants were selected by a phone interview on the basis of the Mini International Neuropsychiatric Interview script (M.I.N.I.; Lecubier et al., 1997). Written informed consent was obtained from all participants after the nature of the study had been explained to them; the protocol was approved by the local ethical committee.

Approach–Avoidance Task (AAT)

During this reaction time (RT) task, participants were asked to classify stimuli on the basis of an aspect (color) that was orthogonal to the aspect of interest (facial emotion). Participants responded to emotional face pictures presented on a computer screen, by pulling a joystick either towards their body (approach movement) or pushing it away from their body (avoidance movement) (task adapted from (Heuer et al., 2007). Pulling or pushing the joystick increased or decreased the size of the picture respectively. The speed of the size change was proportional to the amplitude of the joystick movement. As soon as the joystick reached its target position (i.e. the required direction; full movement involved a 30° rotation from the upright position) the picture disappeared from the screen. The time between the onset of the stimulus and its disappearance from the screen was recorded with >1 ms accuracy. After each completed trial, the participant moved the joystick back to its central position and initiated a new trial by pressing the fire button near the top of the joystick. Face stimuli were selected from the Karolinska Directed Emotional Faces database (Lundqvist, Flykt, & Öhman, 1998). Happy, Angry, Neutral, and

Disgusted¹ facial expressions were taken from the same model (five male and five female models in all) and each picture was presented either with a yellowish or a greyish filter. In addition, checkerboards (10 yellow, 10 grey) were included as non-facial control stimuli. This resulted in a total of 100 different stimuli, which were presented in random order. All participants were instructed to push away yellow stimuli and to pull grey stimuli towards them, and to respond as fast and as accurately as possible. Usually, response latencies are shorter for affect-congruent (e.g., happy-approach; angry-avoid) as compared to affect incongruent response conditions (e.g., angry-approach; happy-avoid). Before the real test started, participants were presented with 18 practice trials, which were similar to the test trials except for the fact that the pictures showed different models.

DNA laboratory analysis

Genomic DNA was extracted from saliva samples by means of the Oragene™ DNA self-collection kit, and following the manufacturer's instructions (DNA Genotek, Inc., Kanata, Ontario, Canada; 2006). The DAT1 polymorphism was amplified on an MJ DNA engine thermal cycler (MJ Research), with an initial denaturation at 94°C for 4 min, followed by 32 cycles of 45 s at 94°C, 45 s at 68°C, 60 s at 72°C, and a final elongation of 5 min at 72°C. The 25 ml reaction mixture consisted of 50 mM Tris (pH 9.0), 20 mM NH₄SO₄, 3 mM MgCl₂, 200 mM dNTPs, 0.5 mM primers, and 1U Taq polymerase (Invitrogen, Carlsbad, CA, USA). Products were electrophoresed on 2% agarose gel and visualized by means of ethidium bromide. The oligo primer sequences used to amplify the VNTR are DAT1-F: 5'-TGT GGT GTA GGG AAC GGC CTG AG-3' DAT1-R: 5'-CTT CCT GGA GGT CAC GGC TCA AGG, as originally described in Waldman et al. (1998). Each individual was genotyped twice.

Procedure

All participants were tested individually. They completed a 30 min reasoning-based intelligence test (SPM; Raven, Court, & Raven, 1988), and subsequently performed the AAT, which took about 10 min.

Statistical analyses

Independent samples *t*-tests were performed for analyses of age and gender between 10/10 homozygous and 9-repeat carriers. RT outliers were filtered using a <150 and >1500 ms cut-off. For each participant, the median of the remaining RTs (97%) for the correct responses was calculated per cell (defined by: Emotion and Movement). Following previous studies (Heuer et al., 2007; Roelofs, Elzinga, & Rotteveel, 2005; Roelofs et al.,

1 The disgusted face stimuli have not yet been validated for the AAT (see also Seidel, Habel, Kirschner, Gur, & Derntl, 2010), and were added for the benefit of a research question beyotond the scope of this study. These stimuli were therefore not included in the analyses but treated as filler stimuli.

2010; Roelofs, van Peer, et al., 2009; Roelofs, Minelli, et al., 2009; Rotteveel & Phaf, 2004; van Peer et al., 2007; van Peer, Spinhoven, van Dijk, & Roelofs, 2009; Volman, Roelofs, Koch, Verhagen, & Toni, 2011) the analysis was based on RTs for happy and angry faces, both known to elicit reliable approach–avoidance effects. RTs for neutral faces were used for baseline correction. Median RTs were baseline corrected by subtracting the corresponding RTs for neutral faces (e.g., RT angry push–RT neutral push; RT angry pull–RT neutral pull; RT happy push–RT neutral push; RT happy pull–RT neutral pull). Corrected RTs for angry and happy faces were entered in a three-way repeated-measures Analysis of Variance (rmANOVA), with as between-subject factor Group (DAT1 9-repeat carriers; DAT1 10/10 homozygotes) and within-subject factors Valence (angry; happy) and Movement (push; pull). Alpha was set at .05, and effect sizes are reported in partial eta squared (η_p^2).

For display purposes (Figure 2.1), AAT-effect scores were calculated for each participant and for each emotion separately by subtracting median pull RTs from the corresponding median push RTs (e.g., RT angry push–RT angry pull; RT happy push–RT happy pull). These AAT-effect scores for angry and happy faces were baseline-corrected by subtracting the AAT-effect scores for neutral faces (e.g., AAT-effect angry–AAT-effect neutral; AAT-effect happy–AAT-effect neutral). Corrected AAT-effect scores were entered in a two-way repeated measures Analysis of Variance (rmANOVA), with as between-subject factor Group (DAT1 9-repeat carriers; DAT1 10/10 homozygotes) and within-subject factor Emotion (happy; angry). AAT-effect scores with a negative sign (push is faster than pull) reflect a relative avoidance tendency, and AAT-effect scores with a positive sign (pull is faster than push) reflect a relative approach tendency.

Results

Genotypes

The genotype distribution of the DAT1 polymorphism in our population was 61 (16 males; age \pm SD: 23.6 \pm 2.5) 10/10 homozygous subjects (62.9%) and 36 (9 males; age \pm SD 22.8 \pm 2.0) 9-repeat carrier subjects (37.1%). The allelic distribution of the gene corresponded to the Hardy Weinberg equilibrium ($\chi^2 = 2.77$, $P = 0.096$). No significant differences among genotype frequencies were found with respect to age ($t(93) = -1.57$, $p = 0.121$) or gender ($t(95) = 0.13$, $p = 0.895$).

Approach–Avoidance Task (AAT)

Owing to technical problems, data were missing for 4 participants, leaving 97 participants for the analyses. Table 2.1 provides an overview of the outcomes for RTs. Error rates were low: 3.3% for 10/10-homozygotes, and 3.7% for 9-repeat carriers.

Table 2.1. Genotype-specific mean (ms ± SEM) reaction times for approach (pull) and avoidance (push) responses to angry and happy faces.

Valence	9-repeat carriers		10/10 homozygotes	
	Push	Pull	Push	Pull
Angry	515 (11)	549 (12)	526 (9)	541 (10)
Happy	534 (14)	535 (12)	538 (10)	549 (10)

The three-way (Group (10/10 homozygotes, 9-repeat carriers) × Valence (happy, angry) × Movement (push, pull)) rmANOVA for the RTs showed a main effect of Valence ($F(1,95) = 4.36, p = 0.039, \eta_p^2 = 0.044$), a main effect of Movement ($F(1,95) = 5.63, p = 0.020, \eta_p^2 = 0.065$), and a Valence × Movement interaction ($F(1,95) = 6.80, p = 0.011, \eta_p^2 = 0.067$). Most critically, there was a significant Group × Valence × Movement interaction ($F(1,95) = 4.09, p = 0.046, \eta_p^2 = 0.041$). In order to explore the nature of this group interaction, we conducted separate two-way (Valence (happy, angry) × Movement (push, pull)) rmANOVAs for each group, demonstrating a significant Valence × Movement interaction in the 9-repeat carriers group ($F(1,35) = 5.92, p = 0.020, \eta_p^2 = 0.145$), but not in the 10/10 homozygous group ($F(1,60) = 0.312, p = 0.578, \eta_p^2 = 0.005$). Further one-way rmANOVAs for the 9-repeat carriers group revealed a significant effect for movement for happy faces ($F(1,35) = 7.54, p = 0.009, \eta_p^2 = 0.177$), but not for angry faces ($F(1,35) = 0.21, p = 0.653, \eta_p^2 = 0.006$). However, no Group × Movement effects were found when testing effects for happy and angry faces separately (happy: $F(1,95) = 1.12, p = 0.292, \eta_p^2 = 0.012$; angry $F(1,95) = 1.49, p = 0.225, \eta_p^2 = 0.015$). Together, these findings indicate that 9-repeat carriers show increased social approach–avoidance tendencies, as compared to 10/10 homozygous subjects (Figure 2.1).

In order to control whether the RTs were affected by individual differences related to physical characteristics of the joystick movement (unrelated to stimulus valence) we added the AAT-effect scores for the control stimuli (checkerboards) as covariate in the analyses and found that the Group × Valence × Movement interaction remained unaffected: $F(1,94) = 4.11, p = 0.045, \eta_p^2 = 0.042$. When we checked for Gender we found that the Group × Valence × Movement interaction also remained significant ($F(1,94) = 4.03, p = 0.048, \eta_p^2 = 0.041$), and no significant effects for Gender emerged (all $ps > 0.700$).

Discussion

The aim of this study was to explore the possible role of DAT1 polymorphisms in social-motivational behavior, as assessed by an objective measure of approach–avoidance tendencies.

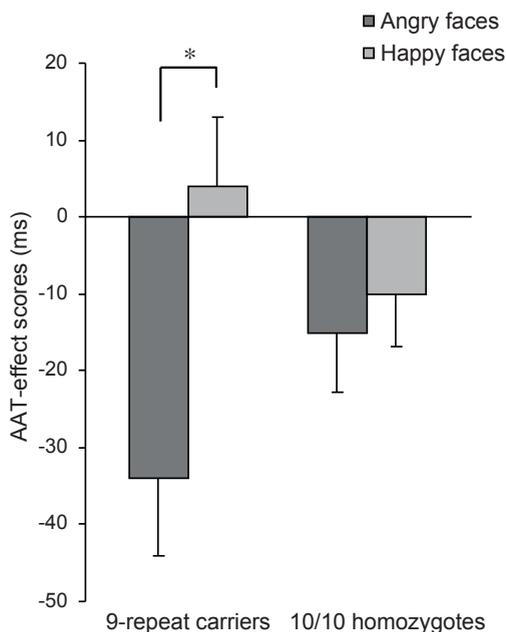


Figure 1. Median AAT-effect scores (ms) for approach and avoidance movements to happy and angry faces for the 9-repeat carriers group and 10/10 homozygotes. DAT1 9-repeat carriers show increased avoidance tendencies to angry faces, but not happy faces, compared to 10-repeat homozygotes. $*p \leq .05$.

Results indicated that DAT1 9-repeat carriers show increased approach-avoidance tendencies as compared to 10-repeat homozygotes. These increased approach-avoidance effects of the DAT1 9-repeat carriers were particularly reflected in a significantly stronger approach vs. avoidance tendency of happy faces. The effects remained significant after controlling for possible confounding factors such as gender and individual differences in joystick handling, and suggest that striatal dopaminergic polymorphisms do play a role in social motivation.

Increased synaptic DA availability is generally associated with increased reward-related activity, and usually linked to augmented motivational behavior (see for a review Cools, 2008). In our study, the DAT1 9-repeat carriers showed increased emotion-driven action tendencies in reaction to stimuli that communicate a motivational drive to the observer, and with which their response was immediately rewarded in the sense that the speed by which the happy faces grew or the angry faces shrank and disappeared was a direct function of the speed with which participants pulled or pushed the joystick respectively. These findings correspond to a study by Talmi and colleagues (2008) showing a positive relation between the vigor of an action and motivation to obtain a reward, which was also related to an increased blood oxygen level-dependent signal in the Nucleus Accumbens, suggestive of increased DA signaling. In addition, Huys and

colleagues (2011) have shown that the motivation of healthy individuals to acquire a reward equally influences both approaching of appetitive and avoiding of aversive stimuli. Note that the concept of avoidance used here does not imply a response that prevents exposure to the feared stimulus, but rather the tendency to withdraw as fast as possible when it appears, and relative to the tendency to approach the feared stimulus (e.g., Chen & Bargh, 1999; Rinck & Becker, 2007).

Interestingly, in our study the increased approach–avoidance effects of the DAT1 9-repeat carriers, compared to the 10-repeat homozygotes, were particularly reflected in a significantly stronger approach vs. avoidance of happy faces, whereas the relative avoidance tendency for angry faces did not reach significance. This relatively strong effect for happy faces in the DAT1 9-repeat carriers is in line with findings relating striatal dopamine, thought to be specifically affected by DAT1 polymorphisms (Heinz et al., 2000; VanNess et al., 2005), particularly to the approach of appetitive stimuli (Boureau & Dayan, 2011; Huys et al., 2011). However, it should be noted that the group difference was only significant when the approach–avoidance tendencies for happy faces were contrasted with the approach–avoidance tendencies for angry faces, suggesting a general increase in motivational behavior in 9-repeat carriers.

Our findings indicate that the responses of individuals carrying the 9-repeat polymorphism are affected by task irrelevant social-emotional features, whereas the responses of 10-repeat homozygotes are not. It has been suggested that the 9-repeat allele is a vulnerability factor for psychopathologies such as PTSD (Segman et al., 2002). But alternatively, our findings might signal increased sensitivity to both positive and negative contextual conditions (Belsky et al., 2009). Belsky and Pluess (2009) argued that an individual with such increased sensitivity may not only be more vulnerable to the negative effect of an adverse environment, but may also be more susceptible to beneficial consequences of a positive context. For example, an aversive context may enhance social avoidance, which might explain (mixed) results from association studies of DAT1 with anxiety (Kennedy et al., 2001; Segman et al., 2002), and be in line with earlier findings of a relation between DAT alterations and the presence of social anxiety such as reduced density of DA uptake sites in the striatum of social phobic patients relative to healthy controls (Tiihonen et al., 1997), or low DAT binding in healthy participants, which was associated with the personality trait of detachment (Laakso et al., 2000; Schneier, Liebowitz, & Laruelle, 2001).

Although DAT1 polymorphisms are typically associated with deviations in striatal DA transmission and reward processing (Dreher et al., 2009; Sesack et al., 1998), we cannot rule out that our behavioral results may also have been affected by DA signaling in other relevant brain structures involved in social approach–avoidance behavior, such as the amygdala or frontal areas (Kienast et al., 2008; Volman, Roelofs, et al., 2011).

Our findings may also benefit the quest for clarifying issues concerning DAT expression by the DAT1 repeat polymorphism. Whereas imaging studies show mixed results for

the influence of the number of repeats in the DAT1 VNTR (see for a review Costa, Riedel, Müller, Möller, & Ettinger, 2011), several molecular genetic studies point to the possibility of an increase in DAT1 gene expression depending on the number of repeats (Fuke et al., 2001; Michelhaugh, Fiskerstrand, Lovejoy, Bannon, & Quinn, 2001; Mill, Asherson, Browes, D'Souza, & Craig, 2002; VanNess et al., 2005; but see Greenwood & Kelsoe, 2003). These last findings support the idea of the 9-repeat allele accounting for decreased DAT1 availability when compared to the 10-repeat allele and are in line with our study, although much more research is necessary to elucidate the functional effects of the DAT1 repeat polymorphism and possible modulating factors (e.g., Shumay, Chen, Fowler, & Volkow, 2011; Shumay, Fowler, & Volkow, 2010).

We acknowledge that complex human social behavior can never be attributed to one single genetic polymorphism, and that our results should therefore be considered preliminary, and for the benefit of generating hypotheses only. However, in our attempt to measure one single aspect of social behavior (e.g., behavioral approach–avoidance), we selected a sensitive, validated task that objectively measures subtle differences in implicit approach–avoidance tendencies (Heuer et al., 2007; Rinck & Becker, 2007; Roelofs et al., 2010; Wiers, Rinck, Dictus, & van den Wildenberg, 2009). The use of well-controlled sensitive paradigms such as fMRI or implicit RT tasks, has previously been shown to result in replicable results for serotonergic transporter gene polymorphisms associated with angry face processing, where earlier self-report studies failed to find relations (Bertolino et al., 2005; Hariri et al., 2002, 2005; Heinz et al., 2005; Pezawas et al., 2005; Smolka et al., 2007). For these reasons, we believe that our findings may be relevant in the selection of candidate genes in future studies involving social motivational behavior, especially since previous studies using self-report measures of social anxiety and avoidance failed to find such correlations (e.g., Schneier et al., 2009; van der Wee et al., 2008).

In summary, this is the first attempt to relate striatal dopaminergic polymorphisms to an objective implicit measure of social approach–avoidance behavior. The findings demonstrate that DAT1 9-repeat carriers show increased approach–avoidance tendencies to social-emotional cues and may help the selection of candidate genes in future studies concerning social behavior.



Alleviating social avoidance:
Effects of single dose testosterone
administration on approach-avoidance
action

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Hormones and Behavior, 65(4), 351–354.

Abstract

Testosterone is an important regulator of social-motivational behavior and is known for its dominance-enhancing and social-anxiolytic properties. However, to date no studies have systematically investigated the causal effect of testosterone on actual social approach-avoidance behavior in humans. The present study sets out to test the effects of testosterone administration in healthy female volunteers using an objective implicit measure of social motivational behavior: the social Approach- Avoidance Task, a reaction time task requiring participants to approach or avoid visually presented emotional (happy, angry, and neutral) faces. Participants showed significantly diminished avoidance tendencies to angry faces after testosterone administration. Testosterone did not affect approach-avoidance tendencies to social affiliation (happy) faces. Thus, a single dose testosterone administration reduces automatic social threat avoidance tendencies in healthy females. These findings further the understanding of the neuroendocrine regulation of social motivational behavior and may have direct treatment implications for social anxiety, characterized by persistent social avoidance.

Introduction

The Hypothalamus-Pituitary-Gonadal (HPG) axis with its end product testosterone plays an important role in the regulation of social motivational behavior. Testosterone is associated with social dominance and approach behavior, and has socially anxiolytic effects (Eisenegger et al., 2011; Maner et al., 2008; Mazur & Booth, 1998; Mehta & Josephs, 2010; Honk et al., 1999). According to the challenge hypothesis testosterone levels rise in preparation to a challenging encounter in which social status might be threatened, thereby initiating approach motivation and simultaneously reducing fear (Archer, 2006; Bos et al., 2012).

In the past decade, several studies of testosterone administration to healthy female participants confirmed the causal relationship between testosterone and its dominance-enhancing and social-anxiolytic properties. Concerning the latter, testosterone administration has been shown to reduce fear-potentiated startle reflexes in highly anxious participants, attentional bias to fearful facial expressions, and conscious recognition of threat-related facial expressions (Hermans, Putman, Baas, Koppeschaar, & van Honk, 2006; Hermans et al., 2007; van Honk & Schutter, 2007; van Honk, Peper, & Schutter, 2005). Enhancement of social dominance was indicated by increased heart rate acceleration, slower gaze aversion, and by an increase of activity in a social approach related brain circuit when viewing angry faces (Hermans, Ramsey, & van Honk, 2008; Terburg, Aarts, & van Honk, 2012; Honk et al., 2001).

Together, these findings indicate that testosterone administration reduces fear and sensitivity to threat, and enhances social dominance related behavior. However, so far evidence is constrained to processing of emotion and gaze behavior. No studies have systematically tested effects on actual social approach-avoidance actions. Elucidating the effects of testosterone on actual approach behavior would not only advance our theoretical understanding of steroid involvement in social emotional behavior, but it would also have great implications for the treatment of social avoidance related disorders associated with reduced testosterone levels such as social phobia and depression (Gerra et al., 2000; Giltay et al., 2012).

This study sets out to test whether testosterone administration diminishes threat avoidance and promotes threat approach, using an objective implicit measure of social motivational behavior: the Approach-Avoidance Task (AAT). The AAT is a valid and reliable measure of social approach-avoidance action tendencies (Heuer et al., 2007; Roelofs et al., 2010; Roelofs, van Peer, et al., 2009; Roelofs, Minelli, et al., 2009a; Volman, Roelofs, et al., 2011; Volman, Toni, et al., 2011). This reaction time (RT) task involves participants to approach and avoid socially aversive and appetitive visually presented stimuli (angry and happy faces, respectively) by pulling a joystick towards themselves (approach) or pushing the joystick away from themselves (avoidance). Angry faces with direct gaze constitute

a potent threat stimulus potentially signaling impending aggression, and elicit increased avoidance tendencies in high socially anxious individuals in particular (Adams, Gordon, Baird, Ambady, & Kleck, 2003; Öhman, 1986; Roelofs et al., 2010).

Based on the role of testosterone in social dominance and in enhancement of action motivation, we predict that administration of testosterone would reduce threat avoidance and increase threat approach tendencies to angry faces on the AAT.

Methods

Participants

Twenty-four healthy females with a mean age of 29 years ($SD = 8.4$, range 20-50), served as participants for partial fulfillment of course credit or financial compensation. Only female participants were included, because there are as yet no known parameters (e.g., dose and time course) for inducing psychological effects in men after administration of a single dose of testosterone (Tuiten et al., 2000). The sample was recruited via advertisement in the Leiden metropolitan area (The Netherlands). Exclusion criteria were age <18 and >50 , use of medication, somatic illnesses, neurological conditions, recent or past psychiatric problems, history of head injury, left-handedness, peri- or postmenopause, and pregnancy or breast feeding. Fourteen women were using single-phase contraceptives, whereas ten women were normally cycling and evenly distributed over menstrual cycle phases.² All participants had normal or corrected-to-normal vision, were unaware of the aim of the study and provided written informed consent. The study was approved by the Medical Ethics Committee of the Leiden University Medical Centre, and was in accordance with the declaration of Helsinki.

Testosterone administration

In a double-blind, randomized, placebo-controlled, crossover design participants received a single dose of 0.5 mg testosterone suspended in a clear solution (0.5 ml) with 0.5 mg hydroxypropyl-beta-cyclodextrin, 0.005 ml ethanol 96%, and distilled water. The matched placebo contained the same ingredients, except the testosterone. Participants were asked to hold the liquid under their tongue for 60 s. During sublingual administration of 0.5 mg testosterone cyclodextrin, testosterone is directly absorbed into the

2 Of the fourteen women using contraceptives, nine were using single-phase estrogen/progestogen contraceptives, two were using a progestogen-only intra-uterine device, and three were using an estrogen/cyproterone acetate contraceptive. Of the other ten – normally cycling – women, three were likely in the menstrual phase, two in the follicular phase, two in the ovulatory phase and three in the luteal phase during testing. Phase was defined by cycle day (Colzato, Hertsig, Van Den Wildenberg, & Hommel, 2010).

bloodstream. In females, such a dose yields a sharp increase of 20-25 nmol/l in plasma testosterone levels within 15 min, which declines to baseline levels within the next 90 min. Subsequently, pharmacodynamic effects are measurable approximately 4 to 6 h after testosterone intake (Tuiten et al., 2000).

Approach-Avoidance Task (AAT)

During this reaction time (RT) task, participants responded to emotional face pictures presented on a computer screen, by pulling a joystick either towards their body (approach movement) or pushing it away from their body (avoidance movement) (task adapted from Heuer et al., 2007). Pulling or pushing the joystick increased or decreased the size of the picture respectively, giving the impression of moving towards or moving away from the participant. The speed of the size change was proportional to the amplitude of the joystick movement. As soon as the joystick reached its target position (i.e., the required direction; full movement involved a 30° rotation from the upright position) the picture disappeared from the screen. The time between the onset of the stimulus and its disappearance from the screen was recorded with <1 ms accuracy. After each completed trial the participant moved the joystick back to its central position and initiated a new trial by pressing the fire button near the top of the joystick. Face stimuli were selected from the Karolinska Directed Emotional Faces database based on quality of emotional expression (Goeleven, De Raedt, Leyman, & Verschuere, 2008; Lundqvist et al., 1998). Happy, Angry, and Neutral facial expressions were taken from the same model (five male and five female models in all) and each picture was presented either with a yellowish or a grayish filter. In addition, checkerboards (10 yellow, 10 gray) were included as non-facial control stimuli.³ This resulted in a total of 80 different stimuli, which were presented in random order. All participants were instructed to push on yellow stimuli and to pull on gray stimuli, and to respond as fast and as accurately as possible. Usually, response latencies are shorter for affect-congruent (e.g., happy-approach; angry-avoid) as compared to affect incongruent response conditions (e.g., angry-approach; happy-avoid). Before the real test started, participants were presented with eighteen practice trials, which were similar to the test trials except for the fact that the pictures showed different models.

3 Checkerboard stimuli yielded significantly slower reaction times ($p \leq .009$; RTs (ms \pm SEM) for placebo condition: push 569 (22) and pull 542 (19); testosterone condition: push 548 (21) and pull 520 (16)). It is likely that this slowing is caused by an oddball effect as they were outnumbered by the facial stimuli (i.e., 20 checkerboards to 60 emotional faces). Therefore the checkerboard stimuli have not been included in the main analyses.

Social Phobia and Anxiety Inventory

Social Phobia and Anxiety Inventory (SPAI; Turner, Beidel, Dancu, & Stanley, 1989; validated Dutch version (Bögels & Reith, 1999) has good reliability ($\alpha = .99$), and was used to assess the severity of social anxiety on a separate day prior to participation. It features 45 items in total, of which 32 items assess social phobia, and 10 items measure agoraphobia. Following van Peer et al., (van Peer et al., 2009) only the social phobia score ($\alpha = .99$; mean \pm *SD*: 49.6 (25.3)) was added to the statistical analysis of the RTs.

Procedure

Participants were tested individually at two identical testing sessions with two days in between. Testing sessions started at either 930h or 1330h, and participants were tested on the same time of day on both sessions. Four and a half hours after administration of testosterone or placebo participants performed the AAT in a dimly lit and sound attenuated room. There was no significant difference between the early and late testing sessions for the AAT-effect scores, $F(1, 22) = 0.37, p = .549$, nor SPAI-SP scores, $t(22) = 0.67, p = .511$, nor was there an influence of testing order, $F(2, 42) = 0.82, p = .449$. After completion of the two sessions participants had to indicate in which session they thought to have had testosterone or placebo. Thirteen of the 24 participants were correct, which is at chance-level, Binomial $P(X = 13) = 0.149$, confirming that participants were unaware of the condition.

Statistical analyses

RT outliers were filtered using a <150 and a >1500 ms cut-off. A cut-off of three standard deviations from the mean was used for defining outliers in the remaining RTs. Error rates were calculated after removal of outliers. For each participant, the median of the remaining RTs (97%) for the correct responses was calculated per cell (defined by: Emotion and Movement). AAT effect scores were computed for each participant and for each emotion separately by subtracting median pull RTs from corresponding median push RTs (e.g., RT angry push – RT angry pull; RT happy push – RT happy pull; RT neutral push – RT neutral pull). As a result, more negative AAT-effect scores (push is faster than pull) reflect a relative avoidance tendency.

AAT effect scores were entered in a two-way repeated measures Analysis of Variance (rmANOVA) with Condition (testosterone; placebo) and Valence (angry; happy; neutral) as within-subject factors. Given the moderating role of social anxiety in social approach-avoidance behavior (Radke, Roelofs, & de Bruijn, 2013; Karin Roelofs, van Peer, Berretty, de Jong, et al., 2009; van Peer et al., 2009), we controlled for the possibly confounding effects of social anxiety by adding the SPAI-scores as a continuous variable (ANCOVA) in the analyses. In addition we tested whether AAT-effect scores were significant in certain conditions (i.e., differed from zero) using one sample t-tests. Alpha was set at .05 and effect sizes are reported in eta squared (η^2).

Results

Due to technical problems data were missing for two participants. Table 3.1 provides an overview of the outcomes for RTs. The task was well performed, as indicated by low error rates (3.9%).

The two-way rmANOVA for the AAT-effect scores, with Condition (Placebo, Testosterone) and Valence (angry, happy, neutral) as within-subject factors, and SPAI-SP score as a continuous variable, revealed a significant Condition \times Valence interaction, $F(2, 44) = 3.69, p = .033, \eta^2 = .05$.⁴ To explore the nature of this interaction, we conducted one-way rmANOVA's with SPAI-SP as covariate for each valence separately, demonstrating a significant main effect for Condition for angry faces, $F(1, 22) = 5.67, p = .026, \eta^2 = .18$, but not for happy, $F(1, 22) = 0.78, p = .386, \eta^2 = .03$, and neutral faces, $F(1, 22) = 1.14, p = .298, \eta^2 = .05$ (see Figure 1). Avoidance of angry faces was significant in the Placebo condition (AAT-effect score differed significantly from zero: $t(23) = -2.22, p = .037$), but not in the testosterone condition (see figure 3.1). Taken together, these results suggest a significant reduction of avoidance of angry faces by testosterone.

Table 3.1. Mean RTs (ms \pm SEM) for approach (pull) and avoidance (push) responses to angry, happy, and neutral faces per condition.

Valence	Placebo		Testosterone	
	Push	Pull	Push	Pull
Angry	529 (15)	545 (17)	520 (15)	522 (18)
Happy	524 (16)	534 (16)	517 (16)	530 (18)
Neutral	524 (17)	531 (17)	517 (16)	531 (19)

In order to check whether the effects of testosterone on AAT-effect scores were affected by individual differences related to physical characteristics of the joystick movement (unrelated to stimulus valence) we added the AAT-effect scores for the control stimuli (checkerboards) as covariate and found that these had no influence (all $ps > .127$). Similar analyses checking for age (all $ps > .130$) showed that this variable did not affect the results. And finally, when we included use of contraceptives in the model, the main effect

4 The same Condition \times Valence rmANOVA for the AAT effect scores, without SPAI-SP scores added as a covariate, showed no significant Condition \times Valence interaction [$F(2,46) = 1.04, p = .363, \eta^2 = 0.02$]. In addition, no significant Condition effects for angry, happy and neutral faces emerged without taking SPAI-SP in account (all $ps > .224$). These findings suggest that the reported Valence \times Condition effect was modulated by social anxiety (Simmons, Nelson, & Simonsohn, 2011).

of contraceptive use did not reach significance ($p = .078$), no interactions between contraceptive use and valence and/or condition emerged (all $p > .529$), and most importantly the Condition \times Valence interaction remained unaffected, $F(2, 42) = 3.52, p = .039, \eta^2 = .05$.

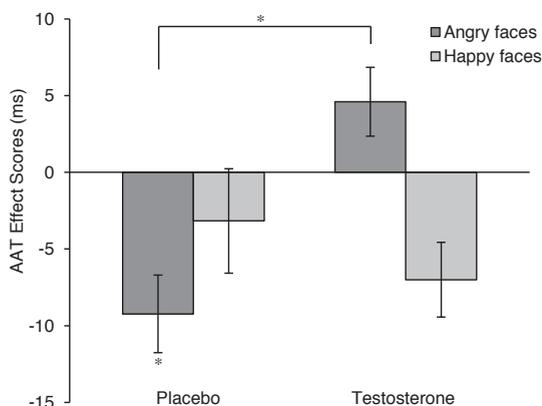


Figure 3.1. Mean AAT effect scores (ms) for approach and avoidance movements to angry and happy faces in the placebo and testosterone conditions. Participants show significant avoidance of angry faces in the placebo condition, but not in the testosterone condition. For display purposes, mean scores are baseline corrected (i.e., AAT effect scores for Neutral faces subtracted from the AAT effect scores of Angry and Happy faces respectively). * $p \leq .05$.

Discussion

The current study sets out to test whether a single administration of testosterone promotes threat approach relative to threat avoidance, using an objective implicit measure of social motivational behavior.

Participants showed significant avoidance of angry faces in the placebo condition and this effect shifted after testosterone administration, leading to a reduction of threat avoidance and an increase in relative threat approach tendencies. The finding of avoidance of social threat in the placebo condition is in line with earlier findings on the AAT (e.g., Heuer et al., 2007a; Roelofs et al., 2010; von Borries et al., 2012). Testosterone administration significantly diminished avoidance tendencies to angry faces, but not to happy faces, a finding that concurs with a body of research on testosterone administration showing dominance-enhancing effects (e.g., Hermans et al., 2008; Terburg et al., 2012; van Honk et al., 2001). In addition, our results are in line with the challenge hypothesis, which states that endogenous testosterone levels rise in preparation to a socially challenging encounter, thereby initiating approach motivation and simultaneously reducing fear (Archer, 2006; Bos et al., 2012). The finding that significant avoidance of social threat faces in the placebo condition shifts to relative approach after administration of testosterone not only may be caused by fear reduction, but also could be the result of an increased

aggressive tendency, although the two explanations are not mutually exclusive. This is the first study showing that testosterone directly influences behavioral approach actions that have an immediate effect on the relative distance of the social threat stimulus (Rinck & Becker, 2007). The current findings extend our knowledge by confirming the causal effect of testosterone on automatic social motivational action (Terburg & van Honk, 2013).

Previous work on the relation between endogenous testosterone and social approach action has shown that testosterone modulates prefrontal brain activity and prefrontal–amygdala connectivity when people had to control their automatic social approach–avoidance tendencies. Higher endogenous testosterone levels were associated with reduced negative connectivity between the anterior prefrontal cortex and the amygdala (Volman, Toni, et al., 2011). Indeed performance on the AAT relies on prefrontal-amygdala crosstalk (Volman, Verhagen, et al., 2013; Volman, Roelofs, et al., 2011), but future investigations featuring testosterone administration should confirm whether the effect of testosterone indeed influences this neural mechanism. Testosterone administration has shown to enhance neural responsiveness of subcortical brain areas involved in social approach towards socially salient stimuli (Hermans et al., 2008; van Wingen et al., 2010). Together these findings suggest that testosterone induces automatic approach behavior, driven by subcortical brain areas (Bos et al., 2012).

The current findings may have implications for social psychopathologies such as social phobia or psychopathy. Concerning the latter, criminal offenders diagnosed with psychopathy, a condition associated with increased testosterone levels and reduced prefrontal inhibition, also showed reduced threat avoidance tendencies on the AAT (von Borries et al., 2012). As far as social avoidance related disorders are concerned, previous studies have indicated that social phobia and depression are associated with reduced testosterone (Gerra et al., 2000; Giltay et al., 2012; Haglund, Nestadt, Cooper, Southwick, & Charney, 2007). Therefore, future research may explore whether testosterone administration reduces social fear and avoidance in social anxiety and may increase therapy efficacy (e.g., Hofmann, Fang, & Gutner, 2014).

A limitation of the present study was that only female participants were tested, which was due to the restrictions of the administration method used (Tuiten et al., 2000). Future research should investigate whether testosterone administration to men has similar effects as suggested by similarities in social behavior related to endogenous and exogenous testosterone across sexes (Hermans et al., 2007; van Honk et al., 2001; van Honk et al., 1999; van Honk & Schutter, 2007).

In sum, the current study shows that a single administration of testosterone directly diminishes automatic avoidance of social threat and promotes relative increase of threat approach tendencies. These findings may be particularly relevant for social psychopathologies such as social anxiety disorder.



Salivary testosterone: Associations with depression, anxiety disorders, and antidepressant use in a large cohort study

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Abstract

Low circulating levels of testosterone have been associated with major depression, but there is more limited evidence for differences in patients with anxiety disorders. The use of selective serotonin reuptake inhibitors (SSRIs) and other antidepressants is associated with sexual side effects, warranting testing for interactions with testosterone. Data are from 722 male and 1380 female participants of The Netherlands Study of Depression and Anxiety (NESDA), who were recruited from the community, general practice care, and specialized mental health care. Depressive and anxiety diagnoses were assessed using the DSM-IV Composite International Diagnostic Interview. To smooth the episodic secretion, the four morning saliva samples per participant and the two evening samples were pooled before testosterone analysis. Morning median testosterone levels were 25.2 pg/ml in men and 16.2 pg/ml in women, with lower evening levels of 18.2 and 14.1 pg/ml, respectively. Significant determinants of testosterone levels were sex, age, time of the day, use of contraceptives, and smoking status. Female patients with a current (1-month) depressive disorder (effect size 0.29; $p = 0.002$), generalized anxiety disorder (0.25; $p = 0.01$), social phobia (0.30; $p < 0.001$), and agoraphobia without panic disorder (0.30; $p = 0.02$) had lower salivary testosterone levels than female controls. Higher testosterone levels were found in male and female participants using SSRIs than in non-users (effect size 0.26; $p < 0.001$). Salivary testosterone levels are lower in female patients with a depressive disorder, generalized anxiety disorder, social phobia, and agoraphobia as compared to female controls. SSRIs may increase salivary testosterone in men and women.

Introduction

There are important sex differences in prevalence proportions of psychiatric disorders, with women having a more than double the risk of depressive disorder, generalized anxiety disorder, social phobia, and panic disorders with or without agoraphobia (Angst et al., 2002; Kessler, McGonagle, Swartz, Blazer, & Nelson, 1993; Kessler, Chiu, Demler, Merikangas, & Walters, 2005; Kornstein, Sloan, & Thase, 2002; Marcus et al., 2005; van Noorden et al., 2010). Differences between the sexes have also been found for symptom profiles of depression, with an earlier age of onset, a higher symptom severity, more atypical depression, and a diminished libido in affected women compared to men (Angst et al., 2002; Kornstein et al., 2002; Smith et al., 2008). Libido and other sexual functions may further worsen under the influence of antidepressant use (Gregorian et al., 2002; Serretti & Chiesa, 2009). Especially selective serotonin reuptake inhibitors (SSRI), generally preferred as the first-line pharmacotherapy treatments for mild to moderate outpatient depression and anxiety disorders, are associated with sexual problems such as diminished libido and difficulties with sexual arousal and orgasm in at least 30% of patients (Clayton, Keller, & McGarvey, 2006; Gregorian et al., 2002; Serretti & Chiesa, 2009).

Testosterone is an important circulating androgen. Its secretion is under the rapid pulsatile control of gonadotropin-releasing hormone (GnRH), which in turn stimulates the production of luteinizing hormone. Consequently, there are cyclic changes in serum testosterone levels (with a slight nocturnal rise), and therefore ideally at least several morning blood samples separated by 30-minute intervals are to be used to assess androgenic status in a meaningful way (Brambilla et al., 1996). Salivary testosterone levels are strongly correlated with free serum testosterone levels in men (Arregger, Contreras, Tumilasci, Aquilano, & Cardoso, 2007; Cardoso et al., 2011), but less strongly in women, for whom there is less evidence (Granger, Shirtcliff, Booth, Kivlighan, & Schwartz, 2004; Wood, 2009).

Testosterone is involved in male secondary sexual characteristics, reproduction, and sexual function. The brain contains receptors for testosterone (Bao, Meynen, & Swaab, 2008; Cooke, 2006; Sarkey, Azcoitia, Garcia-Segura, Garcia-Ovejero, & DonCarlos, 2008; Wang, Kamphuis, Huitinga, Zhou, & Swaab, 2008), and is capable of synthesizing and metabolizing testosterone to, for instance, estradiol (Poletti & Martini, 1999; Puy et al., 1995; Stoffel-Wagner, 2003). In some studies, a low androgen status has been associated with symptoms of fatigue, irritability, dysphoria, declining vigor, low vitality, sexual dysfunction in men (Barrett-Connor, Von Mühlen, & Kritz-Silverstein, 1999; Rhoden & Morgentaler, 2004; Seidman, 2003; Wang et al., 2009) and women (Bolour & Braunstein, 2005), and an increased risk of depressive symptoms and depression in most (Almeida, Yeap, Hankey, Jamrozik, & Flicker, 2008; Hintikka et al., 2009; Shores, Mocerri, Sloan, Matsumoto, & Kivlahan, 2005) but not all studies (Araujo, Durante, Feldman, Goldstein, & McKinlay, 1998;

Berglund, Prytz, Perski, & Svartberg, 2011). Findings were mixed, however, as several other epidemiological studies did not show or showed non-linear relationships between testosterone levels and depressive symptoms (Amiaz & Seidman, 2008). Inconsistencies may partly be due to the link or interaction with the genetic cytosine–adenosine–guanine (CAG) repeat length polymorphism. Longer CAG repeats may diminish the androgen receptor transactivation capacity and has been linked to the vulnerability to depression and anxiety (Härkönen et al., 2003; Seidman, Araujo, Roose, & McKinlay, 2001), though not consistently (Colangelo et al., 2007; Schneider et al., 2011; T'Sjoen et al., 2005). Evidence on the role of testosterone in women is scarce, but there is some evidence that low DHEA(S) is involved in depression in women (Maninger, Wolkowitz, Reus, Epel, & Mellon, 2009; Schmidt et al., 2005). Androgen deprivation therapy in men with prostate cancer induces many of these symptoms, which are reversible after cessation of therapy (Cherrier, Aubin, & Higano, 2009; Shahinian, Kuo, Freeman, & Goodwin, 2006). However, androgen withdrawal induced adverse mood symptoms only in a subgroup of 10% (3 out of 31) of healthy male volunteers (Schmidt et al., 2004). Low testosterone levels have also been found in men with dysthymic disorder (Seidman et al., 2002). In a large community sample of almost 4000 elderly men, free testosterone concentration in the lowest quintile was associated with more depressive symptoms (Almeida et al., 2008). The evidence for a potential role of testosterone in anxiety disorders is much more limited. Low salivary testosterone levels were associated with depressive and anxiety symptoms in 106 adolescent boys but not in 107 girls (Granger et al., 2003) and weakly with anxiety symptoms in 3413 men (Berglund et al., 2011), but other studies found no relationship between social anxiety and basal salivary testosterone levels in 20 adolescents (Gerra et al., 2000) and 58 students (Maner et al., 2008). Moreover, the urine testosterone level was comparable in 16 patients with panic disorder versus 13 controls (Bandelow et al., 1997). Even so, testosterone has been associated with many behavioral aspects of dominance, competition, aggression, and sociality (Archer, 2006; Bos, Terburg, & van Honk, 2010; Gleason, Fuxjager, Oyegbile, & Marler, 2009; Hermans et al., 2008; Liening & Josephs, 2010; Sapolsky, 1991; van Honk et al., 1999; van Honk, Harmon-Jones, Morgan, & Schutter, 2010).

Data on serum testosterone level in anxiety disorders is scarce, whereas salivary testosterone levels have even less often been studied in small groups of patients with psychiatric disorders (Davies et al., 1992). Also, there are few studies on the effects of antidepressants on the hypothalamic–pituitary–gonadal (HPG) axis (Hendrick, Gitlin, Altshuler, & Korenman, 2000). We hypothesized that low salivary testosterone levels are associated with both depressive and anxiety disorders. Second, treatment of depressive and anxiety disorders with antidepressants decreases libido and this may be due to a reduction in testosterone levels. We measured salivary testosterone levels in the large Netherlands Study of Depression and Anxiety (NESDA). A number of 4 morning and 2 evening salivary samples were pooled before testosterone was quantified in order to

minimize the effects of the pulsatile release of testosterone (Brambilla et al., 1996; Wood, 2009). The use of a number of saliva samples had advantages over blood samples as they are more easily collected by participants themselves at several time points at their homes; it requires no pain- and stress-inducing venipunctures; and salivary testosterone reflects the free bio-available serum fraction.

Methods

Participants

Participants took part in NESDA (Penninx et al., 2008), a large longitudinal cohort study on the course of depressive and anxiety disorders. The NESDA sample consists of 2981 participants (mean age 41.9 years, range 18–65; 1002 men and 1979 women), of whom 807 persons were recruited through mental health care organizations, 564 persons through the community setting and the remaining 1610 through primary care. Exclusion criteria were a primary diagnosis of psychotic, obsessive-compulsive, bipolar or severe addiction disorder and not being fluent in Dutch. The baseline assessment was constituted by a medical exam, a face-to-face interview, saliva collection and several written questionnaires. Detailed objectives and methods of NESDA are described elsewhere (Penninx et al., 2008). The research protocol was approved by the ethical committee of participating universities, and all of the respondents provided written informed consent.

There were 2329 participants with a lifetime diagnosis of depressive and/or anxiety disorder and 652 controls without a lifetime psychiatric diagnosis. Controls were defined as having no prior lifetime history of anxiety disorder (i.e., panic disorder, generalized anxiety disorder, or social phobia) or depressive disorder (i.e., major depressive disorder (MDD) or dysthymia).

Assessment of psychopathology and antidepressant use

Several instruments were used to assess psychopathology. The presence of a DSM-IV major depressive disorder or anxiety disorder (i.e., panic disorder, social phobia, generalized anxiety disorder, and agoraphobia) was assessed by the DSM-IV based Composite Interview Diagnostic Instrument (CIDI, WHO Version 2.1). The presence of psychiatric disorders within the past 12 months was used to categorize participants into those with no lifetime anxiety or depressive disorder ($n = 522$), those with a remitted (<1 year) disorder ($n = 461$), with a recent (1-year) or current anxiety disorder ($n = 276$), with a recent (1-year) or current depressive disorder ($n = 412$), and a recent or current comorbid disorder ($n = 471$). We also analyzed current psychiatric disorders within the preceding month for depressive disorder and anxiety disorders (i.e., social phobia, panic disorder and/or agoraphobia, and generalized anxiety disorder). The total score of the Inventory

of Depressive Symptomatology Self Report (IDS-SR) was used to assess overall depression severity (Rush, Gullion, Basco, Jarrett, & Trivedi, 1996), and subdivided into five severity groups, i.e., low (total score 0–13), mild (14–25), moderate (26–38), severe (39–48), and very severe (49–84). Item 22 (i.e., interest in sex) from the IDS-SR was analyzed separately as an internal validation of the salivary testosterone assessment, that was rated on a four-point scale: 0 (i.e., usual interest), 1 (i.e., somewhat less interest or pleasure), 2 (i.e., little interest or pleasure), and 3 (i.e., no interest or pleasure). The total score of the Beck Anxiety Inventory was used to assess affective and somatic symptoms of anxiety (Beck, Epstein, Brown, & Steer, 1988), and subdivided into four severity groups, i.e., normal (total score 0–9), mild (10–18), moderate (19–29), and severe (30–63) (Kabacoff, Segal, Hersen, & Van Hasselt, 1997). For 15-item Fear Questionnaire cut-off scores of 19 for the 5-item agoraphobia AG subscale and 18 for 5-item social phobia (SO) subscale were used (Van Zuuren, 1988).

Medication use during the month prior to baseline interview was registered by observation of drug containers brought to the interview or self-report. Using the World Health Organization Anatomical Therapeutic Chemical classification (WHO, 2009), psychoactive medication was categorized into antidepressants (i.e., tricyclic antidepressants [TCA; N06AA], SSRIs [N06AB], and other antidepressants [N06AF, N06AG, N06AX]), and benzodiazepine use (N03AE, N05BA, N05CD, N05CF) was dichotomized.

Salivary testosterone measurement

At baseline, respondents were instructed to collect saliva samples at home on a regular (preferably working) day shortly after the interview. The median time between the interview and saliva sampling was 9 days (25th–75th percentile, 4–22 days). Instructions prohibited eating, smoking, drinking, or brushing teeth within 15min. Saliva samples were obtained using Salivettes (Sarstedt AG and Co., Nümbrecht, Germany) at 6 time points: four morning samples (at awakening and at 30, 45, and 60 min later) and 2 evening samples (at 22:00 h and 23:00 h). Indirect evidence suggests that compliance of participants to the protocol was good, as the cortisol awakening response showed the characteristic curve within the first hour of awakening in the large majority of participants, with a steep decline in both evening samples (Vreeburg et al., 2009). Samples were stored in refrigerators and returned by regular mail. After receipt, Salivettes were centrifuged at 2000 g for 10min, aliquoted, and stored at –80 °C. Samples had been thawed once for the assessment of salivary cortisol, in which results have been published previously (Vreeburg et al., 2009). While storage at –80 °C of salivary samples does not affect testosterone levels during at least two years, the extra freeze thaw cycle may have contributed some error to testosterone measurement (Granger et al., 2004). Salivary testosterone levels have been shown to be relatively stable over time, with a test–retest stability of 0.65 in men and 0.78 in women over two weeks (Liening, Stanton, Saini, & Schultheiss, 2010).

To smooth the episodic secretion (Brambilla et al., 1996; Wood, 2009), 75 μ l of each of the 4 samples collected in the morning (at wake-up and after 30, 45 and 60 min) were mixed to yield one morning sample, and 150 μ l of each of the 2 evening samples collected at 22:00 and 23:00 h were mixed to yield also one evening sample. So for every participant, one mixed morning sample and one mixed evening sample were assayed. If one of the samples was missing, a corresponding volume of the other sample(s) was taken. The 11 subjects who did not provide any evening sample were excluded. For every other subject who provided saliva, both a morning and evening sample could be combined. Biochemical analysis of free testosterone in saliva was measured in duplo by the testosterone in saliva assay from Diagnostic Biochem Canada (EiAsy Testosterone Saliva, DBC: CAN-TE-300) using 2 \times 100 μ l material. The sensitivity of the kit is 1.0 pg/ml and there are hardly cross-reactivities with other steroids. In every assay a standard control was used, with a mean of 26.9 pg/ml (SD 2.1) that was reproducible with a coefficient of variation (CV) of 7.8%. The intra assay precision was 7.1, 3.4, and 6.7% at the concentrations of resp. 14, 38 and 123 pg/ml ($n = 10$). In our study about 120 kits were used, all of the same lot. The CV over all testosterone measurements was 10.2%.

To validate the use of Salivettes for testosterone measurement, we also compared saliva obtained though Salivettes with those obtained through Salicaps (a passive drooling device) in a repeated-measures design. We included 10 healthy volunteers (age 35 [SD 12] year; 5 females). Saliva was collected at 6 time-points with alternating Salicaps (IBL International GMBH, Hamburg, Germany) (3 times) and Salivettes (Sarstedt, Newton, NC, USA) (3 times) with 10 min in between and always starting with a Salicap. Participants were instructed to minimize physical exercise and not to eat, drink or smoke, 1 h before the start and during the sampling period. Moreover, to avoid cross-contamination, participants were instructed to rinse the mouth with pure water immediately after each sample (always 10 min before the next sampling). All samples were frozen and after a single freeze–thaw cycle centrifuged for 10 min at 5000 g to remove mucins and to extract the saliva from the Salivettes. The three Salivette samples were also combined to one sample (mixed sample), and the same was done for the three Salicap samples. Testosterone was measured in duplo in every sample as well as the mixed samples using the method described above. There was a significant higher testosterone level in saliva obtained by Salivettes than by Salicaps (as was shown previously) (Granger et al., 2004), but this difference was highly constant. Pearson's correlation coefficients between the individuals' mean values obtained with Salivettes and Salicaps were $r = 0.87$ ($p < 0.001$) for the calculated mean and $r = 0.87$ ($p < 0.001$) for the measured value from the mixed samples. Using ranked-order Spearman's correlation coefficients similar findings were obtained ($r = 0.82$ and $r = 0.82$, respectively).

Potential covariates

Sociodemographic factors included sex, age, and educational level (years of attained education). Age was categorized by decade. Body mass index (BMI) was calculated as weight divided by length squared, and categorized into 4 groups ($< 20 \text{ kg/m}^2$ / 20 to 25 kg/m^2 / 25 to 30 kg/m^2 / $\geq 30 \text{ kg/m}^2$). Smoking status was dichotomized into nonsmoker or current smoker. Alcohol consumption was categorized into 3 groups (i.e., non-user/ a number of units of alcohol of less than 2 per day/ 2 or more per day). Physical activity was assessed using the International Physical Activity Questionnaire (Craig et al., 2003) and expressed per 1000 Metabolic Equivalent of Task (MET)/min a week, and subsequently categorized into 3 groups (i.e., < 3 / 3 to 6 / ≥ 6 of 1000MET/min per week). A MET/min is defined as the metabolic equivalent of the number of calories consumed by a person (of 60 kg) per minute in an activity relative to the basal metabolic rate (www.ipaq.ki.se), and ≥ 3 indicates activities of at least moderate intensity. Respondents reported working status on the sampling day (i.e., whether (61.8%) or not (38.2%) the participant went to their job that day). Sampling data was used to categorize weekday versus weekend day and season, which was categorized into dark months (i.e., October to February) and months with more daylight (i.e., March through September).

For subgroup analyses in women, information on the use of oral contraceptives and on menstrual phase was obtained. Duration of the menstrual cycle and the number of days since the last menstruation were used to yield the phase of the menstrual cycle at the time of saliva sampling for women reporting a menstrual cycle between 28 and 32 days (i.e., 0–3 days of cycle was regarded as the early follicular phase, 4–13 days as the late follicular phase, and 14–32 days as the luteal phase). Remaining women were categorized as either using oral contraceptives or being post-menopausal.

Prevalent cardiovascular diseases (i.e., coronary disease, cardiac arrhythmia, angina pectoris, heart failure, and myocardial infarction) were ascertained using an algorithm based on self-report data and medication use.

Statistical analysis

The distributions of morning and evening salivary testosterone levels were strongly positively skewed and therefore naturally log transformed values were used for analyses. Back-transformed geometric mean values are presented in tables and figure. Pearson's correlation coefficients were used to analyze the relationship between morning and evening salivary testosterone levels. Univariate analysis of variance was performed to evaluate the effects of sociodemographic factors, health indicators, and sampling factors on salivary testosterone levels, for morning and evening testosterone levels separately.

Because the morning and evening samples were highly inter-correlated, showing highly comparable associations with psychiatric characteristics, analyses reported in the paper were conducted over individual means of the z-scores of morning and evening

samples. The effects of psychiatric characteristics and psychotropic medication on testosterone levels were analyzed in unadjusted and adjusted analyses of (co)variance (adjusted for sex, age, menstrual status, years of education, smoking status, prevalent cardiovascular disease, and northern European ancestry), in men and women separately. When analyzing the associations with psychopathology measures, we also adjusted for SSRI use, because of its strong association with salivary testosterone levels.

Two-tailed *p*-values of less than 0.05 were considered to indicate statistical significance. For significant findings, effect sizes were calculated with Cohen's *d*. All of the analyses were conducted using SPSS version 17.0 statistical software (SPSS Inc, Chicago, Illinois).

Results

Participants

The 722 men were on average 45.0 (range 18 to 64) years old and the 1380 women 42.7 (range 18 to 65) (Table 4.1). The mean BMI was 26.2 kg/m² for men and 25.3 for women, and around 17% of men and women were obese (i.e., BMI ≥ 30 kg/m²). Men were more often current smokers and consumed more often ≥ 2 units of alcohol per day than women. The prevalence of cardiovascular disease was approximately double in men versus women. There was a large range of salivary testosterone levels, that importantly overlapped in men and women. Nevertheless, men had higher median salivary testosterone levels than women both in the morning and evening samples (Table 4.1). Testosterone levels showed rank-order stability within each gender between morning and evening samples, with Spearman's rank correlation coefficients of 0.62 in men and also 0.62 in women.

Determinants of salivary testosterone

The associations between age, menstrual status in women, health indicators (BMI, smoking, alcohol intake, and physical activity), and sampling factors (working status and season with more daylight) on salivary testosterone levels were analyzed as potential correlates may be considered as covariates in epidemiological association studies. In men and women, age was strongly and inversely associated with both morning and evening salivary testosterone levels (all *ps* < 0.001). The lowest levels were found in men and women over age 50. Current smoking was associated with high salivary testosterone levels, especially for evening levels and more strongly so in women than in men. Although the menstrual status showed an association with salivary testosterone, this did not persist after adjustment for covariates (e.g., age, smoking status, and SSRI use; *p* = 0.20), but in post-hoc comparisons women using oral contraceptives (that increases SHBG which binds testosterone) had lower testosterone levels than premenopausal women not using oral contraceptives (*p* = 0.03). No univariate associations were found with other potential determinants.

Table 4.1. Characteristics in 2102 participants, according to gender.

Variables	Men (N = 722)	Women (N = 1380)
Age — yr	44.9 ± 12.6	42.8 ± 13.2
Education — yr	12.2 ± 3.3	12.4 ± 3.3
Northern European ancestry (no., %)	692 (95.8%)	1313 (95.1%)
Body-mass index — kg/m ²		
Mean	26.2 ± 4.4	25.3 ± 5.1
≥30 — no. (%)	127 (17.6%)	226 (16.4%)
Current smoker (no., %)	272 (37.7%)	426 (30.9%)
Alcohol consumption ≥2 units/d (no., %)	181 (25.1%)	166 (12.0%)
Physical activity — 1000 MET-min/wk (median, IQR)	2.83 (1.37–4.88)	3.08 (1.53–4.95)
Prevalent cardiovascular disease (no., %)	70 (9.8%)	63 (4.6%)
Salivary testosterone level — pg/mL		
Morning (geometric mean, 95% CI)	25.7 (24.5–27.1)	17.7 (17.0–18.5)
Evening (geometric mean, 95% CI)	19.4 (18.4–20.5)	14.9 (14.1–15.6)

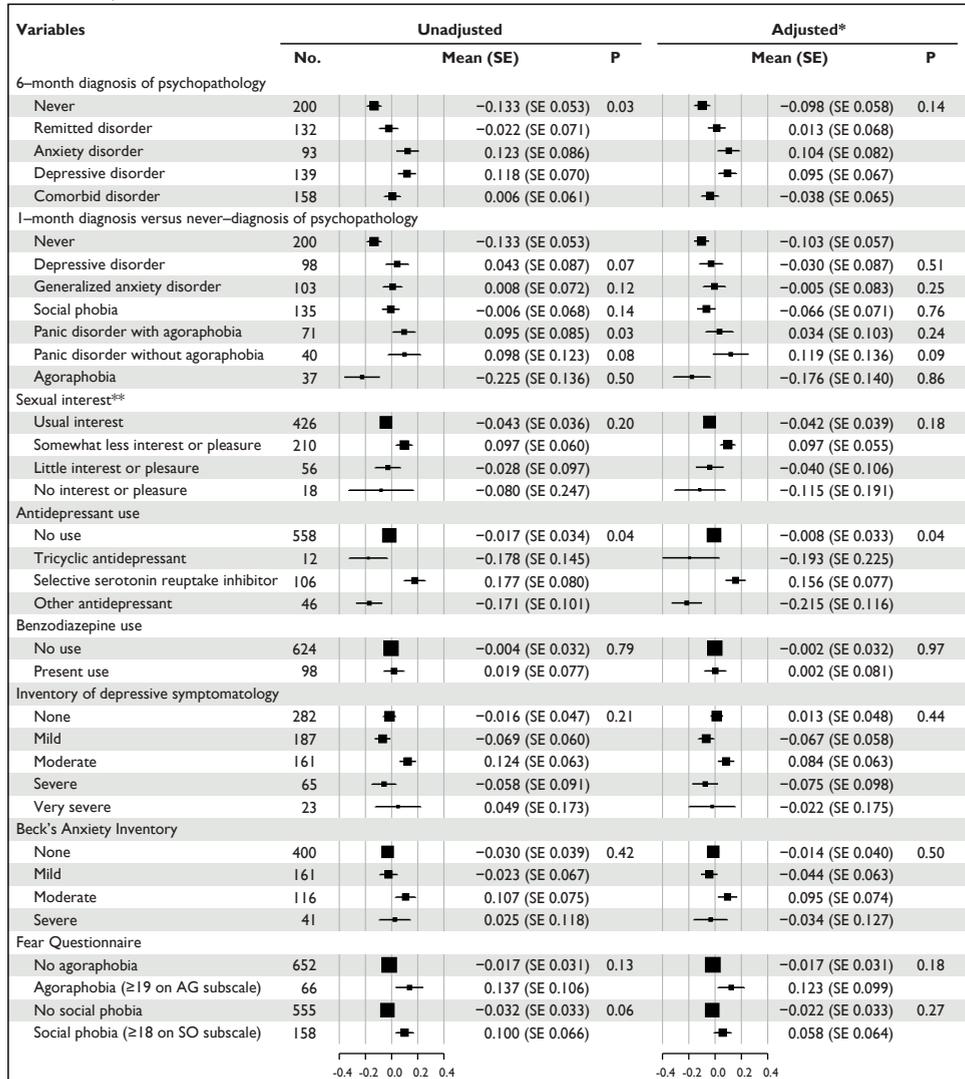
Plus-minus values are means ±SD, CI denotes confidence interval. P-values are for the difference between the groups by analysis of variance or chi-squared test, when appropriate

Salivary testosterone, psychopathology and antidepressant use

We combined the morning and evening testosterone levels using the mean of z-scores, because morning and evening levels were intercorrelated and associations with measures of psychopathology were highly consistent for morning and evening testosterone levels in both men and women (data not shown). Tables 4.2 and 4.3 show the associations with measures of psychopathology and medication use separately in men and women, respectively.

In adjusted models in men, there were no associations with measures of psychopathology (Table 4.2). Those 106 men using SSRIs had higher testosterone levels than 558 men not using antidepressants (effect size 0.207; $p = 0.05$). When repeating our analysis in men with recent (1 year) or current psychopathology the mean difference did not importantly change (92 versus 245 men; effect size 0.192).

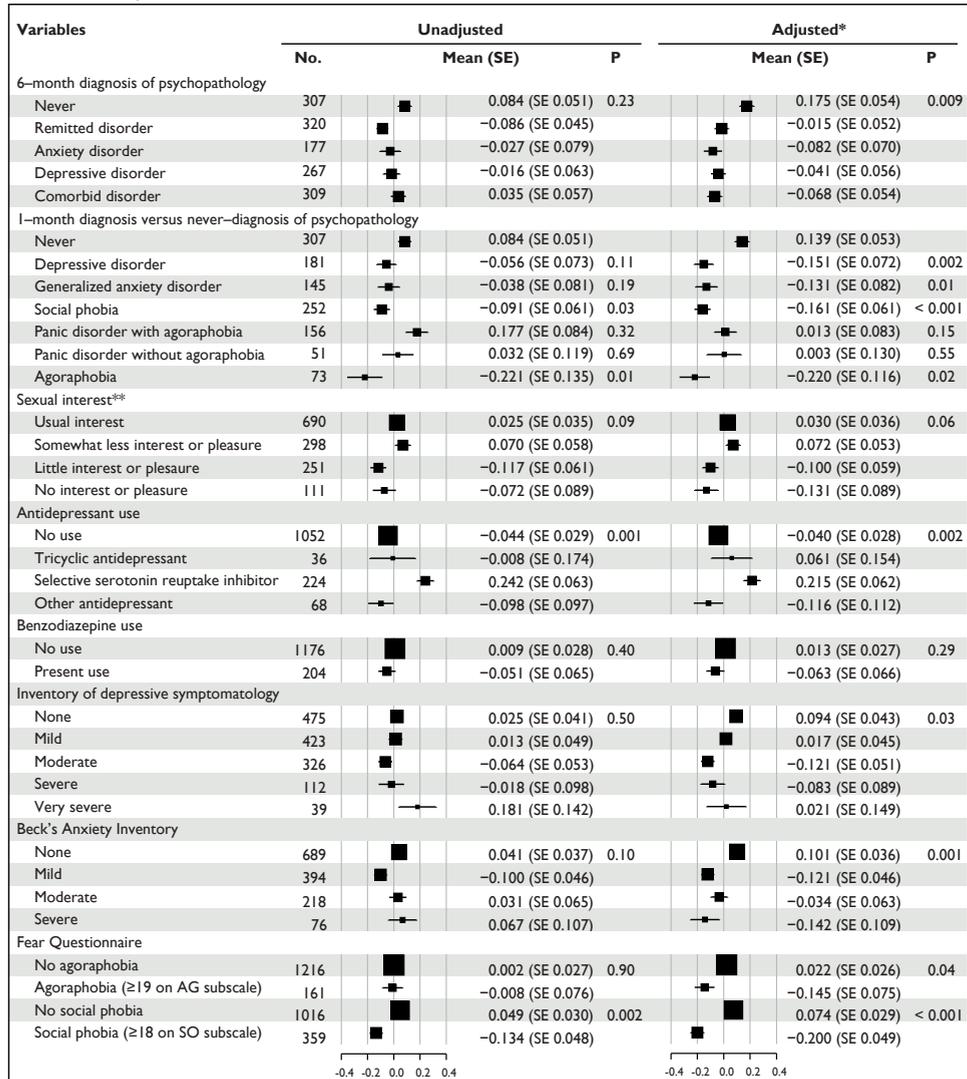
In adjusted models in women, there was a group difference when taking into account the 12-month diagnostic status in adjusted analyses, indicating that women with any psychiatric disorder had on average a lower testosterone level. When zooming in on the 1-month diagnostic categories, the female patients with a current depressive disorder ($p = 0.002$), current generalized anxiety disorder ($p = 0.01$), current social phobia ($p < 0.001$), current agoraphobia (without panic disorder; $p = 0.02$) had lower testosterone levels than the reference group of female participants having no lifetime history of depressive or anxiety disorder. The effect sizes were 0.287, 0.251, 0.299 and 0.304 for the depressive disorder, generalized anxiety disorder social phobia, and agoraphobia, respectively, indicating modest strengths. Again, more markedly increased testosterone levels were found in 224 women using SSRIs than 1052 non-users of antidepressants (effect size: 0.273; $p < 0.001$), but not in women using TCAs or other antidepressants. When repeating our analysis in women with recent (1 year) or current psychopathology the mean difference

Table 4.2. Forest plot for mean standard scores of salivary testosterone levels according to psychiatric characteristics in 722 male subjects.

The size of each square is proportional to the number of participants; horizontal lines indicate standard errors. Standard scores were calculated as the mean of z -scores of the morning and evening testosterone levels, within each sex.

*: Adjusted for age, years of education, smoking status, prevalent cardiovascular disease, and northern European ancestry (as well as SSRI use [2 categories] when appropriate).

**.: Sexual interest was assessed with item 22 of the Inventory of Depressive Symptomatology Self Report (IDS-SR). p -values are for the difference between the groups by analysis of variance.

Table 4.3. Forest plot for mean standard scores of salivary testosterone levels according to psychiatric characteristics in 1380 female subjects.

The size of each square is proportional to the number of participants; horizontal lines indicate standard errors.

Standard scores were calculated as the mean of z-scores of the morning and evening testosterone levels, within each sex.

*: Adjusted for age, years of education, smoking status, prevalent cardiovascular disease, and northern European ancestry (as well as SSRI use [2 categories] when appropriate).

** : Sexual interest was assessed with item 22 of the Inventory of Depressive Symptomatology Self Report (IDS-SR).

P-values are for the difference between the groups by analysis of variance.

did increase slightly (184 versus 475 women; effect size 0.335). In women, the severity of anxiety symptoms ($p = 0.001$), agoraphobia (effect size 0.175; $p = 0.04$), and social phobia (effect size 0.294; $p = 0.001$) was related to lower testosterone levels. There was a linear trend for decreasing testosterone levels with increased social phobia scores (Figure 4.1). The comparison between the groups with severe anxiety versus no anxiety yielded an effect size of 0.254. Although, there was also a difference between categories of severity of depressive symptoms ($p = 0.03$), this was not a linear association.

Because associations between SSRI use and salivary testosterone levels were comparable in men and women (combined effect size 0.258), we further explored the effects of antidepressants in all subjects combined. In adjusted analyses, SSRI users but not users of TCAs or other antidepressants showed higher salivary testosterone levels than non-users of antidepressants (Figure 4.1). In post-hoc tests, we found that differences were statistically significant for fluvoxamine and paroxetine, although differences for sertraline, fluoxetine and citalopram showed a similar tendency that did not reach significance. The mean testosterone difference between SSRI users and non-users of antidepressants was of similar strength in subjects with remitted disorders and current disorders (0.234 versus 0.268, respectively).

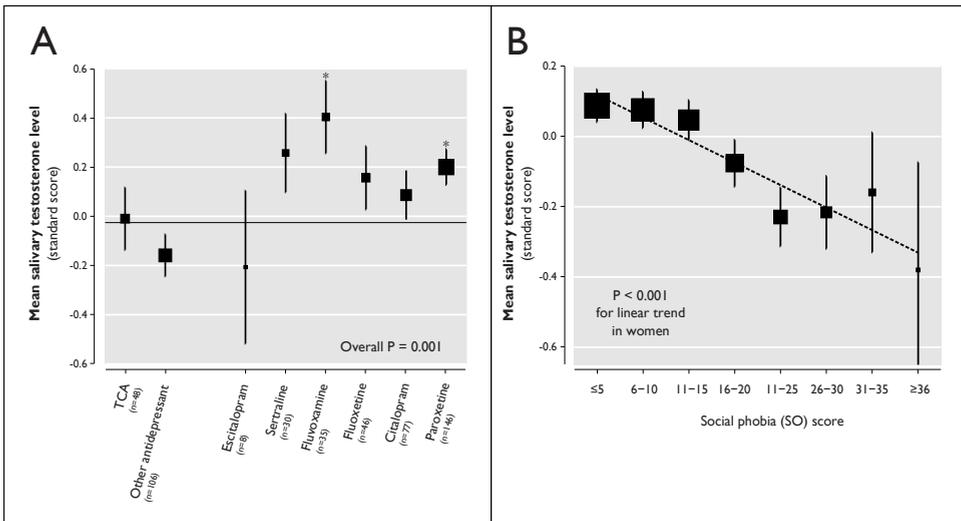


Figure 4.1. The mean standard scores (with error bars representing standard errors) for morning and evening salivary testosterone levels, according (**Box A**) the kind of antidepressant used in men and women combined, and (**Box B**) social phobia (SO) subscale score (part of the Fear Questionnaire) in women only. The black reference line shows the mean score in nonusers of antidepressants. The size of each square is proportional to the number of participants. Vertical lines indicate standard errors. Scores are adjusted for sex, age, menstrual status, years of education, smoking status, prevalent cardiovascular disease, and northern European ancestry. In **Box B**, testosterone values were additionally adjusted for SSRI use. P values are (**Box A**) by analysis of covariance (with * $P < 0.01$ for the difference with non-users of antidepressants), or (**Box B**) for linear trend in analysis of covariance.

Discussion

We found that depressive disorder, generalized anxiety disorder, social phobia, and agoraphobia were associated with lower salivary testosterone levels in women, but not in men. Remarkably, SSRI use was associated with elevated testosterone levels in both men and women. While morning testosterone levels were higher than evening testosterone levels, we found that within-person correlation indicated rank-order stability over the day. Our study therefore provides support for the usefulness, validity and reliability of the non-invasive method of testosterone measurement in saliva. It also points to the importance of taking confounding variables into account, especially of sex, age, menstrual cycle, time of the day, and smoking status. The associations between smoking and physical activity with higher testosterone levels (Shiels et al., 2009) and between oral contraceptive use and lower testosterone levels (Edwards & O'Neal, 2009; Lienen et al., 2010) are in line with previous findings.

Testosterone levels were lower in female patients with current depression versus female controls. Previous studies in women are relatively scarce (Bolour & Braunstein, 2005), in part because blood levels of free testosterone in women were rather unreliable (Bachmann et al., 2002), until recently. Previous studies in men (Almeida et al., 2008; Barrett-Connor et al., 1999; Hintikka et al., 2009; Rhoden & Morgentaler, 2004; Seidman, 2003; Shores et al., 2005; Wang et al., 2009) found lower testosterone levels in men with depressive symptoms compared to male controls, which we could not confirm. Some previous epidemiological studies found no relationship between low testosterone and depressive symptoms (Araujo et al., 1998; Berglund et al., 2011). However, androgen activity is not only the effect of bioavailable androgens but also the responsiveness of the androgen receptor in target cells. The androgen receptor gene (located on the X chromosome) contains a polymorphic CAG repeat sequence affecting androgen sensitivity. Previous studies yielded inconsistent results; CAG repeat length and depressive symptoms were positively associated (Härkönen et al., 2003), unrelated (T'Sjoen et al., 2005), or associated only in subgroups of black men (Colangelo et al., 2007) or patient groups (Schneider et al., 2011). In another study, low testosterone levels were associated with depressive symptoms in men with short CAG repeat lengths only (Seidman et al., 2001). As we did not take into account the CAG repeat length polymorphism, this may explain our null-finding in depressed men. Trials with testosterone administration showed inconsistent evidence for antidepressant properties, more so in hypogonadal than in eugonadal men (Amiaz & Seidman, 2008). These studies were done in participants who did (Harrison G. Pope et al., 2010; Harrison G. Pope, Cohane, Kanayama, Siegel, & Hudson, 2003; Zarrouf, Artz, Griffith, Sirbu, & Kommor, 2009) or did not (Giltay et al., 2010) suffer from a depressive disorder. In supraphysiologic dosages, testosterone may induce euphoria and other

symptoms of hypomania (Pope, Kouri, & Hudson, 2000; Yates, Perry, MacIndoe, Holman, & Ellingrod, 1999).

Yet, we included twice as many women than men in our study. Our findings of low testosterone levels in social anxiety disorder, generalized anxiety disorder, and agoraphobia are not in line with the previous smaller studies that found no relationship between social anxiety and basal salivary testosterone levels (Gerra et al., 2000; Maner et al., 2008). Yet, among the psychiatric disorders under study, particularly those that were characterized by social withdrawal (i.e., depression, social anxiety and agoraphobia) and high social anxiety scores were associated with low testosterone levels in women. No association with panic disorder was found. These findings fit previous notions that reduced testosterone was particularly evident for those primates and humans who behaved socially submissive and showed increased levels of social fear behavior, such as social withdrawal (Archer, 2006; Bos et al., 2010; Gleason et al., 2009; Liening & Josephs, 2010; Sapolsky, 1991; van Honk et al., 1999, 2010). This could suggest that low testosterone is rather a consequence than a cause of the affective symptoms.

Nevertheless, there are putative biological mechanisms that point to a causal role of low testosterone. First, affective symptoms are importantly mediated by the 'fear network' involving the amygdala, hippocampus, and prefrontal cortex, while exogenous and endogenous testosterone significantly modulated local activity and interregional connectivity in the prefrontal cortex and the amygdala during social affective behavior (Hermans et al., 2008a; Volman, Toni, et al., 2011). Neurons in the amygdala (Cooke, 2006; Sarkey et al., 2008), hippocampus (Bao et al., 2008; Wang et al., 2008) and neocortex (Sarkey et al., 2008) indeed specifically express androgen receptors. Second, there may be an imbalance between testosterone and cortisol (van Honk et al., 2010; Viau, 2002). Androgens may downregulate the hypothalamo–pituitary–adrenal (HPA) axis in a direct way via androgen receptors which are expressed in corticotropin-releasing factor (CRF) producing neurons in the hypothalamus and which suppressed the promoter region of the CRF gene (Bao et al., 2008; Wang et al., 2008). Elevated plasma cortisol levels and hyperactivity of CRF neurons in the paraventricular nucleus (PVN) of the hypothalamus are consistent neurobiological findings in depressive and anxiety disorders (Belmaker & Agam, 2008; Vreeburg et al., 2009, 2010; Wang et al., 2008), and the expression of androgen receptors is low in post-mortem brain tissue from patients with depression (Wang et al., 2008). Therefore, our findings fit with the idea that low bioavailable testosterone increases the risk to several psychiatric disorders, that social anxiety and submission induces a lowering of androgens, or both.

The use of SSRIs is associated with sexual problems, such as lowered libido and difficulties with sexual arousal or orgasm (Clayton et al., 2006; Gregorian et al., 2002; Serretti & Chiesa, 2009). These side effects normally continue for as long as the individual is taking the medication and may partly be explained by increased prolactin levels in some cases

(Madhusoodanan, Parida, & Jimenez, 2010), that subsequently may also induce gynecomastia and galactorrhoea. As androgens are pivotal for sexual function, we were interested in the relationship between antidepressant use and testosterone. Remarkably, we found that SSRI users had higher salivary testosterone levels versus participants who did not use antidepressants, which is hard to reconcile with their sexual side effects, although SSRIs may have induced androgen antagonism or the higher testosterone levels may be associated with treatment-induced increases in prosocial behavior. The mechanisms of these side effects are likely complex, involving not only endocrine (e.g., prolactin and sex hormonal) but also directly effects on the central and peripheral nervous systems involved in sexual function. Besides systemic effect on the circulating free testosterone level, SSRIs (and current smoking) may have had a local effect on salivary glands, as antidepressants reduce the salivary flow through muscarinic acetylcholine receptor occupancy, inducing the common complaint of a dry mouth. But effects such as a dry mouth are more commonly observed during TCA use, which in our study was not associated with higher testosterone levels (Scully, 2003).

Our study had some limitations. First, Salivettes were used for the assessment of salivary testosterone, where a passive drooling device would have been preferable. Since we observed a systematic elevation, all Salivette samples need to be collected and handled in the same way in order to yield reliable statistical results as done in the present study. As the difference in results was non-random (i.e., higher levels of testosterone using Salivettes as compared to passive drool saliva samples but with a strong correlation), the internal validity of this study was high. Second, our analyses were cross-sectional and therefore cannot indicate the causal direction. Third, we needed to rely on saliva sampling at the homes of the participants, but as we pooled either four or two saliva samples, compliance and timing with the sampling instructions seemed less of an issue. Fourth, most participants had “(either remitted or current)” anxiety or depressive disorders, and therefore the control group without any lifetime psychiatric diagnosis was relatively small. Nevertheless, noncompliance with instructions could have resulted in some measurement error. The CV of testosterone was around 10%, which error may have biased our findings somewhat toward the null hypothesis. All saliva samples were visually inspected for potential discoloration but not tested biochemically for potential blood contamination (Granger et al., 2004). The strengths of our study included the large sample size, the ‘gold standard’ use of multiple samples that were combined (Brambilla et al., 1996), and the inclusion of patients with several anxiety disorders. We also confirmed findings of low testosterone levels found in participants who were female, using oral contraceptives, and were older, and high testosterone levels found in participants who smoke, which support the validity of salivary testosterone as a measure of androgenic activity.

We conclude that saliva samples are useful for non-invasive screening purposes. Salivary testosterone levels were significantly lower in female patients with depression,

generalized anxiety disorder, social anxiety disorder, and agoraphobia. These findings stress the role of testosterone in social affective symptomatology in women. Future studies should explore whether testosterone treatment or augmentation is beneficial and safe not only in male but also in female patients with low androgenic status.



Dare to approach: Single dose
testosterone administration promotes
threat approach in patients with Social
Anxiety Disorder

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Abstract

Persistent fear and avoidance in patients with Social Anxiety Disorder (SAD) has been associated with reduced testosterone levels. Because threat avoidance is a major maintaining factor in SAD, and because testosterone administration promotes social approach, we tested whether testosterone administration can directly facilitate threat approach behavior in SAD. In a double-blind, placebo-controlled study, 17 female participants with SAD received a single dose of testosterone before performing a well-established social Approach–Avoidance Task. This objective implicit measure of social motivational action-tendencies requires participants to approach or avoid visually presented emotional faces. After testosterone administration, the patients showed increased approach-tendencies to angry facial expressions. These results suggest that testosterone can counteract persistent automatic social avoidance-tendencies in SAD. This finding advances our understanding of steroid involvement in the regulation of social motivational action in general and in SAD in particular, and may have important clinical implications, promoting testosterone's candidacy for pharmacological treatment-enhancement studies.

Introduction

Social Anxiety Disorder (SAD) is the most common anxiety disorder, characterized by an intense fear of social situations in which the individual may be scrutinized by others (American Psychiatric Association, 2013). Avoidance behavior is one of the core characteristics of SAD and plays a crucial role in its persistence as it hinders extinction of fear in social situations (Clark & Wells, 1995). Previous work showed that women with SAD have reduced endogenous testosterone levels, which were also related to the severity of social phobia symptoms (Giltay et al., 2012). Despite mounting evidence for the social approach facilitating properties of testosterone administration (Enter, Spinhoven, & Roelofs, 2014; Terburg et al., 2012), to date no studies have tested whether testosterone administration can alleviate actual social avoidance behavior in SAD.

Testosterone is important in the regulation of social motivational behavior, and enhances social approach motivation while reducing social fear in a socially challenging environment (Archer, 2006; Bos et al., 2012; Radke et al., 2015; Terburg & van Honk, 2013). Also in socially anxious individuals it can increase fixations to the eyes of emotional faces (Enter, Terburg, Harrewijn, Spinhoven, & Roelofs, 2016). Recently, we have shown that testosterone promotes actual social approach behavior in healthy women (Enter et al., 2014). After a single dose testosterone administration the participants showed reduced automatic avoidance of social threat (i.e., angry facial expressions) and relative increased threat approach-tendencies. Such effects would be of particular interest for SAD, as social avoidance is a major characteristic and an important factor in the persistence of the disorder. Therefore, testing whether testosterone can alleviate social avoidance-tendencies in SAD would be of theoretical and clinical importance.

We set out to test whether testosterone administration promotes threat approach in SAD by using a well-established objective and implicit measure of social motivational behavior: the Approach-Avoidance Task (AAT; Heuer et al., 2007; Roelofs et al., 2010; Roelofs, Minelli, et al., 2009; Roelofs, van Peer, 2009; Volman, Roelofs, et al., 2011; Volman, Toni, et al., 2011). This reaction time task requires participants to approach and avoid socially aversive and appetitive visually presented stimuli (angry and happy faces, respectively) by pulling a joystick towards themselves (approach) or pushing the joystick away from themselves (avoidance). Angry faces with direct gaze constitute a potent threat stimulus, that elicit avoidance responses, particularly in socially anxious individuals (Adams et al., 2003; Öhman, 1986; Roelofs et al., 2010).

Based on the approach-enhancing and social-anxiolytic role of testosterone in social motivational behavior, we predict that administration of testosterone to SAD participants would reduce threat avoidance and increase threat approach-tendencies to angry faces on the AAT.

Methods

Participants

Participants with SAD were recruited from outpatient anxiety departments of mental health centers, through advertisements on the internet, and in local newspapers. Inclusion criterion was a total score of >60 on the Liebowitz Social Anxiety Scale (LSAS; Liebowitz, 1987), representing the cut-off score for the presence of social anxiety disorder (Rytwinski et al., 2009).⁵ In addition, participants were screened with the Mini International Neuropsychiatric Interview script (M.I.N.I.; Lecubier et al., 1997) to determine the presence of a DSM-IV diagnosis of generalized SAD. Only female participants were included, because there are as yet no known parameters (e.g., dose and time course) for inducing neurophysiological effects in men after administration of a single dose of testosterone cyclodextrin (Tuiten et al., 2000). Both women using single-phase contraceptives and normally cycling women participated in the study (Hermans et al., 2010). Exclusion criteria were age < 18 and > 50, use of (psychotropic) medication, somatic illnesses, neurological conditions, psychotic disorder, current comorbid diagnosis of mood or anxiety disorders other than SAD, history of head injury, left-handedness, peri- or postmenopause, and pregnancy or breast feeding. After initial screening of 24 socially anxious participants, 17 participants fulfilled all DSM-IV criteria for generalized SAD at the time of testing and were selected for this study. This group had a mean age of 22.8 years ($SD = 5.0$), and a LSAS total score of 78.6 ($SD = 14.3$), see Table S5.1 for additional participant characteristics. All participants had completed or were following higher education, had normal or corrected-to-normal vision, were unaware of the aim of the study, provided written informed consent, and received a financial compensation of €80 and a travel allowance. Data on ethnicity were not recorded. The study was approved by the Medical Ethics Committee of the Leiden University Medical Centre, and was in accordance with the declaration of Helsinki.

Testosterone administration

In a double-blind, randomized, placebo-controlled, crossover design participants received a single dose of 0.5 mg testosterone suspended in a clear solution (0.5 ml) with 0.5 mg hydroxypropyl-beta-cyclodextrin, 0.005 ml ethanol 96%, and distilled water. The matched placebo contained the same ingredients, except testosterone. Participants were asked to hold the liquid under their tongue for 60 seconds. During sublingual administration of 0.5 mg testosterone with cyclodextrin as carrier, testosterone is directly absorbed into the bloodstream. In females, such a dose yields a sharp increase of 20-25 nmol/l in plasma testosterone levels within 15 minutes, which declines to baseline levels within

5 N.B. One participant scored 56 on the LSAS but was included because she met the DSM-IV diagnostic criteria.

the next 90 minutes (van Rooij et al., 2012). Pharmacodynamic effects are measurable approximately four to six hours after testosterone intake (Bos et al., 2012; Tuiten et al., 2000).

Approach-Avoidance Task (AAT)

During this reaction time (RT) task participants responded to happy, angry, and neutral facial expressions presented on a computer screen, by pulling a joystick either towards their body (approach movement) or pushing it away from their body (avoidance movement). Pulling or pushing the joystick increased or decreased the size of the picture respectively, giving the impression of moving towards or moving away from the participant. Usually, response latencies are shorter for affect-congruent (e.g., happy-approach; angry-avoid) as compared to affect incongruent response conditions (e.g., angry-approach; happy-avoid). This task has previously been used by Enter and colleagues (2014). A detailed description is available in the Supplemental Information.

Procedure

Participants were tested individually at two identical testing sessions with two days in between. Testing sessions started at either 930h or 1330h, and participants were tested on the same time of day on both sessions. Four and a half hours after administration of testosterone or placebo participants performed the AAT in a dimly lit and sound attenuated room. After completion of the two sessions participants had to indicate in which session they thought to have had testosterone or placebo. Responses of six of the 17 participants were correct, which is at chance-level, Binomial $P(X = 6) = 0.094$, confirming that participants were unaware of the condition. Before the first testing day the participants completed additional questionnaires, including the Dutch versions of the Beck Depression Inventory (BDI: Beck, Rush, Hollon, & Emery, 1979) and the Social Phobia and Anxiety Inventory (SPAI: Turner et al., 1989; see Table S6.1).

Statistical analyses

RT outliers were filtered using a <150 and >1500 ms cut-off. A cut-off of three standard deviations from the mean was used for defining outliers in the remaining RTs. Error rates were calculated after removal of outliers. For each participant, the median of the remaining RTs (91%) for the correct responses (RT1) was calculated per cell (defined by: Emotion and Movement). Following Enter and colleagues (Enter et al., 2014) AAT-effect scores were computed for each participant and for each emotion separately by subtracting median pull RTs from corresponding median push RTs (e.g., RT angry push – RT angry pull; RT happy push – RT happy pull; RT neutral push – RT neutral pull). As a result, more negative AAT-effect scores (push is faster than pull) reflect a relative avoidance tendency.

To test effects of testosterone administration on approach-avoidance tendencies to emotional faces, AAT-effect scores were entered in a two-way repeated measures Analy-

sis of Variance (rmANOVA) with Condition (testosterone, placebo) and Emotion (angry, happy, neutral) as within-subject factors. Greenhouse-Geisser correction was used when appropriate (uncorrected degrees of freedom are reported together with the correction factor epsilon (ϵ)). In addition, we tested whether AAT-effect scores were significant in certain conditions (i.e., differed from zero) using one sample *t*-tests. Alpha was set at .05 and effect sizes are reported in partial eta squared (η_p^2). Finally, we checked whether avoidance behavior to angry faces in the placebo condition was associated with severity of social anxiety symptomatology by computing Spearman's correlation coefficient between baseline corrected AAT-effect scores and SPAI total scores.

Results

Table 5.1 provides an overview of the outcomes for RTs. The task was well performed, as indicated by low error rates (9%).

Table 5.1. Mean RTs (*ms* \pm SEM) for approach (pull) and avoidance (push) responses to angry, happy, and neutral faces per condition.

Valence	Placebo		Testosterone	
	Push	Pull	Push	Pull
Angry	425 (38)	429 (33)	447 (55)	438 (63)
Happy	431 (44)	424 (35)	435 (45)	447 (51)
Neutral	421 (35)	428 (32)	431 (47)	447 (51)

In line with our hypotheses, the two-way rmANOVA for the AAT-effect scores, with Condition (Placebo, Testosterone) and Emotion (angry, happy, neutral) as within-subject factors, revealed a significant Condition x Emotion interaction, $F(1,22,19.5) = 4.95$, $p = .032$, $\epsilon = .608$, $\eta_p^2 = .236$ and a significant effect of Emotion, $F(1,16) = 6.25$, $p = .024$, $\eta_p^2 = .281$. There were no significant Condition effects for each Emotion separately (all $F_s < 2.6$, $p_s > .13$), but separate two-way rmANOVAs for each pair of emotions demonstrated a significant Condition x Emotion interaction for angry vs happy faces, $F(1,16) = 6.25$, $p = .024$, $\eta_p^2 = .281$, and for angry vs neutral faces, $F(1,16) = 4.61$, $p = .047$, $\eta_p^2 = .224$, but not for happy vs neutral faces, $F(1,16) = 0.561$, $p = .465$, $\eta_p^2 = .034$. As expected, these findings pointed towards a specific effect of testosterone on the AAT-effect scores for angry faces, compared to happy and neutral faces. Indeed, when looking into each condition separately, one-way rmANOVAs for Emotion (angry, happy, neutral) revealed a significant effect for Emotion in the testosterone condition, $F(1.43,22.95) = 4.90$, $p = .026$, $\epsilon = .717$, $\eta_p^2 = .234$, but not in the placebo condition, $F(1.44,23.0) = 1.22$, $p = .300$, $\epsilon = .718$, $\eta_p^2 = .071$. Separate follow-up analyses for each pair of emotions showed that the AAT-effect scores

in the testosterone condition were markedly higher for angry faces compared to happy and neutral faces, by revealing a significant main effect of Emotion for angry vs happy, $F(1,16) = 6.94, p = .018, \eta_p^2 = .303$, and angry vs neutral, $F(1,16) = 16.14, p = .001, \eta_p^2 = .502$, but not for happy vs neutral faces, $F(1,16) = 0.098, p = .759, \eta_p^2 = .006$. Taken together, these results suggest a significant increase of approach-tendencies towards angry faces by testosterone in SAD participants, see Figure 5.1.

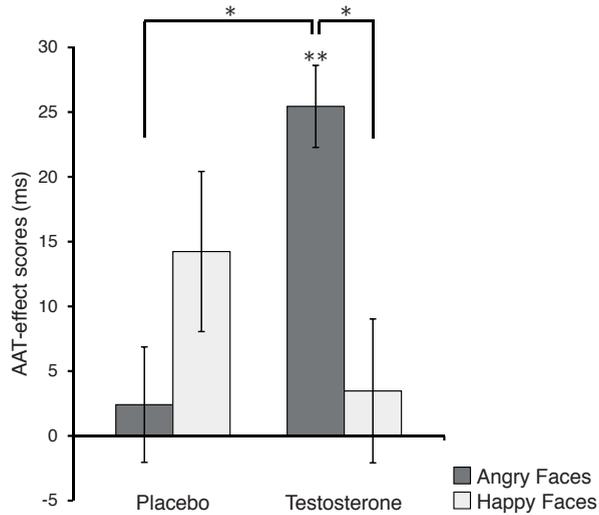


Figure 5.1. Mean Approach-Avoidance Task (AAT) effect scores (ms) for approach and avoidance movements to angry and happy faces in the placebo and testosterone condition. Participants show significant approach tendencies toward angry faces after testosterone administration, compared with happy faces. For display purposes, mean scores are baseline corrected (i.e., AAT effect scores for neutral faces subtracted from the AAT effect scores of angry and happy faces, respectively). Of note, baseline corrected AAT effect scores for angry faces showed a significant treatment effect, $F(1, 16) = 4.61, p = .047, \eta_p^2 = .224$, and differed significantly from zero in the testosterone condition, $t(16) = 4.02, p = .001$, indicating threat approach. * $p < .05$. ** $p < .01$.

Next we checked whether avoidance behavior to angry faces in the placebo condition was associated with social anxiety symptomatology. A significant negative correlation between the baseline corrected AAT-effect scores and the SPAI scores, $r = -.660, p = .004$, indicated that those patients who had stronger avoidance tendencies also had more severe social anxiety symptoms. Social anxiety scores did not modulate the testosterone effects (SPAI: all $F_s < 1.81, p_s > .199$). There were also no such effects when taking LSAS as symptom severity measure (All $F_s < 0.757, p_s > .398$).

In order to test whether this threat-approach enhancing effect of testosterone is comparable to the effect previously observed in healthy participants, we added the AAT-effect scores based on the reaction times (operationalized as movement initiation

times at 7° displacement instead of 30°) of the previously published group of healthy participants (Dorien Enter et al., 2014c) as a control group (HC).⁶ We conducted a three-way rmANOVA, with Condition (placebo, testosterone) and Emotion (angry, happy, neutral) as within-subject factors, and Group (SAD, HC) as between-subjects factor. Age was added as a standardized covariate because the HC-group has a higher age-range. Similar to the previous analyses in both groups separately, this analysis revealed a significant Condition x Emotion interaction, $F(2,76) = 4.61, p = .013, \eta_p^2 = .108$. There were no significant effects of Age (all $ps > .37, \eta_p^2 \leq .02$) or Group (all $ps > .33, \eta_p^2 < .03$), the latter indicating that SAD participants benefitted to a similar extent from testosterone as the non-anxious group. Separate follow-up analyses for each pair of Emotions showed that the AAT-effect scores in the testosterone condition tended to be higher for angry faces compared to happy and neutral faces, by revealing a significant main effect of Emotion for angry vs neutral, $F(1,38) = 9.52, p = .043, \eta_p^2 = .200$, a trend-level significant effect of Emotion for angry vs happy, $F(1,38) = 3.19, p = .082, \eta_p^2 = .077$, but a non-significant effect of Emotion for happy vs neutral faces, $F(1,38) = 0.284, p = .597, \eta_p^2 = .007$. There were no effects of Group (all $ps > .17, \eta_p^2 < .04$), nor age (all $ps > .27, \eta_p^2 < .03$). Therefore, these results indicate that testosterone promotes similar threat approach-tendencies in non-anxious healthy participants and participants suffering from generalized SAD.

The effects of testosterone on AAT-effect scores were not affected by administration order, time of testing, or use of contraceptives (see supplemental information).

Discussion

The present findings show that single dose testosterone administration can lead to increased approach of social threat cues in women with SAD. This is the first study in SAD showing that testosterone directly influences automatic approach-avoidance actions and promotes approach behavior that directly affects the perceived distance of the social threat (Rinck & Becker, 2007).

Angry faces play an important role in social communication as they convey social dominance and are being perceived as a threat, especially by socially anxious individuals (Adams et al., 2003; Öhman, 1986). In our study, testosterone administration leads to a significant increase in approach of angry faces, relative to happy and neutral facial expressions. This finding is in agreement with literature that shows that testosterone reduces fear and sensitivity to threat, and enhances social dominance related behavior (Archer, 2006; Bos et al., 2012; Enter et al., 2014; Terburg & van Honk, 2013). Our previous study showed that testosterone promotes actual threat approach behavior in healthy

6 This group was tested using similar inclusion criteria and the same study-setup.

participants (Enter et al., 2014), and the current study extends these findings by showing that testosterone also promotes social threat approach in participants with generalized SAD. Socio-neuroendocrine models of SAD propose that individuals with SAD display exaggerated socially submissive behavior, which is associated with social avoidance and low endogenous testosterone levels (Gilbert, 2001; Giltay et al., 2012; Hermans & van Honk, 2006; Öhman, & Wiens, 2003; Sapolsky, 1991; Weisman, et al., 2011). An increase in threat approach behavior after administration of testosterone would be the result of an enhanced motivational tendency towards social dominance in this socially submissive group (Archer, 2006; Bos et al., 2012; Enter et al., 2014; Terburg & van Honk, 2013). This interpretation fits recent findings that testosterone can promote gaze behaviour towards the eye-regions in healthy and socially anxious individuals (Enter et al., 2016; Terburg et al., 2012).

Performance on the AAT depends on the coordinating role of the anterior prefrontal cortex and its connection with the amygdala and other brain regions (Volman, Verhagen, et al., 2013; Volman, Roelofs, et al., 2011). This neural circuit is important for the regulation of social emotional behavior, and frontal-amygdala crosstalk during operation of the AAT is influenced by endogenous testosterone levels (Volman, Toni, et al., 2011). Studies featuring testosterone administration showed that testosterone affects neural responsiveness of brain areas involved in social approach such as amygdala and striatum towards socially salient stimuli (Hermans et al., 2008; van Wingen et al., 2010). Moreover, the way testosterone affects the amygdala depends on motivational context and it specifically promotes threat approach during performance of the AAT (Radke et al., 2015). Typically, these neural circuits show altered functioning in SAD. Neuroimaging studies in SAD have consistently shown increased activation of the amygdala and connected frontal-striatal circuits when participants were exposed to threatening facial expressions (Fouche et al., 2013), and it has been proposed that the amygdala is not properly regulated by cortical areas during social threat in SAD (Cremers et al., 2015; Freitas-Ferrari et al., 2010). Future neuroimaging research should elucidate whether testosterone administration also promotes threat approach in SAD by influencing activity in these neural circuits underlying social motivational behavior.

A few methodological issues are relevant to consider with regard to this study. First, following previous hormone administration studies featuring clinical participants on the AAT (van Peer et al., 2009), we selected the movement initiation time (7° joystick rotation) and not the full movement time (30° joystick rotation) as an outcome measure. It has been proposed that the initiation time reflects the time necessary for neural processes involved in stimulus evaluation, response selection, and programming the execution of motor movements while the movement time includes the neuro-muscular response (Rotteveel & Phaf, 2004). The latter usually shows a general slowing in patient samples with affective disorders, such as anxiety and depression, compared to HC (Sabbe, Hulstijn,

van Hoof, Tuynman-Qua, & Zitman, 1999; Volman, Toni, et al., 2013). Since we aimed to test effects on the cognitive affective processes reflected in the initiation time, we selected this measure for our analyses instead of the actual movement execution which may be confounded by general slowing in SAD. Second, previous studies featuring the AAT found that socially anxious participants show typical social avoidance behavior in response to angry faces (Heuer et al., 2007; Roelofs et al., 2010; van Peer et al., 2009, but see Roelofs et al., 2009). Although our results seem to point towards a relatively stronger tendency to avoid angry compared to happy faces in the placebo condition (see figure 5.1), this effect did not reach significance in the present study. Despite this absence of avoidance of angry faces on a group level, we found that avoidance of angry faces was stronger for more severely affected patients, as indicated by a significant correlation between baseline corrected AAT-effect scores for angry faces and SPAI scores. In addition, it should be noted that testosterone can evoke strong placebo responses (Handelsman, 2011), therefore we cannot exclude the possibility that placebo effects have contributed to decreased avoidance of angry faces in the placebo condition. Third, our sample size is small, even though it falls within a range that is common for SAD studies, and is employed in a powerful within subjects design. Future studies including a larger number of participants should replicate these findings and would also have the power to investigate the role of individual differences in endogenous testosterone levels. Finally, as a consequence of the testosterone administration method, only female participants were tested in this study (Tuiten et al., 2000). Future research should investigate whether testosterone administration to men has similar effects as suggested by similarities in social behavior related to endogenous and exogenous testosterone across sexes (Goetz et al., 2014; Hermans et al., 2008).

The finding that testosterone promotes social approach motivation in SAD may have clinical implications. A large proportion of SAD patients (up to 50%) does not recover after current evidence based psychological and pharmacological treatments (Hofmann & Bögels, 2006; Stein & Stein, 2008), and there is a need for new treatment strategies that enhance remission rates. A novel line of research has shown that treatment effects are augmented by pairing exposure therapy with a pharmacological agent (Hofmann et al., 2014; Singewald, Schmuckermair, Whittle, Holmes, & Ressler, 2015). Given our promising findings showing that testosterone promotes actual social approach behavior in SAD during a social challenge, and since exposure therapy is aimed at reduction of social avoidance behavior, it would be interesting for future investigations to explore whether adding testosterone as a pharmacological enhancer in the first few therapy sessions can boost efficacy of exposure therapy in SAD. In addition, future studies should test for dose-response relationships before administering testosterone in clinical practice.

In conclusion, this is the first study in SAD that shows that testosterone counteracts automatic avoidance behavior by promoting social approach-tendencies that have a direct effect on the perceived distance of the social threat. These findings support theories

on the role of testosterone in the regulation of social motivational behavior and concur with the predictions of socio-neuroendocrine models of SAD. These findings also may have clinical implications as social avoidance plays a crucial role in the persistence of SAD. The finding that this core characteristic can directly be influenced by single dose testosterone administration may encourage exploration of testosterone administration as a means to enhance therapy efficacy.

Supplemental Information

Supplemental Methods

Table S5.1: *Participant Characteristics (n = 17).*

	<i>M</i>	<i>SD</i>
Age (years)	22.8	5.0
LSAS total	78.6	14.3
SPAI total	99.84	21.0
BDI	16.5	11.5

Data are presented in mean and standard deviation. Abbreviations: LSAS, Liebowitz Social Anxiety Scale; SPAI, Social Phobia and Anxiety Inventory; BDI, Beck Depression Inventory.

Approach-Avoidance Task (AAT)

During this reaction time (RT) task participants responded to emotional face pictures presented on a computer screen, by pulling a joystick either towards their body (approach movement) or pushing it away from their body (avoidance movement; task adapted from (Heuer et al., 2007)). Pulling or pushing the joystick increased or decreased the size of the picture respectively, giving the impression of moving towards or moving away from the participant. The speed of the size change was proportional to the amplitude of the joystick movement. As soon as the joystick reached its target position (i.e., the required direction; full movement involved a 30° rotation from the upright position) the picture disappeared from the screen. The time between the onset of the stimulus and its disappearance from the screen was recorded with <1 ms accuracy at 7°, 15°, 22°, and 30° rotation, with the reaction time set to 7° displacement. After each completed trial the participant moved the joystick back to its central position and initiated a new trial by pressing the fire button near the top of the joystick. Face stimuli were selected from the Karolinska Directed Emotional Faces database based on quality of emotional expression (Goeleven et al., 2008; Lundqvist et al., 1998). Happy, angry, and neutral facial expressions were taken from the same model (five male and five female models in all) and each picture was presented either with a yellowish or a grayish filter. In addition, checkerboards (10 yellow, 10 gray) were included as non-facial control stimuli.⁷ This resulted in a

⁷ Checkerboard stimuli yielded significantly slower reaction times ($p \leq .04$; RTs (ms \pm SEM) for placebo condition: push 451 (10), pull 436 (9); testosterone condition: push 462 (12), pull 245 (15)). It is likely that this slowing is caused by an oddball effect as they were outnumbered by the facial stimuli (i.e., 20 checkerboards to 60 emotional faces). Therefore the checkerboard stimuli have not been included in the main analyses.

total of 80 different stimuli, which were presented in random order. All participants were instructed to push on yellow stimuli and to pull on gray stimuli, and to respond as fast and as accurately as possible. Usually, response latencies are shorter for affect-congruent (e.g., happy-approach; angry-avoid) as compared to affect incongruent response conditions (e.g., angry-approach; happy-avoid). Before the real test started, participants were presented with eighteen practice trials, which were similar to the test trials except for the fact that the pictures showed different models.

Supplemental Results

To check whether the effects of testosterone on AAT-effect scores were affected by administration order, time of testing or use of contraceptives, we separately added these factors as between-subjects factors to the model. These analyses showed no influence of contraceptive use (all $ps > .25$, $\eta_p^2 < .09$). Nor were there main or interaction effects for Order (all $ps > .39$, $\eta_p^2 < .05$), or time of testing (all $ps > .23$, $\eta_p^2 \leq .09$). Most importantly, the Condition \times Emotion interaction remained significant after including these factors into the model (all $ps > .04$, $\eta_p^2 > .20$); and trend-level significant after including the factor Order into the model ($p = .057$, $\epsilon = .595$, $\eta_p^2 = .21$).



Single dose testosterone
administration alleviates gaze
avoidance in women with Social
Anxiety Disorder

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Abstract

Gaze avoidance is one of the most characteristic and persistent social features in people with Social Anxiety Disorder (SAD). It signals social submissiveness and hampers adequate social interactions. Patients with SAD typically show reduced testosterone levels, a hormone that facilitates socially dominant gaze behavior. Therefore we tested as a proof of principle whether single dose testosterone administration can reduce gaze avoidance in SAD. In a double-blind, within-subject design, 18 medication-free female participants with SAD and 19 female healthy control participants received a single dose of 0.5 mg testosterone and a matched placebo, at two separate days. On each day, their spontaneous gaze behavior was recorded using eye-tracking, while they looked at angry, happy, and neutral facial expressions. Testosterone enhanced the percentage of first fixations to the eye-region in participants with SAD compared to healthy controls. In addition, SAD patients' initial gaze avoidance in the placebo condition was associated with more severe social anxiety symptoms and this relation was no longer present after testosterone administration. These findings indicate that single dose testosterone administration can alleviate gaze avoidance in SAD. They support theories on the dominance enhancing effects of testosterone and extend those by showing that effects are particularly strong in individuals featured by socially submissive behavior. The finding that this core characteristic of SAD can be directly influenced by single dose testosterone administration calls for future inquiry into the clinical utility of testosterone in the treatment of SAD.

Introduction

Social Anxiety Disorder (SAD) is a common anxiety disorder, characterized by persistent fear and avoidance of social situations (American Psychiatric Association, 2013). SAD has been related to a ubiquitous social hierarchy system, with individuals with SAD displaying socially submissive as opposed to socially dominant behavior (Hermans & van Honk, 2006; Maner et al., 2008; Weisman et al., 2011). Typical submissive behavior, such as avoidance of eye contact plays a crucial role in the persistence of the disorder by hindering extinction of fear in social situations (Clark & Wells, 1995; Stein & Stein, 2008). Especially angry facial expressions with direct gaze signal social scrutiny or a potential dominance challenge and elicit avoidance tendencies in highly socially anxious individuals (Öhman, 1986; Roelofs et al., 2010). Indeed, eye-tracking studies investigating gaze behavior in SAD, have demonstrated avoidance of the eye-region of angry faces (Horley, Williams, Gonsalvez, & Gordon, 2004; Moukheiber et al., 2010; Moukheiber, Rautureau, Perez-Diaz, Jouvent, & Pelissolo, 2012). Because avoidance behavior is the major maintaining factor in SAD, it is relevant to develop interventions that directly target this behavior (Clark & Wells, 1995; Gamer & Büchel, 2012; Hofmann et al., 2014; Roelofs et al., 2010).

SAD is associated with reduced endogenous testosterone levels (Giltay et al., 2012), and because testosterone is known to reduce social avoidance (Enter et al., 2014; Terburg et al., 2012), it is striking that so far no studies have tested the direct effects of testosterone administration in SAD. Testosterone has an important role in the regulation of social motivational behavior: It has socially anxiolytic effects, and is associated with social dominance and approach behavior (Bos et al., 2012; Enter et al., 2014; Terburg & van Honk, 2013). Based on recent findings indicating that testosterone administration in healthy females promotes reactive social dominant gaze behavior to angry faces (Terburg et al., 2012; Terburg, Hooiveld, Aarts, Kenemans, & van Honk, 2011), we predicted that testosterone administration would alleviate submissive gaze avoidance to angry faces in individuals with SAD.

We tested this hypothesis in a double-blind and placebo controlled within-subject study. A total of 18 medication-free participants with SAD and 19 healthy control participants received a single dose of 0.5 mg testosterone and a matched placebo in two sessions. In each session, their spontaneous gaze behavior was recorded while they looked at angry, happy, and neutral facial expressions. Gaze avoidance of eye contact was reliably indexed as relative reduction of initial gaze fixations on the eye-region (Becker & Detweiler-Bedell, 2009; Gamer & Büchel, 2012; Gamer, Zurowski, & Büchel, 2010; Garner, Mogg, & Bradley, 2006). We predicted that testosterone administration in contrast to placebo would reduce gaze avoidance and increase the number of first fixations to the eyes of angry faces in particular in SAD.

Method

Participants

Characteristics of the participant groups are presented in Table 6.1 (see also Table S6.1 and S6.2, Supplemental Information). Participants with social anxiety disorder (SAD) were recruited from outpatient anxiety departments of mental health centers, through advertisements on the internet, and in local newspapers. Inclusion criterion was a total score of > 60 on the Liebowitz Social Anxiety Scale (Liebowitz, 1987; Rytwinski et al., 2009). In addition participants were screened with the Mini International Neuropsychiatric Interview script (Lecubier et al., 1997) to determine the presence of a DSM-IV diagnosis of generalized Social Anxiety Disorder. Healthy control (HC) participants were recruited via advertisements in community centers, on the internet, and in local newspapers. Only female participants were included, because there are as yet no known parameters (e.g., dose and time course) for inducing neurophysiological effects in men after administration of a single dose of testosterone (Tuiten et al., 2000). Both women using single-phase contraceptives (11 HC, 15 SAD), and normally cycling women (8 HC, 3 SAD) participated in the study (e.g., Hermans et al., 2010). Exclusion criteria were age < 18 and > 50, use of (psychotropic) medication, somatic illnesses, neurological conditions, psychotic disorder, history of head injury, left-handedness, peri- or postmenopause, and pregnancy or breast feeding (for both HC and SAD groups), recent or past psychiatric problems (only HC group), and current comorbid diagnosis of mood or anxiety disorders other than SAD (only SAD group). After initial screening of 24 subjects for both groups, 19 SAD and 19 HC participants were selected on basis of matching for age and level of education (all participants were following or completed higher education). Data of one SAD participant was lost due to technical failure, leaving 18 SAD and 19 HC participants for analyses. Thirteen of the 18 SAD participants met full DSM-IV criteria for gSAD at the time of testing; the other five had sub-syndromal SAD (i.e., they did no longer fulfill DSM-IV criterion E: the symptoms lead to significant burden in social or occupational functioning at time of testing). See Table S6.2 for the demographic characteristics of this group of 13 participants with syndromal SAD. Our primary aim was to test effects in participants who fulfill all DSM-IV criteria for generalized SAD (SAD syndromal group) but for transparency reasons we will also report analyses for all participants, including the five who were in remission (SAD combined). All participants had normal or corrected-to-normal vision, were unaware of the aim of the study, provided written informed consent, and received financial compensation. The study was approved by the Medical Ethics Committee of the Leiden University Medical Centre, and was in accordance with the declaration of Helsinki.

Table 6.1. Group Characteristics for the Healthy Control (HC) group and the combined group of participants with syndromal and sub-syndromal Social Anxiety Disorder (SAD).

	HC (n = 19)	SAD (n = 18)	t(35)	p-Value
Age (years)	25.2 (4.0)	23.1 (4.6)	1.49	.145
LSAS anxiety	9.5 (7.2)	41.5 (6.2)	-14.49	<.001
LSAS avoidance	7.8 (6.3)	35.4 (7.4)	-12.21	<.001
LSAS total	17.3 (12.9)	76.8 (12.5)	-14.25	<.001
BDI	2.5 (2.2)	13.5 (11.9)	-3.86	.001

Data are presented in mean and standard deviation. Abbreviations: LSAS, Liebowitz Social Anxiety Scale; BDI, Beck Depression Inventory.

Testosterone administration

In a double-blind, randomized, placebo-controlled, cross-over design participants received a single dose (0.5 ml) of 0.5 mg testosterone suspended in a clear solution with 0.5 mg hydroxypropyl-beta-cyclodextrin, 0.005 ml ethanol 96%, and distilled water. The matched placebo contained the same ingredients, except the testosterone. Participants were asked to hold the liquid under their tongue for 60 seconds. During sublingual administration of 0.5 mg testosterone cyclodextrin, testosterone is directly absorbed into the bloodstream. In females, such a dose yields a sharp increase of 20-25 nmol/l in plasma testosterone levels within 15 minutes, which declines to baseline levels within the next 90 minutes (van Rooij et al., 2012). Previous research applying this method has convincingly shown consistent psychophysiological and behavioral effects approximately 4-6 hours after administration, therefore this time interval was also applied in the current study (Bos et al., 2012; Enter et al., 2014; Tuiten et al., 2000).

Passive Viewing Task

Face stimuli were selected from the NimStim set of facial expressions (Tottenham et al., 2009). Happy, Angry, and Neutral facial expressions were taken from the same model (four male and four female models), cut out in an oval shape (368 x 515 pixels) to remove distracting features, and presented with a grayish filter on an equiluminant gray background. One face at the time was shown on the middle of the screen (9.3° x 12.9° visual angle; screen resolution 1280 x 1024 pixels), in such a way that the pre-trial fixation cross was situated on the nasal bridge below the eyes. Stimuli were repeated three times, resulting in 72 randomized trials in total. Trials started when the participant maintained a fixation on the central fixation cross for 1000 ms. Stimulus presentation time was 5000 ms, followed by an intertrial interval (blank gray screen) of 4000-7000 ms. Three breaks were offered throughout the task, and could be terminated by a button press. Participants sat at a distance of 60-65 cm from the screen and were instructed to look at the fixation cross, and then look at the pictures without further instructions except for not moving their head.

Procedure

Participants were tested individually at two identical testing sessions with two days in between. Testing sessions started at either 0930 or 1330 h, and participants were tested on the same time of day on both sessions. Four hours after administration of testosterone or placebo participants were seated in a dimly lit and sound attenuated room, where they performed a standard nine-point calibration procedure, followed by the Passive Viewing Task. In addition to gaze behavior, EEG and facial mimicry were recorded, results of which will be reported elsewhere.

Data acquisition and statistical analyses

Eye movements were recorded with a Tobii T120 binocular infrared eye tracker (Tobii Technology, Danderyd, Sweden), sampling at 120 Hz, with 0.5° accuracy. Oval Areas of Interest (AOIs) were created around each eye for each picture separately and excluded the inter-ocular space (Figure 2c). Gaze fixations were defined as the average location of all subsequent gaze points within 1.5° visual angle, with a minimal duration of 100 ms excluding the initial gaze points on the central fixation cross (Tobii Technology, Danderyd, Sweden). Calculated parameters were Percentage First Fixations (i.e., 100 times the amount of first fixations in the eye AOI divided by the total amount of first fixations) and the duration of first fixations. In addition, Percentage Total Fixations (i.e., 100 times the amount of fixations in the eye AOI divided by the total amount of fixations) and Fixation Duration were calculated for fixations during the total stimulus presentation time.

To account for data loss due to eventual motion and eye blink artifacts, we applied a cut-off of at least 150 data points per trial (25% of the possible 600 data points in each trial, 120Hz sampling-rate and 5 second presentation) for trial inclusion in the analysis. Using this filter criterion 4.7% of the trials were discarded which was not different between groups, treatment condition or their interaction (all $ps > .23$).

Parameters were entered in a three-way repeated measures Analysis of Variance (rmANOVA) with Treatment (placebo, testosterone) and Emotion (angry, happy, neutral) as within-subject factors, and Group (HC, SAD) as between-subjects factor. Alpha was set at .05 (two-tailed) and effect sizes are reported in partial eta squared (η_p^2). Greenhouse-Geisser correction was used when appropriate (uncorrected degrees of freedom are reported together with the correction factor epsilon (ϵ)). The statistics will be first reported for the combined group of participants with syndromal and sub-syndromal SAD participants (SAD combined, $n = 18$), and will be subsequently repeated for the target group of participants who still fulfilled all criteria for SAD at the time of testing (SAD syndromal, $n = 13$).

Table 6.2. First fixations (percentage \pm SEM) towards the eye-region of angry, happy, and neutral facial expressions after administration of placebo and testosterone for Healthy Control participants ($n = 19$) and the combined group of participants with syndromal and sub-syndromal Social Anxiety Disorder ($n = 18$).

Emotion	Healthy Controls		Social Anxiety Disorder	
	Placebo	Testosterone	Placebo	Testosterone
Angry	43 (6)	36 (6)	30 (6)	37 (8)
Happy	49 (7)	42 (6)	36 (7)	37 (9)
Neutral	48 (7)	40 (6)	41 (8)	40 (9)

Results

Percentage first fixations

The percentage first fixations to the eye region for each condition, emotion and group are presented in Table 6.2. First we conducted a three-way rmANOVA with all emotions in the analysis for the percentage first fixations, with Treatment (placebo, testosterone) and Emotion (angry, happy, neutral) as within-subject factors, and Group (HC, SAD) as between-subjects factor. There was no significant Treatment \times Emotion \times Group interaction; this counted both when including the SAD (combined) group, $F(2,70) = 1.04$, $p = .360$, and when including the SAD (syndromal) group, $F(2,60) = 1.13$, $p = .331$. However, a significant Treatment \times Group interaction was present both when including the SAD (combined) group, $F(1,35) = 6.67$, $p = .014$, $\eta_p^2 = .16$, and when including the SAD (syndromal) group, $F(1,30) = 7.39$, $p = .011$, $\eta_p^2 = .20$, indicating that testosterone affects gaze behavior in the HC and SAD groups in a differential manner (Figure 6.1). There were no significant post-hoc effects involving Group with all emotions in the analyses, all $F_s < 1.83$, $p_s > .285$, but a main effect of Emotion (both with the SAD (combined) group, $F(2,70) = 7.32$, $p = .002$, $\eta_p^2 = .17$, and with the SAD (syndromal) group, $F(2,60) = 5.12$, $p = .009$, $\eta_p^2 = .15$), indicated relative avoidance of angry versus happy faces (both with SAD (combined), $F(1,35) = 10.52$, $p = .003$, $\eta_p^2 = .23$, and with SAD (syndromal), $F(1,30) = .841$, $p = .007$, $\eta_p^2 = .22$) and angry versus neutral faces (both with SAD (combined), $F(1,35) = 10.46$, $p = .003$, $\eta_p^2 = .23$, and with SAD (syndromal), $F(1,30) = 8.06$, $p = .008$, $\eta_p^2 = .21$) across groups.

Because of our specific hypothesis on the effects of testosterone on the first fixations towards the eyes of angry faces in SAD, we checked whether the Treatment \times Group effect would hold for angry faces alone. Indeed, the significant Treatment \times Group interaction remained for angry faces. This occurred both when including the SAD (combined) group, $F(1,35) = 8.41$, $p = .006$, $\eta_p^2 = .19$, and when including the SAD (syndromal) group, $F(1,30) = 9.53$, $p = .004$, $\eta_p^2 = .24$ (Figure 6.2a). A similar rmANOVA did not reveal significant Treatment \times Group effects for first fixations on the eyes of happy and neutral faces (again

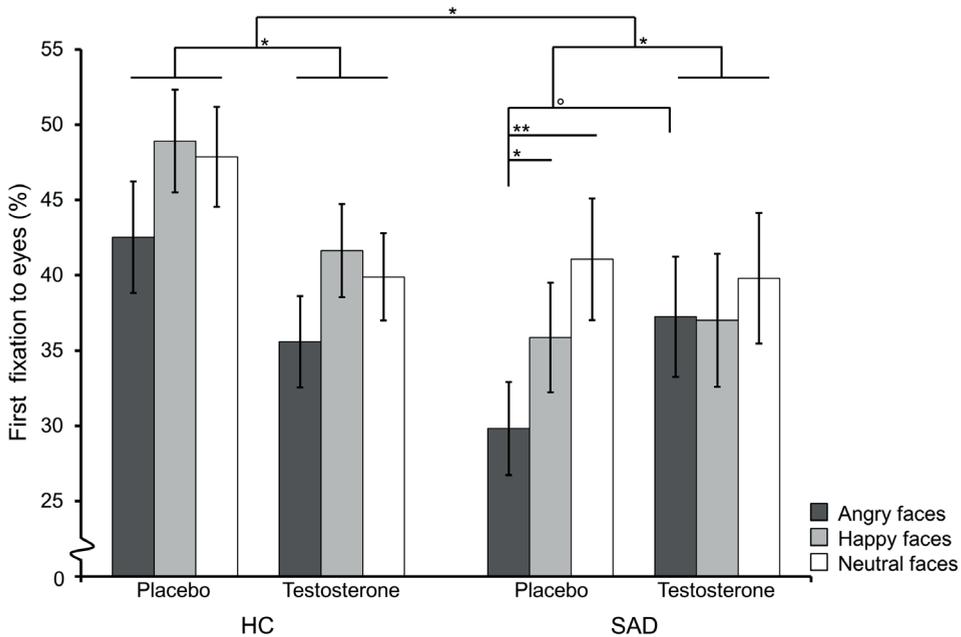


Figure 6.1. First fixation data for angry, happy, and neutral facial expressions. Percentage (mean \pm SEM) first fixations towards the eyes of angry, happy and neutral facial expressions after administration of placebo and testosterone for Healthy Control participants (HC, $n = 19$) and the combined group of participants with syndromal and sub-syndromal Social Anxiety Disorder (SAD combined, $n = 18$). A significant Treatment \times Group interaction indicates that testosterone differentially affects gaze behavior in SAD and HC groups. The Treatment \times Group effect remains significant when tested for angry faces alone but not when tested for happy or neutral faces. ** $p < .01$, * $p < .05$, $\circ p < .06$.

both when including the SAD (combined) group, happy: $F(1,35) = 3.22, p = .085$, neutral: $F(1,35) = 1.61, p = .213$; and when including the SAD (syndromal) group, happy: $F(1,30) = 3.40, p = .075$, neutral: $F(1,30) = 1.63, p = .211$). These findings confirm that testosterone increases the first fixations towards the eye region of angry faces in SAD versus HC.

To further explore the Treatment \times Group effect of the omnibus analysis, we conducted analyses for each group separately. A two-way rmANOVA with Treatment (placebo, testosterone) and Emotion (angry, happy, neutral) for the percentage first fixations in the SAD group revealed a significant Treatment \times Emotion interaction. This counted both for the SAD (combined) group, $F(2,32) = 4.43, p = .020, \eta_p^2 = 0.21$, and for the SAD (syndromal) group, $F(2,24) = 3.56, p = .044, \eta_p^2 = 0.23$. In addition, there was a significant main effect of Emotion in the SAD (combined) group, $F(1.5,25.8) = 5.54, p = .016, \epsilon = .759, \eta_p^2 = .25$, but not in the SAD (syndromal) group: $F(1.4,16.9) = 2.76, p = .105, \epsilon = .702, \eta_p^2 = .19$, nor did this analysis yield a significant main effect of Treatment in either group, all $F_s < 1.28$, all $p_s > .280$. Subsequent one-way rmANOVA's for each emotion separately yielded a trend towards a significant main effect of Treatment for Angry faces, $F(1,17) = 4.09, p = .059, \eta_p^2 = .19$, in the SAD (combined) group. Critically, the Treatment effect of testosterone for

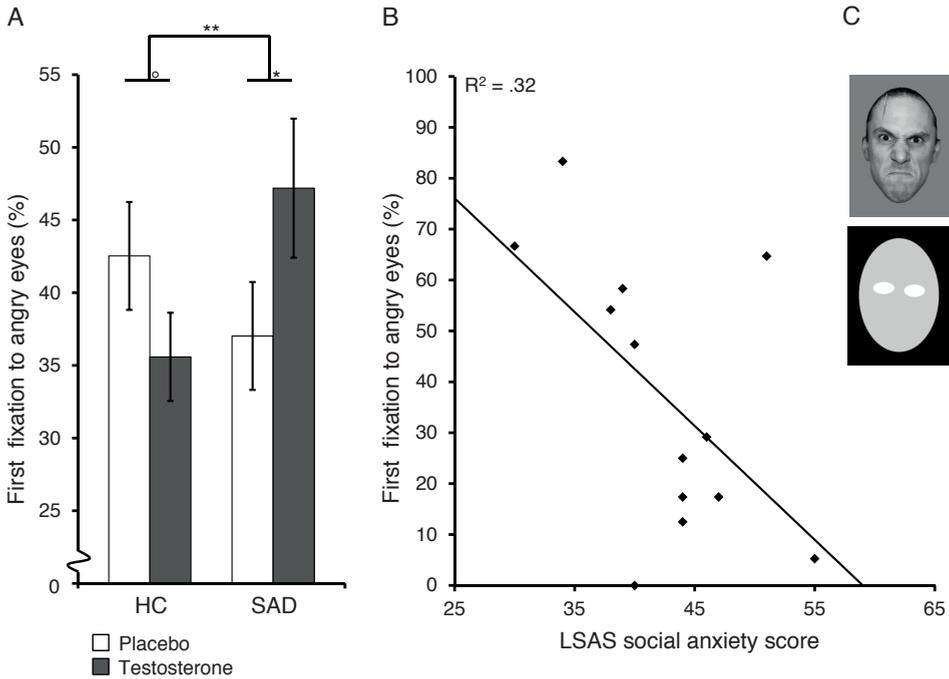


Figure 6.2. First fixation data for angry facial expressions and correlation with social anxiety scores in SAD. **A**) Percentage (mean \pm SEM) first fixations towards the eyes of angry facial expressions after administration of placebo and testosterone for Healthy Control participants (HC, $n = 19$) and participants with generalized Social Anxiety Disorder who met the full DSM-IV criteria on the day of testing (SAD syndromal, $n = 13$). In SAD participants testosterone enhanced the percentage of first fixations to the eyes of angry faces, whereas HCs showed the opposite pattern. $**p < .01$, $*p \leq .05$, $^{\circ}p < .06$. **B**) A significant correlation between percentage first fixations on angry eyes and LSAS social anxiety scores in the placebo condition indicated that SAD participants ($n = 13$) with stronger anxiety symptomatology showed increased gaze avoidance of angry eye contact. **C**) Example of an angry facial expression and corresponding Area of Interest (AOI) map, with the eye AOI depicted in white.

angry faces was significant in the SAD (syndromal) group, $F(1,12) = 4.90$, $p = .047$, $\eta_p^2 = .29$, suggesting that testosterone increased first fixations to the eye-region of angry faces within the SAD patients (Figure 2a). There was no such effect for Happy ($F(1,17) = 0.12$, $p = .729$ (SAD combined); $F(1,12) = 0.39$, $p = .543$ (SAD syndromal)), or Neutral faces ($F(1,17) = 0.15$, $p = .706$ (SAD combined); $F(1,12) = 0.02$, $p = .968$ (SAD syndromal)).

Separate analyses for the SAD participants per condition showed significant avoidance of the eyes of angry faces, when compared to neutral faces in the placebo condition. This Emotion effect was present both in the SAD (combined) group, $F(1,17) = 11.73$, $p = .003$, $\eta_p^2 = .41$, and in the SAD (syndromal) group, $F(1,12) = 7.01$, $p = .021$, $\eta_p^2 = .37$. There was also significant avoidance for angry faces when compared to happy faces, in the SAD (combined) group, $F(1,17) = 6.07$, $p = .025$, $\eta_p^2 = .26$, which was a trend in the SAD (syndromal) group, $F(1,12) = 3.95$, $p = .070$, $\eta_p^2 = .25$. There were no such effects for happy

versus neutral faces, not when including the SAD (combined) group, $F(1,17) = 4.08$, $p = .059$, nor when including the SAD (syndromal) group, $F(1,12) = 2.02$, $p = .180$. These gaze avoidance effects for the eyes of angry faces in the SAD group were no longer present in the testosterone condition, all $F_s < 0.89$, $p_s > .398$. To further explore the nature of the avoidance effects in the placebo condition in the subgroup of participants who met the full DSM-IV criteria for SAD, we tested whether gaze avoidance towards the eye-regions was correlated to the symptom severity. A significant correlation between percentage first fixations on angry eyes and social anxiety scores in the placebo condition, $r = -.561$, $p = .046$, indeed indicated that participants with stronger social anxiety symptomatology showed relatively greater gaze avoidance of angry eye contact (Figure 6.2b). This effect was no longer present after testosterone administration, $r = -.384$, $p = .195$. A similar correlation existed for the first fixations towards the eyes of happy faces, $r = -.575$, $p = .040$, and neutral faces, $r = -.590$, $p = .034$, which became a trend after testosterone administration: happy, $r = -.498$, $p = .083$, and neutral, $r = -.502$, $p = .080$. Interestingly, none of these effects was significant in the SAD (combined) group, all $r_s < -.404$, all $p_s > .097$.

Together these analyses within the SAD group indicate that initial gaze avoidance of eye contact is related to the severity of social anxiety symptomatology in individuals with SAD, and that the avoidance of angry eyes in particular can be alleviated by single dose testosterone administration.

In contrast, in the HC group the two-way rmANOVA with Treatment (placebo, testosterone) and Emotion (angry, happy, neutral) showed no significant Treatment x Emotion interaction, $F(2,36) = 0.03$, $p = .974$. However, a main effect of Treatment, $F(1,18) = 9.06$, $p = .008$, $\eta_p^2 = .34$, indicated a relative decrease in the number of first fixations on the eye-regions of faces after testosterone (Figure 1). A main effect of Emotion, $F(2,36) = 3.60$, $p = .038$, $\eta_p^2 = .17$, showed that the HC, like the SAD group, showed relatively less first fixations to the eye-region of angry faces, relative to happy, $F(1,18) = 4.80$, $p = .042$, $\eta_p^2 = .21$, but not relative to neutral faces, $F(1,18) = 1.88$, $p = .187$. However, avoidance of angry eye-contact in the HC group in the placebo-condition was not related to the level of social anxiety (all $r_s < .328$; all $p_s > .170$), as was observed for the syndromal SAD group.

First fixation duration, percentage total fixations, and total fixation duration

Separate three-way rmANOVAs with Treatment (placebo, testosterone) and Emotion (angry, happy, neutral) as within-subject factors, and Group (HC, SAD) as between-subjects factor, revealed neither Treatment, nor Group effects for first fixation duration, percentage total fixations, or total fixation duration, not when including the SAD (combined) group, all $F_s < 1.53$, $p_s > .225$, nor when including the SAD (syndromal) group, all $F_s < 2.25$, $p_s > .148$ (see Table S6.3 and S6.4). In addition, none of these measurements was significantly related to symptom severity in the SAD (combined) group (all $p_s > .391$), nor the SAD (syndromal) group (all $p_s > .05$).

Checks for confounding factors

No significant relationship between depression symptoms (Beck Depression Inventory score, (Luteijn & Bouman, 1988) and percentage first fixations emerged in the SAD (combined) group, all $r_s < -.230$, $p_s > .358$, nor in the SAD (syndromal) group, all $r_s < -.454$, $p_s > .119$. Finally, the effects of testosterone on the percentage first fixations in the SAD participants remained after controlling for testing order and other possibly confounding variables such as time of testing and use of contraceptives (see Supplemental Information).

Discussion

This study shows that a single dose testosterone administration can alleviate gaze avoidance, which is one of the core communicative features of social anxiety and Social Anxiety Disorder (SAD) in particular (Weeks, Howell, & Goldin, 2013). In accordance with previous research, avoidance of eye contact was correlated to severity of social anxiety symptoms. Critically, we showed that administration of testosterone led to an increase of initial fixations to the eyes of facial stimuli in SAD compared to healthy control (HC) participants. This finding supports theories on the anxiolytic and social dominance enhancing properties of testosterone and extends those models by indicating that the avoidance-reducing effect of testosterone is context dependent and only present in individuals featured by clinical avoidance behavior.

The first eye movement or first fixation has previously been used as a reliable measure of overt attention towards or away from a social threat stimulus (Becker & Detweiler-Bedell, 2009; Gamer et al., 2010; Garner et al., 2006). The latter authors argued that initial orienting to the eyes is relevant for not only adequate social information processing, but also for preparation of approach and avoidance behavior (Bradley, 2009). In our study, participants showed significant avoidance of the eyes of angry faces when compared to happy and neutral facial expressions. It is probable that they quickly detected the threatening eyes of angry faces preceding their first avoiding eye movement upon stimulus onset (Armstrong & Olatunji, 2012; Becker & Detweiler-Bedell, 2009; Garner et al., 2006). This behavior can be defined as gaze avoidance (i.e., the prevention of threatening eye contact by avoiding to directly gaze at the dominant threat), and is in concurrence with the hypervigilance-avoidance theory (Bögels & Mansell, 2004). Most importantly, in our sample we see the clinical relevance of first fixations on the eye-region in patients with SAD confirmed by the correlation between this measure and the severity of anxiety symptoms in the placebo condition: increased social anxiety scores were associated with reduced initial fixations on the eyes of the facial stimuli in patients with SAD and not in HC. This finding concurs with literature that shows that gaze avoidance of eye contact is a

typical feature of SAD, and is related to symptom severity (Clark & Wells, 1995; Moukheiber et al., 2010, 2012; Stein & Stein, 2008). Our finding that testosterone specifically affects the first fixation, and decreases the amount of eye-area avoiding eye movements (by enhancing the amount of first fixations towards the eyes), is in agreement with literature that suggests that testosterone biases the brain towards social dominance by influencing early automatic mechanisms (Bos et al., 2012; Radke et al., 2015; Terburg & van Honk, 2013). Moreover, this finding is in line with earlier findings showing that testosterone influences actual social motivational behavior. In healthy females testosterone promotes relative increase of threat approach action tendencies (Enter et al., 2014) and increases social dominant gaze behavior by prolonging stares into the eyes of angry faces (Terburg et al., 2012). We extend these findings by showing that testosterone administration also leads to a reduction of submissive gaze avoidance of angry eye contact in participants who suffer from social anxiety disorder.

This behavior is likely caused by the influence of testosterone on the neural pathways that mediate automatic motivational tendencies. Testosterone affects the amygdala and connected frontal-striatal circuits (Hermans et al., 2008; Radke et al., 2015), which typically show altered functioning in SAD (Fouche et al., 2013). Interestingly, reduced striatal dopamine (D2) receptor binding is related to subordinate social status in female *Cynomolgus* monkeys (Grant et al., 1998) and similarly, deviating striatal functioning has also been found in SAD (Freitas-Ferrari et al., 2010). It is known that testosterone enhances dopamine levels in the ventral striatum (de Souza Silva et al., 2009), which in turn can lead to increased reward sensitivity and augmented motivational behavior (Cools, 2008; Enter, Colzato, & Roelofs, 2012). Interestingly, and in line with the current findings in SAD, effects of testosterone administration on striatal functioning have previously been found to be particularly pronounced in individuals who are scoring low on reward-seeking behavior (Hermans et al., 2010). This may suggest that the dominance enhancing effect of testosterone in the current study is related to increased reward sensitivity. An increase in eye-contact may lead to an encounter with a possibly rewarding outcome, such as increased social status and its benefits. Indeed, it has been shown that the reward value of a stimulus has an effect on eye-movements: humans automatically orient their eyes towards potentially rewarding stimuli (Hickey, Chelazzi, & Theeuwes, 2010; Hickey & van Zoest, 2012). Hence, it could be that testosterone increases the reward-sensitivity of the socially submissive individual, which leads to augmented behavioral responses towards a potentially rewarding stimulus; in this case in increased first fixations to the eyes of facial stimuli (Bos et al., 2012; van Honk et al., 2004). In addition, the behavioral effects of testosterone may also be influenced by anxiolytic processes via glucocorticoid pathways, steroid receptors, and gamma-aminobutyric acid (GABA) receptors (e.g., Terburg & van Honk, 2013). Future studies combining testosterone administration, eye tracking and neuroimaging techniques should elucidate the neuroendocrine mechanisms in the

gaze enhancing effect of testosterone in SAD and the extent to which such effects could reflect or are mediated by anxiolytic processes.

In contrast to the SAD group, the healthy controls showed a tendency towards diminished first fixations on the eye-region of facial expressions. This finding is consistent with earlier studies showing a testosterone-induced reduction in social cognition: after testosterone administration healthy female participants showed less facial mimicry - which is a measure of empathy - of angry and happy facial expressions (Hermans, Putman, & van Honk, 2006), reduced conscious recognition of angry faces (van Honk & Schutter, 2007), and decreased performance on the Reading the Mind in the Eye Task (RMET), which assesses the ability to infer the mental state of another by reading subtle cues expressed by the eyes (van Honk, Schutter, et al., 2011). In addition, reduced eye-contact has been associated with a decline in empathy (Cowan, Vanman, & Nielsen, 2014). Together, these findings concur with the notion that testosterone promotes approach motivation in order to facilitate self-oriented dominance seeking behavior (Bos et al., 2012).

The differential findings for patients and controls are in line with literature that suggests that social context determines how testosterone affects social status (i.e., social reward) promoting behavior (Eisenegger et al., 2011; Terburg & van Honk, 2013; van Honk, Terburg, & Bos, 2011). Both the social environment and individual differences have been shown to influence the modulatory effects of testosterone (Mehta & Josephs, 2010). Concerning the latter, van Honk and colleagues (van Honk, Schutter, et al., 2011) found that the quantity of prenatal testosterone exposure predicted the effects of administered testosterone on cognitive empathic ability in adult participants. Similarly, social anxiety seemed to modulate the effects of both endogenous and exogenous testosterone on behavioral responses towards social dominance threat (Enter et al., 2014; Maner et al., 2008). Therefore, it is not curious that our study, which entails two disparate groups of participants, finds differential effects of testosterone on gaze behavior towards the eye-region of emotional faces. Future research should address the mechanisms underlying the differential effects of testosterone on gaze behavior in healthy controls and participants with SAD.

A few interpretational issues should be discussed with regard to the present findings. First, one might perceive the result in the healthy controls (i.e., a tendency towards diminished first fixations towards angry eyes) to be in conflict with a study by Terburg and colleagues (2012), who showed that testosterone administration in healthy females prolongs dominant staring into the eyes of angry faces. However, the two studies probably captured two different types of subordinate gaze behavior (Terburg, Aarts, & van Honk, 2012b), namely gaze aversion (i.e., rapid breaking of eye-contact with a more dominant conspecific, and thus signaling submission and ending the threatening encounter; (Terburg et al., 2012), versus gaze avoidance (i.e., the prevention of threatening eye contact by avoiding to directly gaze at the dominant threat in the first place) in the present study.

Crucially, we show that this type of subordinate gaze behavior is also affected by testosterone administration; hence presenting the first evidence that testosterone diminishes not only submissive gaze *aversion* in healthy participants (Terburg et al., 2012), but also submissive gaze *avoidance* in socially anxious individuals. In addition, we show that the effect of testosterone on gaze avoidance is context dependent and only present in individuals featured by clinical avoidance behavior. Second, our findings were specific for the first fixations, whereas the gaze variables during the total stimulus presentation time were not affected by testosterone. This finding concurs with literature that suggests that testosterone biases the brain towards social dominance by influencing automatic processes (e.g., Terburg & van Honk, 2013). In addition, this early automatic measure is probably less vulnerable to other influences, such as effects of top-down control, fatigue, and repetition than the later measures. More research is required to test the specificity of the effects of testosterone for initial gaze behavior. Third, in contrast to our hypothesis the gaze avoidance alleviating effect of testosterone was not specific for angry faces. Nevertheless, although the Treatment x Group effect did not interact with Emotion, separate analyses revealed that the Treatment x Group effect was only significant for angry faces and did not hold when testing this effect for happy or neutral faces in isolation. These results show that the alleviating effect of testosterone on gaze avoidance in SAD patients compared to healthy controls also holds for angry faces, although this effect is not specific for angry faces. Fourth, we found no group-effect in initial gaze-avoidance in the placebo condition (see also Moukheiber et al., 2010, 2012). However, avoidance for angry versus neutral and happy faces was found across groups and replicated in the SAD group. Most interestingly, gaze-avoidance in the placebo condition was significantly associated with social anxiety levels in SAD patients but not in healthy participants. These findings suggest that gaze avoidance was uniquely related to the symptom severity in SAD patients and future research using larger groups is needed to determine whether initial gaze avoidance is a specific trait for SAD. Finally, only female participants were tested in this study, which was a consequence of the testosterone administration method (Tuiten et al., 2000). Future research should not only replicate these results in larger female samples, but also should investigate whether testosterone administration to men has similar effects, as suggested by similarities in social behavior related to endogenous and exogenous testosterone across sexes (Goetz et al., 2014; Hermans et al., 2008). In addition, as is customary with this testosterone administration paradigm, we aimed to control for steroid hormone level fluctuations associated with the menstrual cycle by including women on single-phase contraceptives, and naturally cycling women who were tested in the preovulatory phase (e.g., Hermans et al., 2010; van Honk, Schutter, et al., 2011). Although the effects of testosterone on first fixations remained while statistically controlling for contraceptive use, future studies ideally control for hormone fluctuations by only testing participants who are on single-phase contraceptives, or by directly assess-

ing estradiol levels, especially given the evidence that this hormone possibly mediates testosterone-effects on social dominance (Terburg & van Honk, 2013; Ziomkiewicz, Wichary, Gomula, & Pawlowski, 2015).

In terms of clinical implications, the present results may encourage further investigation of whether testosterone could have clinical utility in the treatment of SAD. Forty to 50% of patients does not benefit from current evidence based psychological and pharmacological treatments (Hofmann & Bögels, 2006; Stein & Stein, 2008), and improving therapy efficacy by pharmacological enhancement seems a promising new venue (Hofmann et al., 2014; Singewald et al., 2015). Testosterone acts on the social motivational system and enhances social approach motivation while reducing social fear and avoidance in a socially challenging environment (Enter et al., 2014). Thus, it may be worthwhile to further explore whether testosterone can act as an adjunct in exposure therapies, where boosting prosocial behavior in the first few sessions is essential for therapy outcome. Nevertheless, it should be noted that there is still much unclear about the working mechanisms of testosterone and of pharmacological add-ons for exposure-based therapy. It is possible that, besides the potentially beneficial effects of testosterone on dopamine transmission, its effects on the GABA system might not only work anxiolytic but could also potentially interfere with extinction learning, something worth investigating in future research (Singewald et al., 2015).

In conclusion, a single dose administration of testosterone alleviates gaze avoidance by increasing initial gaze towards the eye-region of facial expressions in participants with Social Anxiety Disorder. These findings support the role of testosterone in dominance-enhancing behavior and suggest need for study of testosterone administration as a means to enhance therapy efficacy in Social Anxiety Disorder.

Supplemental Information

Supplemental methods

Table S6.1. Additional characteristics for the Healthy Control (HC) group and the combined group of participants with syndromal and sub-syndromal Social Anxiety Disorder (SAD).

	HC (n = 19)	SAD (n = 18)	t(35)	p-Value
SPAI social phobia	47.6 (26.5)	120.4 (22.7)	-8.96	<.001
SPAI agoraphobia	10.9 (10.1)	23.6 (10.8)	-3.67	.001
SPAI difference	36.6 (22.1)	96.9 (19.9)	-8.68	<.001

Data are presented in mean and standard deviation. Abbreviations: SPAI, Social Phobia and Anxiety Inventory.

Table S6.2. Characteristics for the Healthy Control (HC) and the group with syndromal Social Anxiety Disorder (SAD).

	HC (n = 19)	SAD (n = 13)	t(30)	p-Value
Age (years)	25.2 (4.0)	23.0 (4.7)	1.34	.162
LSAS anxiety	9.5 (7.2)	42.7 (6.8)	-13.10	<.001
LSAS avoidance	7.8 (6.3)	35.6 (8.5)	-10.65	<.001
LSAS total	17.3 (12.9)	78.3 (14.3)	-12.53	<.001
SPAI social phobia	47.6 (26.5)	128.2 (20.0)	-9.29	<.001
SPAI agoraphobia	10.9 (10.1)	26.8 (10.1)	-4.34	<.001
SPAI difference	36.6 (22.1)	101.4 (20.0)	-8.45	<.001
BDI	2.5 (2.2)	18.5 (12.3)	-4.66	<.001

Data are presented in mean and standard deviation. Abbreviations: LSAS, Liebowitz Social Anxiety Scale; SPAI, Social Phobia and Anxiety Inventory; BDI, Beck Depression Inventory.

Supplemental results

Checks for confounding factors

To check whether the effects of testosterone on the percentage first fixations was affected by testing order or by time of testing (i.e., testing session starting at either 930h or 1330h), we added these variables as between-subjects factors in separate analyses to the three-way rmANOVA with Treatment (placebo, testosterone) and Emotion (angry, happy, neutral) as within-subject factors, and Group (HC, SAD) as between-subjects factor. This analysis showed that the critical Treatment x Group interaction remained significant in both cases: when including the SAD (combined) group (Order: $F(1,34) = 7.65$, $p = .009$, $\eta_p^2 = .184$, and Time: $F(1,34) = 6.33$, $p = .017$, $\eta_p^2 = .157$), and when including the SAD (syndromal) group (Order: $F(1,29) = 8.67$, $p = .006$, $\eta_p^2 = .230$, and Time: $F(1,29) = 7.00$, $p =$

.013, $\eta_p^2 = .194$). Furthermore, to rule out the possibility that fluctuating hormone levels over the menstrual cycle would account for the observed effects, we checked whether the Treatment x Group interaction remained in the subgroup using single-phase contraceptives, which was the case, both when including the SAD (combined) group ($F(1,24) = 5.76, p = .025, \eta_p^2 = .193$) and when including the SAD (syndromal) group ($F(1,19) = 7.85, p = .011, \eta_p^2 = .292$).

Table S6.3. First fixation duration ($ms \pm SEM$) on the eyes and offset time of first fixations ($ms \pm SEM$) for angry, happy, and neutral facial expressions after administration of placebo and testosterone for Healthy Control participants (HC, $n = 19$) and the combined group of participants with syndromal and sub-syndromal Social Anxiety Disorder (SAD, $n = 18$).

Emotion	HC		SAD	
	Placebo	Testosterone	Placebo	Testosterone
<i>First fixation duration</i>				
Angry	364 (36)	330 (24)	324 (26)	306 (33)
Happy	436 (62)	376 (31)	377 (39)	325 (21)
Neutral	379 (36)	360 (30)	365 (27)	322 (27)
<i>First fixation offset time</i>				
Angry	323 (14)	323 (14)	330 (15)	312 (16)
Happy	326 (15)	336 (14)	336 (16)	312 (16)
Neutral	325 (15)	332 (12)	341 (16)	312 (15)

Table S6.4. Percentage fixations ($percentage \pm SEM$) on the eyes and duration of fixations ($ms \pm SEM$) on the eyes for the total stimulus presentation time for angry, happy, and neutral facial expressions after administration of placebo and testosterone for Healthy Control participants (HC, $n = 19$) and the combined group of participants with syndromal and sub-syndromal Social Anxiety Disorder (SAD, $n = 18$).

Emotion	HC		SAD	
	Placebo	Testosterone	Placebo	Testosterone
<i>% fixations</i>				
Angry	32 (3)	31 (3)	27 (4)	27 (4)
Happy	35 (4)	34 (3)	30 (4)	29 (4)
Neutral	37 (3)	36 (3)	32 (4)	31 (4)
<i>Fixation duration</i>				
Angry	360 (19)	360 (20)	350 (16)	327 (18)
Happy	358 (20)	367 (20)	361 (19)	338 (17)
Neutral	361 (23)	362 (16)	357 (17)	336 (19)

Table S6.5. Total number of fixations (count \pm SEM) and total dwell time (ms \pm SEM) for the total stimulus presentation time for angry, happy, and neutral facial expressions after administration of placebo and testosterone for Healthy Control participants (HC, $n = 19$) and the combined group of participants with syndromal and sub-syndromal Social Anxiety Disorder (SAD, $n = 18$).

Emotion	HC		SAD	
	Placebo	Testosterone	Placebo	Testosterone
<i>Total number of fixations</i>				
Angry	261 (17)	276 (13)	270 (12)	279 (17)
Happy	262 (14)	265 (14)	163 (13)	281 (19)
Neutral	265 (14)	267 (14)	265 (13)	281 (17)
<i>Total dwell time</i>				
Angry	82 (5)	88 (3)	86 (4)	85 (5)
Happy	84 (5)	87 (4)	85 (4)	86 (5)
Neutral	84 (4)	86 (4)	87 (3)	86 (5)



Exogenous Testosterone Affects Early Threat Processing in Socially Anxious and Healthy Women

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Abstract

Testosterone plays an important role in social threat processing. Recent evidence suggests that testosterone administration has socially anxiolytic effects, but it remains unknown whether this involves early automatic or later, more controlled, processing-stages. We investigated the acute effects of testosterone administration on social threat processing in 19 female patients with Social Anxiety Disorder (SAD) and 19 healthy controls. Event-related potentials (ERPs) were recorded during an emotional Stroop task with subliminally presented faces. Testosterone induced qualitative changes in early ERPs (<200 ms after stimulus onset) in both groups. An initial testosterone-induced spatial shift reflected a change in the basic processing (N170/VPP) of neutral faces, which was followed by a shift for angry faces suggesting a decrease in early threat vigilance. These findings suggest that testosterone specifically affects early automatic social information processing. The decreased vigilance for angry faces explains how testosterone can decrease threat avoidance, which is particularly relevant for SAD.

Introduction

Testosterone has an important role in the regulation of social motivational behavior. A surge in testosterone has anxiolytic effects and facilitates social dominance and approach behavior in socially challenging situations (Archer, 2006; Bos et al., 2012; Terburg & Van Honk, 2013). Especially an angry looking face with direct gaze is perceived as a signal of social threat, as it can signal an impending aggressive encounter (Öhman, 1986). In line with this notion, recent single dose administration studies show that testosterone promotes approach action tendencies to angry faces on a social approach-avoidance task (Enter, Spinhoven, & Roelofs, 2014, 2016). In addition it facilitated socially dominant gaze behavior as indicated by increased fixation to the eyes of angry faces (Enter et al., 2016; Terburg et al., 2016; Terburg et al., 2012).

At the neural level, testosterone has been found to enhance the reactivity of the amygdala towards angry facial expressions (Goetz et al., 2014; Hermans et al., 2008), and to reduce its connectivity with circuits involving the orbitofrontal or prefrontal cortex, thalamus, brainstem, and striatum (van Wingen et al., 2010; Volman, Toni et al., 2011). However, using a social approach-avoidance task, in which angry and happy faces have to be approached or avoided by pulling or pushing a joystick, Radke and colleagues (2015) showed that testosterone *increased* amygdala responses specifically during *approach* of angry faces, but *decreased* amygdala responses during angry face *avoidance*, suggesting that testosterone modulates social threat processing in a motivation-specific manner. However, little is known about the temporal dynamics of these effects, and it remains unknown whether they involve early automatic or later, more controlled, stages of social threat processing. Gaining insight into these processes would be of particular interest for Social Anxiety Disorder (SAD), as this frequent and persistent disorder is characterized by increased early automatic vigilance and biased goal-directed processing of social threat (for reviews see e.g., Bar-Haim, Lamy, Pergamin, Bakermans-Kranenburg, & van IJzendoorn, 2007; Gilboa-Schechtman & Shachar-Lavie, 2013; Staugaard, 2010) as well as by decreased salivary levels of basal testosterone (Giltay et al., 2012).

In the current study we therefore investigate the effects of testosterone on social threat processing in participants with SAD and healthy participants, using temporally fine-grained recordings of the event-related brain potentials (ERPs) during an emotional Stroop task with subliminally presented angry, happy and neutral faces. We performed a spatiotemporal clustering analysis (Brunet, Murray, & Michel, 2011; Murray, Brunet, & Michel, 2008) on the ERPs, as this method has several advantages compared to conventional ERP amplitude analyses. Most importantly, it can tease apart the following two ERP effects: 1) topographic modulations, which reflect a change in neural sources, indicating the activation of different cognitive processes (a *qualitative* change in processing), and 2) amplitude modulations, which, in absence of a concurrent topographic modulation,

reflect increases or decreases in response strength of a common cognitive process (a *quantitative* change in processing) (see e.g., Murray et al., 2008; Pourtois, Delplanque, Michel, & Vuilleumier, 2008). Furthermore, this method may be more sensitive in detecting differences between groups or task conditions (Murray et al., 2008), as it includes the full range (instead of only a limited number) of channels and time windows, and it can detect topographic changes even when amplitude is low. Particularly for pharmacological interventions like testosterone, which modulates multiple parts of the emotion circuitry (see e.g., Bos et al., 2012; van Wingen et al., 2010), effects are unlikely to be bound to single ERP peaks.

Based on previous EEG studies showing increased amplitudes for subliminally presented angry (compared to neutral) faces especially on early ERP components such as the frontocentral P2 or VPP, the N2, and the EPN (Balconi & Lucchiari, 2005, 2007; van Peer et al., 2010) we expected increased processing of angry faces in the early (<250 ms) time window. Some studies with supraliminal stimuli suggest that this effect may be amplified in socially anxious compared to non-anxious participants, but overall electrophysiological evidence for hypervigilance to social threat in social anxiety is still inconsistent (see Schulz, Mothes-Lasch, & Straube, 2013 for a review).

Most importantly, based on the social-anxiolytic and approach-promoting effects of testosterone (Archer, 2006; Terburg & Van Honk, 2013) we expected that testosterone administration compared to placebo would reduce processing of angry versus neutral and happy faces, particularly in SAD patients who are characterized by a social threat bias as well as lower endogenous testosterone levels. Finally, based on behavioral findings suggesting that effects of testosterone are most pronounced for preconscious processing of threat (Van Honk et al., 2000) we hypothesized that these effects may be predominantly manifested in the early automatic processing stages.

Methods

Participants

Participant characteristics are presented in Table 7.1. Participants for the Social Anxiety Disorder (SAD) group were recruited from outpatient anxiety departments of mental health centers, through advertisements on the internet, and in local newspapers. Inclusion criterion was a total score of > 60 on the Liebowitz Social Anxiety Scale (Liebowitz, 1987; Rytwinski et al., 2009). In addition these participants were screened with the Mini International Neuropsychiatric Interview script (M.I.N.I.; Lecubier et al., 1997) to verify the DSM-IV diagnosis of generalized Social Anxiety Disorder. One participant (LSAS score 56) scored just below the LSAS cutoff but was included as she did fulfill the DSM-IV diagnostic criteria. Healthy control (HC) participants were recruited via advertisements in community

centers, on the internet, and in local newspapers. Only female participants were included, because the parameters (e.g., dose and time course) for inducing neurophysiological effects in men with a single dose administration of testosterone are as yet unknown (Tuiten et al., 2000). Both women using single-phase contraceptives and normally cycling women participated in the study. All participants had normal or corrected-to-normal vision. Exclusion criteria were age < 18 and > 50, use of (psychotropic) medication, somatic illnesses, neurological conditions, recent or past psychiatric problems (HC only), psychotic disorder, history of head injury, left-handedness, peri- or postmenopause, and pregnancy or breast feeding. Initially, 24 participants were included in each group. However, as these groups differed significantly in age ($F(1,46) = 12.59, p = .001, \eta_p^2 = 0.21$), and there is no appropriate method to statistically control for such an effect in the analyses (Miller & Chapman, 2001), a subset of 19 SAD and 19 HC participants (age $F(1,36) = 2.78, p = .104$, see table 7.1) was selected on basis of matching for age (Field, 2009; see also Enter, Terburg, et al., 2016). Thirteen of the 19 SAD participants met full DSM-IV criteria for generalized SAD at the time of testing; the other five had sub-syndromal SAD (i.e., they fulfilled all criteria at the telephone screening but the symptoms did no longer lead to significant burden in social or occupational functioning [DSM-IV criterion IV] at time of testing). All participants provided written informed consent, and received financial compensation. The study was approved by the Medical Ethics Committee of the Leiden University Medical Centre, and was in accordance with the declaration of Helsinki.

Table 7.1. *Group Characteristics.*

Variable	HC ($n = 19$)	SAD ($n = 19$)	<i>p</i> -Value
Order (testosterone first)	$n = 8$	$n = 11$.330
Age	25.3 (4.1)	23.0 (4.5)	.104
LSAS social anxiety	9.4 (7.1)	43.2 (6.7)	<.001
LSAS avoidance	7.7 (6.2)	37.0 (7.8)	<.001
LSAS total	17.1 (12.8)	80.2 (13.5)	<.001
SPAI social phobia	47.6 (26.6)	122.6 (23.4)	<.001
SPAI agoraphobia	10.9 (10.1)	23.5 (10.2)	.001
SPAI difference	36.7 (22.2)	99.2 (21.5)	<.001
BDI	2.5 (2.2)	14.7 (11.9)	<.001

Note. Data are presented in mean and standard deviation. Abbreviations: HC, Healthy Controls; SAD, Social Anxiety Disorder; LSAS, Liebowitz Social Anxiety Scale; SPAI, Social Phobia and Anxiety Inventory; BDI, Beck Depression Inventory. *P*-values indicate group differences.

Testosterone Administration

In a double-blind, randomized, placebo-controlled, cross-over design participants received a single dose of 0.5 mg testosterone suspended in a clear solution (0.5 ml) with

0.5 mg hydroxypropyl-beta-cyclodextrin, 0.005 ml ethanol 96%, and distilled water. The matched placebo contained the same ingredients, except for the testosterone. Participants were asked to hold the liquid under their tongue for 60 s. The order of placebo and testosterone administration was counterbalanced across subjects and groups (see Table 1). In females, this dose yields a sharp increase of 20–25 nmol/l in plasma testosterone levels within 15 min, which declines to baseline levels within the next 90 min (van Rooij et al., 2012). Previous research applying this procedure (see Bos et al., 2012 for a review; Eisenegger, Naef, Snozzi, Heinrichs, & Fehr, 2010; Enter et al., 2014; Enter, Spinhoven, et al., 2016; Enter, Terburg, et al., 2016; Tuiten et al., 2000) has convincingly shown consistent psychophysiological and behavioral effects approximately 4–6 h after administration, therefore this time interval was also applied in the current study.

Procedure

Participants were tested individually at two identical testing sessions with two days in between. Testing sessions started at either 09:30 or 13:30 h, on the same time at both sessions. Five hours after testosterone or placebo intake, participants were seated in a dimly lit and sound attenuated room, where they performed the Emotional Stroop Task while EEG was recorded simultaneously. Between testosterone or placebo intake and the Emotional Stroop task, participants had a two hour resting period in a private recreation room (without television or internet access) where they could read and rest, followed by a standard lunch and several unrelated tasks of which the results will be reported elsewhere (i.a., Enter et al., 2014; Enter, Spinhoven, et al., 2016; Enter, Terburg, et al., 2016).

Emotional Stroop Task

Face stimuli were selected from the Pictures of Facial Affect (Ekman & Friesen, 1976) and the Karolinska Directed Emotional Faces (Lundqvist et al., 1998) databases. Angry, happy, and neutral facial expressions were taken from the same model (four male and four female models), cut out in an oval shape to remove distracting features, gray-scaled, and presented with a red, green or blue filter on a black background. Masking stimuli consisted of oval configurations of randomly cut and reassembled fragments of face stimuli (Van Honk et al., 1998). The total stimulus set consisted of 72 target face stimuli (8 actors x 3 expressions x 3 colors) and 6 masks (2 versions x 3 colors) (see also van Peer et al., 2010b). Stimulus presentation and response logging were controlled using E-prime software, a Serial Response Box (Psychology Software Tools, inc.) and a custom-made manual response box.

Participants started with a practice block of nine trials in which only masks were presented. Next, they completed the 72 randomized trials. Each trial started with a 750 ms fixation cross, followed by a very brief (16.7 ms, 2 frames at 120 Hz) exposure to a target face, which was replaced by a mask of the same color. Participants were instructed

to categorize this color as fast as possible by pressing the corresponding button, and their response triggered offset of the masks. New trials started after a random inter-trial interval of 2-4 s.

Incorrect responses were excluded from the analyses. Reaction time (RT) outliers were filtered using a < 200 and > 1300 ms cut-off, and subsequently all RTs exceeding 2.5 SD from the individual participants' mean were removed. These trials were also excluded from the EEG analyses. Of the remaining latencies (HC: 94%, SAD: 95%), the means were calculated per group and condition and log-transformed because of a skewed distribution.

To determine whether participants were capable of consciously perceiving the masked facial expressions, they were asked to complete an awareness check at the end of the second testing session (see Supplemental Information).

Electrophysiological Recording and Analyses

The EEG was recorded at 512 Hz with an Active-Two system (BioSemi, Amsterdam, The Netherlands) from 32 active electrodes referenced to an active common mode sense and with a passive driven right leg ground electrode. All electrodes were mounted in an elastic cap and distributed over the head surface according to the international extended 10–20 system. Horizontal and vertical EOGs were recorded using four bipolar electrodes placed on the outer canthi of the eyes and in the inferior and superior areas of the left orbit. Signals were processed offline using Brain Vision Analyzer software (Version 2.1). Bad EEG channels were interpolated using a topographic interpolation (maximum three channels for each individual data set, $M = 0.29$, $SD = 0.57$). Subsequently, EEG data were re-referenced to an average reference, filtered with a 0.1-Hz high-pass filter (24 dB/oct), and epoched from 200 ms before until 800 ms after stimulus onset. After baseline correction on the pre-stimulus interval, data were corrected for the effects of eye blinks and eye movements using a standard procedure (Gratton, Coles, & Donchin, 1983), and epochs containing artifacts (amplitude values > 100 or < -100 μV , a difference of 150 μV between the lowest and the highest amplitude within 200 ms, a difference > 75 μV between two subsequent sampling points, or a period of 100 ms with activity < 0.50 μV), were removed. Finally, data were averaged to individual ERPs for each facial expression type (happy, angry, and neutral, $M = 21.5$, $SD = 1.7$ trials per category), excluding trials with incorrect responses or outlier RTs (see RT analyses in section 2.4). See Supplemental Information for an overview of the number of remaining trials and grand average ERPs per group and condition. The data of two participants (one HC and one SAD) were excluded because of an excessive number of artifacts (< 15 trials left in one or more conditions), resulting in 36 participants (18 HC, 18 SAD) for the statistical analyses.

Spatiotemporal Clustering Spatiotemporal clustering analysis was performed using the Cartool software by Denis Brunet (version 3.53, brainmapping.unige.ch/cartool,

Brunet et al., 2011) to identify dominant topographic maps of the scalp electric field in the grand-averaged ERP data, and to compare the expression of these maps over time and across groups and experimental conditions. Each topographic map, which usually remains stable for several tens of milliseconds, has been proposed to reflect a period of coherent synchronized activation of large-scale neural networks (functional microstate, see e.g., Lehmann, 1987). Different topographic maps reflect the activation of different neural networks or microstates (e.g., Michel, Seeck, & Landis, 1999), and the typical finding of a sequence of different maps is suggested to represent successive information processing steps (see e.g., Lehmann, 1987). Segmentation of the post-stimulus time window was performed using the Topographic - Atomize and Agglomerate Hierarchical Clustering (T-AAHC) procedure, with rejection of segments smaller than 4 time frames (~ 8 ms) and merging of clusters that correlated above 0.92. The optimal spatiotemporal solution explaining the whole data set was determined by using an objective cross-validation (CV) and modified Krzanowski-Lai (KL) criterion (Pascual-Marqui, Michel, & Lehmann, 1995). The resulting dominant topographic maps (see Figure 1) were fitted back to the individual average ERPs, using a noncompetitive spatial fitting procedure with rejection of segments < 4 time frames. This procedure provides a quantitative value (the global explained variance [GEV], reflecting the goodness of fit) for the representation of each map across participants and conditions. The maps were fitted within four different time intervals, based on the conventional time windows of the corresponding ERP components (80-120 ms [P1, Map #2]; 120-200 ms [N170/VPP, Map #3 and #4]; 180-300 ms [P2, Map #5 and #6]; 275-800 ms [P3/LPP, Map#7 to #10]). The first map (Map #1, 0-80 ms) was excluded from the analyses, as the corresponding ERP component (C1) is known to be exogenous and pre-attentive (Pratt, 2011).

Global Field Power Changes in neural response strength were determined by calculating the global field power (GFP) with Cartool (Brunet et al., 2011). GFP is equivalent to the standard deviation of the scalp electric field, with large values corresponding to moments of high synchronized neural activity (e.g., Lehmann, 1987). For each participant and condition, mean GFP was calculated in three time intervals that were symmetrically centered around the peaks in the grand average (see Figure 3): 80-120 ms, 125-185 ms, and 215-275 ms. As for the spatiotemporal results, the first peak (~70 ms), corresponding to the C1 component, was excluded from the analyses.

Statistical Analyses

All data were analyzed with the Statistical Package for the Social Sciences (SPSS 21) using repeated-measures analyses of variance (ANOVA) with Treatment (placebo, testosterone) and Valence (angry, happy, neutral) as within-subject factors, and Group (HC, SAD) as between-subjects factor. Separate ANOVAs were performed on each time interval of the spatiotemporal clustering and GFP data. The spatiotemporal clustering

analyses included the additional factor Map in case more than one map was present in the respective time interval. Significant interactions were followed by tests of simple effects with *rmANOVA*'s at each level of the relevant factors, to determine the nature of the interaction. Finally, several control analyses were conducted, first to check that the findings were not influenced by awareness of the subliminal stimuli, and second to check for the influence of possible confounding factors such as order of treatment, time of testing, or use of contraception. The results of these analyses are reported in the Supplemental Information, and did not differ notably from the results reported below. All statistical analyses used a two-tailed alpha of .05. Effect sizes of significant results are reported as the proportion of explained variance (partial eta squared [η_p^2]).

Results

Behavioral Results

The response latencies for each group and condition are presented in Table 7.2. The statistical analysis revealed a significant main effect of Valence, $F(2,72) = 3.34$, $p = .041$, $\eta_p^2 = .09$. Post hoc pairwise comparisons indicated that, as expected, the response latencies were significantly slower for angry, $F(1,36) = 4.64$, $p = .038$, $\eta_p^2 = 0.11$, and happy, $F(1,36) = 4.90$, $p = .033$, $\eta_p^2 = 0.12$, compared to neutral faces, suggesting an interference effect for emotional faces. Response latencies for happy and angry faces did not differ significantly, $F(1,36) = 0.12$, $p = .73$. In contrast to the EEG findings, the behavioral results showed no significant effects of Treatment or Group (all $ps > .05$).

Table 7.2. Mean (*ms* ± *SEM*) Color Naming Latencies.

Valence	HC (<i>n</i> = 19)		SAD (<i>n</i> = 19)	
	Placebo	Testosterone	Placebo	Testosterone
Angry	458 (14)	451 (15)	453 (14)	442 (15)
Happy	461 (15)	457 (16)	448 (15)	447 (16)
Neutral	451 (13)	435 (14)	448 (13)	437 (14)

Spatiotemporal Clustering

The spatiotemporal clustering procedure revealed ten distinct dominant field topographies (maps) that together explained 92% of the total ERP variance (see Figure 7.1A and B). Results of the subsequent spatial fitting procedure, reflecting the representation of these maps (in terms of global explained variance, GEV) across participants, conditions, and time, are reported below. We report only statistical results including interactions of Treatment or Group with Valence and Map, as these reflect the effects of interest of the

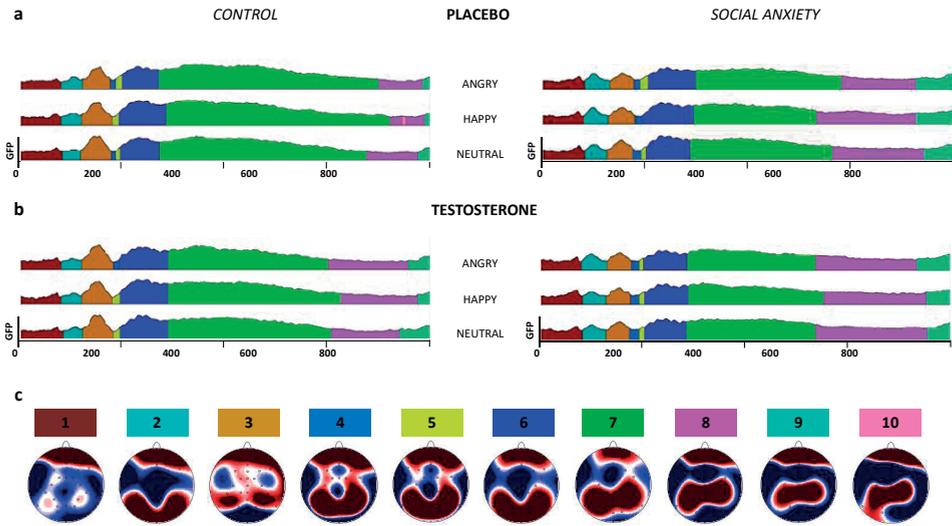


Figure 7.1. Results of the T-AAHC Segmentation Procedure. **a)** and **b)** The sequence of 10 distinct topography solutions are shown under GFP curves for both groups (HC left, SAD right) and all experimental conditions. Each color (and number) labels a period of distinct and stable electric field topography. Time 0 indicates onset of the face stimulus (16.7 ms) followed by a mask. **c)** Topographical properties of the 10 maps, with colors and numbers corresponding to the segmentation in panel a and b. Maps are oriented with the nasion upward and left scalp leftward. Blue denotes negative and red positive scalp potentials. Of note: The timing and topography of these maps suggest they correspond to the C1 (map #1, <80 ms), P1 (map #2, 80-120 ms), N170/VPP (map #3 and #4, 120-200 ms), occipital P2 (map #5 and #6, 180-300 ms), and P3/LPP (maps #7-10, 275-800 ms) ERP components, respectively.

current study: Testosterone- or SAD-related topographic (i.e., qualitative) differences in emotion-related face processing. See Supplemental Information for additional results.

Effects of testosterone administration. In line with the hypothesis that testosterone affects early processing of emotional faces, the rmANOVA of GEV in the N170/VPP time interval (120-200 ms post-stimulus, Maps #3 and #4) showed a significant interaction of Treatment x Valence x Map, $F(2,68) = 5.87, p = .004, \eta_p^2 = 0.147$ (see Figure 2). Follow-up analyses (i.e., rmANOVA Group x Treatment x Valence per map) revealed that the Treatment x Valence interaction was significant for both maps (Map #3, $F(2,68) = 3.61, p = .032, \eta_p^2 = .096$; Map #4, $F(2,68) = 4.09, p = .021, \eta_p^2 = .107$). The first map (Map #3) reflects the typical spatiotemporal pattern of the N170/VPP component (see Figure 1C). Follow-up tests per Valence for this map showed a significant effect of Treatment, reflecting a decrease in GEV after testosterone administration, compared to placebo, for neutral faces, $F(1,34) = 6.84, p = .013, \eta_p^2 = 0.167$, and a trend in the same direction for happy faces, $F(1,34) = 3.91, p = .056, \eta_p^2 = .103$, but not for angry faces, $F(1,34) = 3.15, p = .079, \eta_p^2 = .089$. This finding suggests that testosterone administration resulted in a reduction of the representation (goodness of fit) of the N170/VPP pattern during neutral (and happy) but

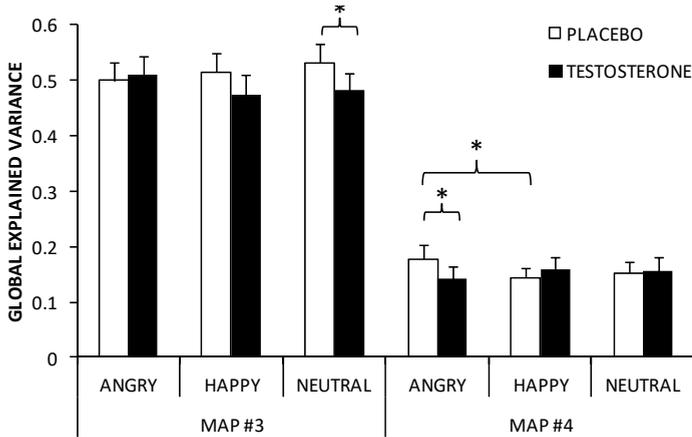


Figure 7.2. Results of the Fitting Procedure. Global Explained Variance of map #3 and map #4 from 120-200 ms post-stimulus (corresponding to the time window of the N170/VPP) for the different experimental conditions, averaged over all participants. Results showed a significant interaction of Treatment x Valence x Map. $*p < .05$

not angry face processing. The effect of Valence was not significant in either Treatment condition (placebo $F(2,68) = 1.57, p = .22$; Testosterone $F(2,68) = 1.99, p = .14$).

The second map in this time interval (Map #4), which has a relatively more positive occipito-parietal topography (see Figure 7.1C), reflects the activation of a different set of neural sources. In contrast to Map #3, post hoc analyses of the significant Treatment x Valence interaction for this map showed a significant effect of Valence in the placebo condition, $F(2,68) = 3.61, p = .05, \epsilon = 0.69, \eta_p^2 = 0.10$, but not in the testosterone condition, $F(2,68) = 0.93, p = .40$. In the placebo condition, GEV was significantly increased for angry compared to happy faces, $F(1,34) = 7.21, p = .011, \eta_p^2 = 0.175$, whereas the difference between angry and neutral or happy and neutral faces were both nonsignificant (both $ps > .05$). Furthermore, follow-up tests by Valence showed that compared to placebo, testosterone administration selectively reduced the GEV for angry faces, $F(1,34) = 7.12, p = .012, \eta_p^2 = .173$. The effect of Treatment was not significant for happy or neutral faces (both $ps > .05$). These findings suggest that this second configuration of neural activity (map #4) was activated mainly during the processing of angry faces in the placebo condition, possibly reflecting an initial threat bias, which disappeared after testosterone administration.

No significant interactions including Treatment and Valence were present in any of the other time intervals (80-120 ms, 180-300 ms, or 275-800 ms), suggesting that testosterone-induced qualitative changes in emotion-related face processing were limited to the N170/VPP processing stage.

Effects of SAD. No significant effects involving the factor Group were present in any of the time windows (all $ps > .05$), suggesting that there were no significant qualitative differences in emotion-related face processing between SAD and HC participants.

Global Field Power

Global Field Power was analyzed to test for differences in response amplitude between groups and conditions, independent of changes in topography. The means for the time intervals of interest are presented in Table 7.3. The results showed a trend towards a main effect of Group in the first time interval (80-120 ms), $F(1,34) = 4.00$, $p = .054$, $\eta_p^2 = 0.105$, suggesting that the P1 amplitude tended to be increased for SAD compared to HC participants, see Figure 3. No other effects reached significance, in any of the time intervals (all $ps > .05$).

Table 7.3. Means (\pm SEM) of the Global Field Power.

Time window	HC (n = 19)			SAD (n = 19)	
	Valence	Placebo	Testosterone	Placebo	Testosterone
80-120 ms (P1)	Angry	3.9 (0.2)	4.1 (0.2)	3.3 (0.2)	3.5 (0.2)
	Happy	4.0 (0.2)	3.9 (0.2)	3.4 (0.2)	3.4 (0.2)
	Neutral	4.1 (0.3)	3.8 (0.2)	3.3 (0.3)	3.5 (0.2)
125-185 ms (N170/VPP)	Angry	4.5 (0.3)	4.5 (0.4)	4.4 (0.3)	4.6 (0.4)
	Happy	4.6 (0.4)	4.5 (0.4)	4.4 (0.4)	4.5 (0.4)
	Neutral	4.6 (0.4)	4.4 (0.4)	4.5 (0.4)	4.6 (0.4)
215-275 ms (P2)	Angry	5.3 (0.5)	5.3 (0.5)	5.1 (0.5)	5.4 (0.5)
	Happy	5.3 (0.6)	5.5 (0.6)	5.3 (0.6)	5.5 (0.6)
	Neutral	5.6 (0.6)	5.2 (0.5)	5.1 (0.6)	5.5 (0.5)

Discussion

In this study we investigated the effects of single dose testosterone administration on social threat processing in socially anxious and non-anxious participants, by recording event-related brain potentials (ERPs) during an emotional Stroop task with subliminally presented angry, happy and neutral faces. The spatiotemporal clustering results show that testosterone selectively affects the early automatic processing of emotional faces, suggesting a reduced initial processing of neutral faces followed by a decreased processing bias for angry faces, while leaving later processes (>200 ms) unaffected. These effects occurred independent of clinical status of the participants and will be discussed in detail below.

The time interval 120-200 ms post-stimulus showed two distinct topographic patterns, which reflect the activity of different neural populations (e.g., Michel et al., 1999) and indicate the presence of two consecutive information processing steps (microstates, e.g., Lehmann, 1987). Both of these were affected by testosterone, but in a different manner. The spatiotemporal characteristics of the first pattern correspond to the N170/VPP

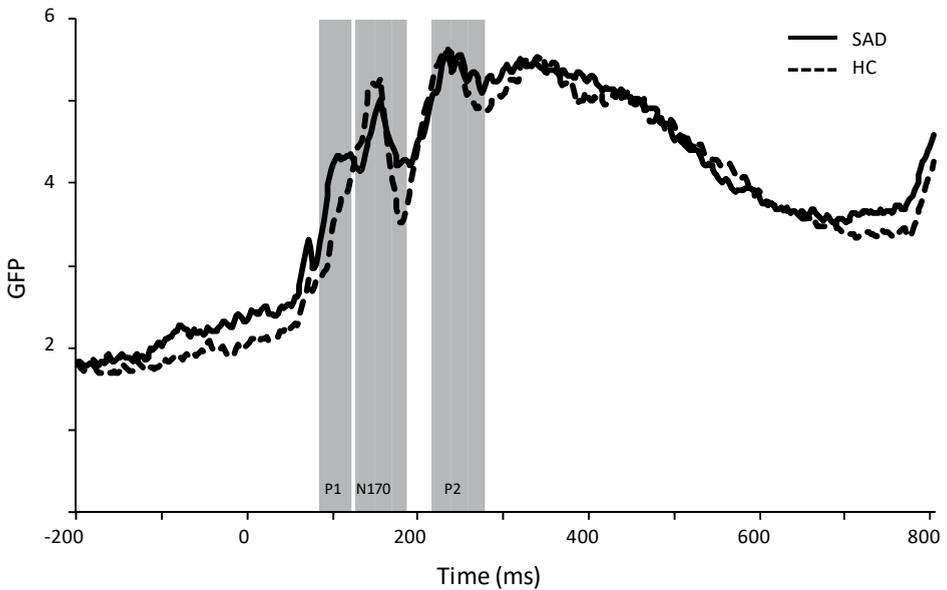


Figure 7.3. Global Field Power (GFP) by Group. Values are averaged per Group (HC, Healthy Controls, $n = 18$; SAD, Social Anxiety Disorder, $n = 18$) over all experimental conditions (Treatment \times Valence). Boxes indicate the time windows used for computing the averages for each ERP component: 80-120 ms (P1), 125-185 ms (N170/VPP), and 215-275 ms (P2) post-stimulus.

ERP complex, which is considered to reflect the early stages of face perception and basic-level categorization (see e.g., Rossion & Jacques, 2011). This pattern showed a significant reduction in global explained variance after testosterone administration, compared to placebo, for neutral faces, which suggests that testosterone changes the neural sources involved in the initial face perception process for neutral but not for angry faces.

The second topographic pattern (map #4), with a relatively more positive occipitoparietal topography, reflects the subsequent activation of a different cognitive process. This finding is consistent with evidence suggesting that the N170/VPP complex on the scalp reflects the activity of multiple neural sources overlapping in time, and represents the intermixed processing of several sources of facial information, including not only basic structural but also high-level (e.g., expression, identity) features (Hinojosa, Mercado, & Carretie, 2015; Rossion & Jacques, 2011). These processes may not be distinguishable with traditional ERP amplitude measures, but can be teased apart by investigating topographic modulations (see e.g., Murray et al., 2008). Moreover, this second pattern was more pronounced during the processing of angry (compared to happy) faces in the placebo condition, suggesting that it may reflect an early processing bias for social threat. This is consistent with recent studies suggesting that the N170 time window is differentially sensitive to emotional expressions, and most strongly responds to angry faces (see

Hinojosa et al., 2015 for a meta-analysis). Some authors (e.g., Del Zotto & Pegna, 2015; Hinojosa et al., 2015) have suggested that this emotional modulation of the N170 may reflect emotional attention processes driven by the amygdala (see also Conty, Dezecache, Hugueville, & Grèzes, 2012), to allow for rapid responses to threat. Most interestingly, this angry face advantage disappeared after testosterone administration, which suggests that testosterone eliminated this early processing bias for social threat.

Taken together, our results reveal that testosterone differentially affects the processing of threatening (angry) and non-threatening (neutral and happy) face stimuli in very early processing stages. These findings may reflect the neural processes underlying previous behavioral findings of threat-specific effects of testosterone (for a review see Bos et al., 2012). In particular, the testosterone-induced reduction in early threat vigilance may explain previous behavioral findings of anxiolytic-like, or approach promoting, effects. For example, studies in healthy participants have shown that testosterone administration reduced the attentional bias to fearful faces (Van Honk et al., 2005), as well as the conscious recognition (Van Honk & Schutter, 2007), behavioral avoidance (Enter et al., 2014), and gaze aversion (Terburg et al., 2012) of angry faces. Testosterone was also found to decrease behavioral avoidance (Enter, Spinhoven, et al., 2016) and gaze aversion (Enter, Terburg, et al., 2016) in patients with SAD. In apparent contrast to these behavioral findings, several fMRI studies have shown that testosterone *increased* amygdala responses during the processing of angry faces (Goetz et al., 2014; Hermans et al., 2008). However, using an approach-avoidance task, Radke et al. (2015) showed that such increased amygdala responses were specifically related to threat approach behavior, while testosterone *decreased* amygdala responses during threat avoidance. Thus, the effects of testosterone on amygdala activity appear to be context-dependent. Based on these findings, and in line with the behavioral findings described above, it was suggested that, by modulating amygdala responses, testosterone biases humans toward approach, and away from avoidance, of social threat (Radke et al., 2015; see also Enter et al., 2014; Enter, Spinhoven, et al., 2016). Our findings are in line with such an approach promoting or threat reducing effect of testosterone, and suggest that testosterone-induced modulations of neural activity may happen already, and predominantly, during the earliest stages of angry face processing. More research is needed to directly investigate the relation between spatial and temporal effects of testosterone on neural activity (e.g., combined fMRI and EEG), and how these relate to behavior, including the role of motivational context.

In addition to these effects of testosterone, our results showed a marginally significant group difference in Global Field Power, reflecting stronger early (80-120 ms post stimulus) neural responses for SAD compared to HC participants. This is consistent with the increased P1 amplitude in high socially anxious participants reported in some previous ERP studies (Peschard, Philippot, Joassin, & Rossignol, 2013; Rossignol, Campanella, Bissot, & Philippot, 2013; Rossignol, Philippot, Bissot, Rigoulot, & Campanella, 2012), which

has been suggested to reflect a general hypervigilance to face stimuli. However, we did not find group differences in the processing of angry faces (see also Mühlberger et al., 2009, but cf. Kolassa & Miltner, 2006; Schulz et al., 2013), or enhanced amplitudes for angry compared to neutral or happy faces (cf., Balconi & Lucchiari, 2005, 2007; van Peer et al., 2010). Overall, ERP evidence for hypervigilance to social threat in social anxiety is still rather inconsistent (see Schulz et al., 2013 for a review). This may be partly due to limitations of the conventional ERP analysis method, such as the inability to differentiate between amplitude and topographic changes (quantitative and qualitative changes, see e.g., Murray et al., 2008), a strong reference-dependence (Murray et al., 2008; Rellecke, Sommer, & Schacht, 2012), and a small (and often different) selection of electrodes and time windows that are included in the analyses. These limitations can be overcome by using reference-free multichannel spatiotemporal clustering methods (e.g., Michel et al., 1999; Murray et al., 2008; Pourtois et al., 2008) as was done in the present study. It would be recommendable for future research to include similar measures to produce more robust findings and further elucidate the nature of social threat processing in SAD and HC.

On a behavioral level, color naming latencies were significantly slower for angry and happy compared to neutral faces, reflecting the typical Emotional Stroop interference-effect (see e.g., Bar-Haim et al., 2007). In contrast to the ERP findings there were no group or treatment effects on this measure, which is not uncommon (although cf. Van Honk et al., 2005). Previous studies have reported significant effects on early ERPs in the absence of behavioral effects with supraliminal (Kolassa & Miltner, 2006; van Peer et al., 2010b) as well as subliminal task versions (van Peer et al., 2010). It has been suggested that reaction times in the Emotional Stroop paradigm result from later processes than attentional capture (see e.g., Bar-Haim et al., 2007), which can explain why they do not reflect the changes in early automatic processing that we found in our ERP measures.

Finally, a few limitations of this study should be discussed. First, as is common in testosterone administration studies, only female participants were tested because the parameters for neurophysiological effects of a single dose of testosterone cyclodextrin in men are as yet unknown (Tuiten et al., 2000b). Future research should investigate whether testosterone administration has similar effects in men, as is suggested by some studies showing similarities in social behavior across sexes related to both exogenous (Goetz et al., 2014) and endogenous testosterone (Van Honk et al., 1999; but cf. Maner, Miller, Schmidt, & Eckel, 2008 for gender differences in testosterone responses to dominance threat in socially anxious men and women). Second, we used a subliminal version of the Emotional Stroop task, as previous studies suggested that effects of testosterone are more pronounced for preconscious processing of threat (Van Honk et al., 2000, 2005). However, the backward masking presumably prevented further cognitive processing of the stimuli (Van Honk et al., 2000; van Peer et al., 2010), which may explain the absence of ERP effects in later processing stages. Third, not all socially anxious participants met the

criteria for a clinical diagnosis of generalized SAD at the time of testing, and the groups were relatively small, which may have attenuated the power to detect group differences. Finally, the electrode configuration used did not allow us to include electrodes at positions P7 and P8 of the extended 10-20 electrode system, which are the sites where the N170 amplitude is typically maximal. This might be an alternative explanation for why we did not find valence or group differences in amplitude (GFP) at the time of the N170 peak.

In conclusion, our findings suggest that testosterone changed the initial basic face perception process for neutral faces, and decreased a subsequent attentional bias for social threat, in socially anxious and non-anxious participants. These findings indicate that testosterone specifically affects the early automatic processing of social cues, and provides support for the notion that testosterone affects biologically prepared motivational processes (e.g., Radke et al., 2015; Van Honk et al., 2005), which may be key to changes in social motivational behavior, such as decreased threat avoidance.

Supplemental Information

Supplemental Results

Grand average ERPs

After artifact rejection, the epoched EEG data were averaged to individual ERPs for each facial expression type, excluding trials with incorrect responses or outlier RTs. Table S7.1 presents the number of remaining trials per group and condition. A rmANOVA showed no significant effect of Testosterone or Group differences on these trial numbers. Figure S7.1 and Figure S7.2 present the average waveforms per group and condition, for a selection of electrodes. The spatiotemporal clustering analysis and calculation of Global Field Power were performed on the basis of the average waveforms from all 32 electrodes.

Table S7.1. Mean (\pm SEM) number of trials per condition for ERPs.

Valence	HC ($n = 18$)		SAD ($n = 18$)	
	Placebo	Testosterone	Placebo	Testosterone
Angry	20.7 (0.4)	21.4 (0.4)	21.5 (0.4)	21.7 (0.4)
Happy	21.3 (0.4)	21.0 (0.4)	21.8 (0.4)	22.0 (0.4)
Neutral	22.2 (0.4)	21.6 (0.4)	21.4 (0.4)	21.3 (0.4)

Awareness Check

To determine whether participants were capable of consciously perceiving the masked facial expressions, they were asked to complete an awareness check at the end of the second testing session. This adapted version of the task comprised a subset of 48 masked faces (each actor and expression was presented twice). The instructions explicitly stated that the stimuli consisted of briefly presented faces, and participants were asked to indicate (if necessary by guessing) whether the emotional expression of these faces was angry, happy or neutral by pressing the corresponding response button. Awareness check data were missing for one participant (SAD), due to technical problems.

Awareness of the subliminal faces was tested with a binomial test ($n = 48$, $p = 0.33$) on the number of correct responses for each participant. Four participants (2 HC, 2 SAD) scored above chance level (≥ 22 correct responses, $p < .05$). The mean number of correct responses of the remaining participants was 15.8 ($SD = 2.5$). To check that the findings reported in the main text of this article were not influenced by awareness of the subliminal stimuli, the analyses were repeated excluding the participants that scored above chance-level ($n=4$) and the participant with missing data on the awareness check (see Results of Control Analyses below).

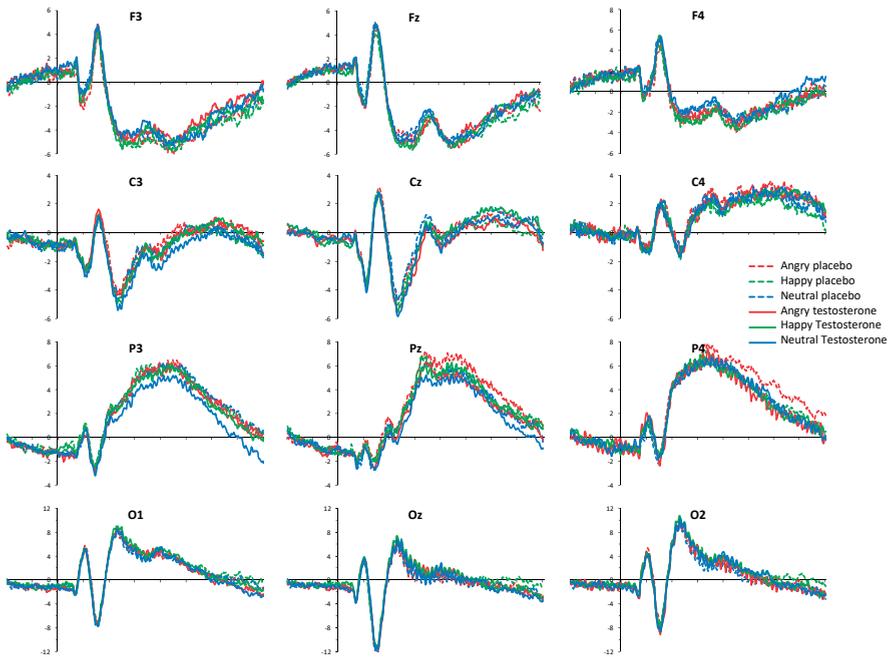


Figure S7.1. Grand average ERP waveforms per condition for healthy control participants on selected electrodes. The x-axis indicates the time interval from -200 until +800 ms relative to the onset of the face stimulus. The y-axis indicates the amplitude in μV , with negative plotted up, and crosses the x-axis at time 0.

Additional Results of Main Analyses

Spatiotemporal clustering In the main text of this article, of the spatiotemporal clustering analyses only the results including interactions of Treatment or Group with Valence and Map are reported, as these reflect the effects of interest of the current study: Testosterone- or SAD-related differences in emotion-related face processing. For the sake of completeness, all other significant results from these analyses are reported below. In addition to the three-way interaction of Treatment \times Valence \times Map reported in the main text, the analysis of the second time window (120-200 ms, Map #3 and #4) showed a significant main effect of Map, $F(1,34) = 81.36$, $p < .001$, $\eta_p^2 = .71$, reflecting a higher GEV for Map #3 compared to Map #4. The following time window (180-300 ms, Map #5 and #6), also showed a significant main effect of Map, $F(1,34) = 57.89$, $p < .001$, $\eta_p^2 = 0.63$, as well as a significant interaction of Valence \times Map, $F(2,68) = 3.99$, $p = .023$, $\eta_p^2 = 0.11$. Follow-up analyses showed that the GEV of Map #6 was significantly higher than the GEV of Map #5 for all faces (all $F_s > 32.50$, $p_s < .001$). Furthermore, results showed a trend effect of Valence for Map #6, $F(2,68) = 2.88$, $p = .063$, $\eta_p^2 = 0.08$, resulting from an increased GEV for happy compared to angry faces, $F(1,34) = 5.55$, $p = .024$, $\eta_p^2 = 0.14$. None of the other follow-up tests reached significance (all $p_s > .05$). Finally, analyses of the last time window

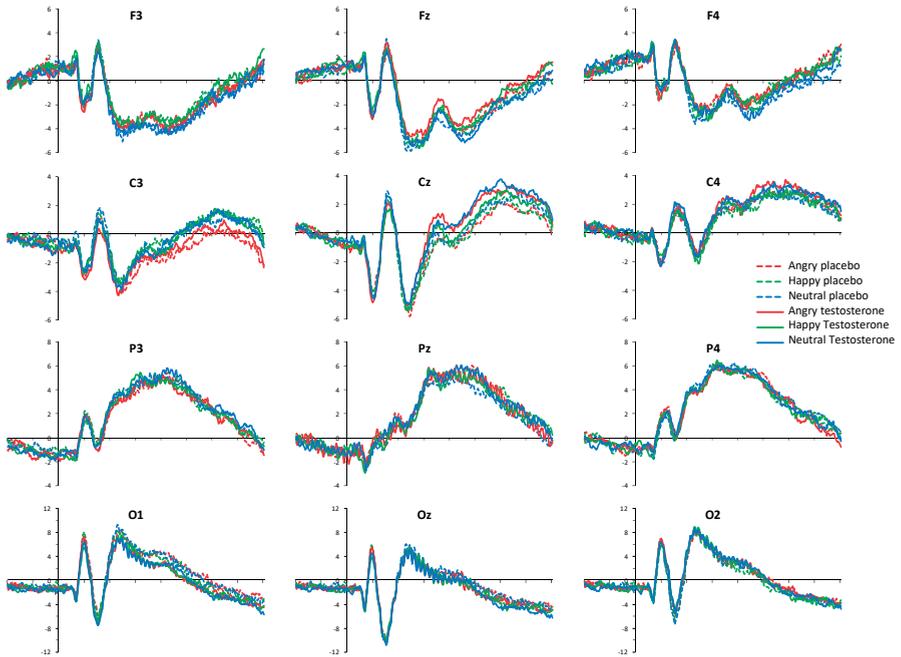


Figure S7.2. Grand average ERP waveforms per condition for Social Anxiety Disorder participants on selected electrodes. The x-axis indicates the time interval from -200 until +800 ms relative to the onset of the face stimulus. The y-axis indicates the amplitude in μV , with negative plotted up, and crosses the x-axis at time 0.

(275-800 ms, Map #7 to #10) showed a significant main effect of Map, $F(3,102) = 42.32, p < .001, \eta_p^2 = 0.55$, as well as a significant interaction of Treatment \times Map, $F(3,102) = 3.40, p = .038, \epsilon = 0.68, \eta_p^2 = 0.09$. Follow-up pair wise comparisons showed that the GEV of Map #7 was higher compared to the GEV of all other Maps (all $F_s > 39.0, p_s < .001$), but the effect of Treatment was not significant for any single Map (all $F_s > 2.98, p_s > .05$).

Results of Control Analyses

In addition to the analyses of interest, several control analyses were conducted. First, to check that the findings were not influenced by awareness of the subliminal stimuli, the analyses were repeated excluding the participants that scored above chance-level ($n = 4$) and the participant with missing data on the awareness check. Second, to verify whether the findings were influenced by possible confounding factors such as Order of treatment (testosterone first, placebo first), Time of testing (morning, afternoon), or use of Contraception (yes, no), the analyses were repeated with these factors (one-by-one) included as an additional between-subject factor. The results of these analyses are reported below.

Spatiotemporal clustering When the participants with above-chance scores or missing data on the awareness check were excluded from the analyses, the interaction of

Treatment x Valence x Map in the N170/VPP time interval, as well as the significant follow-up tests, reported in the main text, remained significant (all $ps < .05$). Furthermore, the decrease in GEV of Map #3 after testosterone administration compared to placebo for happy faces, which was marginally significant ($p=.056$) in the original analysis, became significant in this control analysis, $F(1,29) = 5.47, p = .026, \eta_p^2 = .159$.

The interaction of Treatment x Valence x Map in the N170/VPP time interval, as well as the significant follow-up tests reported in the main text, also remained significant (all $ps < .05$) when time of testing or treatment order were included as a factor in the analysis. Only when use of contraception was included, the interaction of Treatment x Valence for Map #3 in the follow-up tests was no longer significant. More importantly, however, none of these possible confounding factors significantly interacted with Valence and Map (all $ps > .05$), suggesting these factors did not result in qualitative changes in emotion-related face processing.

Global Field Power. Control analyses showed that when the participants with above-chance scores or missing data on the awareness check were excluded from the analyses, the effect of Group in the first time interval (80-120 ms, $p = .054$), reflecting a tendency towards higher P1 amplitudes for SAD patients, remained marginally significant, $F(1,29) = 4.13, p = .052, \eta_p^2 = .13$. Furthermore, this effect became significant, $F(1,32) = 4.59, p = .040, \eta_p^2 = .13$, when treatment order was included as a control factor in the analysis, but was reduced when time of testing, $F(1,32) = 3.34, p = .077, \eta_p^2 = .10$, or use of contraception, $F(1,32) = 1.78, p = .19$, were taken into account. Most importantly, however, none of these factors showed a significant interaction with Group (all $ps > .05$).

Behavioral results. When the participants with above-chance scores or missing data on the awareness check were excluded from the analyses, the main effect of Valence on response latencies, as well as the significant follow-up tests reported in the main text, remained significant (all $ps < .05$). The same was true when order of treatment was included in the analysis (all $ps < .05$). When oral contraception or time of testing were included, the Valence effect was reduced to a marginally significant effect, $F(2,68) = 2.66, p = .077, \eta_p^2 = .07$. More importantly, however, none of these possible confounding variables interacted significantly with the effect of Valence (all $ps > .05$).



General Discussion



Testosterone plays a crucial role in the regulation of social behavior. It promotes social dominance, which entails enhanced approach motivation, and reduced fear and threat sensitivity. Evidence for the causal effects of testosterone on social motivational behavior thus far was scarce, and lacking with regard to SAD. The main aim of this thesis was to elucidate the role of testosterone in the neuroendocrine mechanisms underlying social fear and avoidance in SAD. We hypothesized that 1) SAD is associated with reduced testosterone levels compared to healthy controls, and 2) that administering testosterone to patients with SAD will alleviate social fear and avoidance, and will promote prosocial behavior. The first hypothesis was tested by assessing salivary testosterone levels in a large group of people including SAD patients, and healthy controls. We tested the second hypothesis by investigating the effects of single dose testosterone administration in women with SAD as compared to healthy controls, on several indexes of social motivational behavior. This methodology was also used to investigate underlying neuroendocrinological mechanisms. In addition, considering the interactions between testosterone and striatal dopamine signaling, we explored the interaction between striatal dopaminergic polymorphisms and social approach-avoidance tendencies.

This chapter summarizes the empirical chapters and presents a theoretical integration of the findings. It will conclude with a discussion of the strengths and limitations, and will make suggestions for future research and clinical perspectives.

Summary of empirical chapters

Chapter 2 describes a first study on how genetic variations, which influence the functioning of the mesolimbic reward system, affect social approach-avoidance action tendencies. An implicit social approach-avoidance task (AAT) was performed by two groups of healthy participants carrying different variants of the dopamine transporter (DAT) gene. One group carried the 9-repeat allele, which is associated with increased striatal dopamine levels and has been found to be related to increased sensitivity to reward, the other group consisted of non-9-repeat carriers. Results showed that the DAT1 9-repeat carriers performed increased emotion-driven action tendencies in reaction to stimuli that communicate a motivational drive to the observer: They showed stronger tendencies to approach appetitive social stimuli (i.e., happy expressions), and to avoid aversive stimuli (i.e., angry expressions), compared to non-9-repeat carriers. These results suggest that striatal dopaminergic polymorphisms affect motivational responses to social-emotional cues.

Chapter 3 tested whether single dose testosterone administration diminishes threat avoidance and promotes actual behavioral approach actions that have an immediate effect on the relative distance of the social threat stimulus. Healthy individuals performed

an implicit social AAT featuring angry, happy, and neutral facial expressions. Participants showed significantly diminished avoidance tendencies to angry faces after testosterone administration. Testosterone did not affect approach–avoidance tendencies to social affiliation (happy) faces. Thus, single dose testosterone administration reduces automatic avoidance of social threat and promotes relative increase of threat approach tendencies in healthy females.

Chapter 4 mapped salivary testosterone levels of 722 male and 1380 female participants of The Netherlands Study of Depression and Anxiety (NESDA; Penninx 2008). The results confirmed our first hypothesis, and showed that female patients with social phobia had lower salivary testosterone levels than female controls with no lifetime history of depressive or anxiety disorders. In addition, there was a linear trend for decreasing testosterone levels with increased social phobia scores.

Chapter 5 presents a study that tests the effects of testosterone on actual social approach–avoidance action tendencies in females with SAD. Using the same implicit social AAT as in chapter 3, we investigated whether administering a single dose of testosterone could counteract the automatic social avoidance tendencies in women with SAD. After testosterone administration, the participants showed increased approach tendencies to angry facial expressions, but not happy expressions. These results suggest that testosterone indeed can counteract persistent automatic social avoidance tendencies in SAD, confirming our second hypothesis.

Chapter 6 tested the hypothesis that administering a single dose of testosterone promotes social dominant gaze behavior and thus will be able to alleviate submissive gaze avoidance in individuals with SAD. The spontaneous gaze behavior of participants with SAD and of control participants was recorded using eye-tracking, while they looked at angry, happy, and neutral facial expressions. Testosterone enhanced the percentage of first fixations to the eye-region in participants with SAD compared to healthy controls. In addition, SAD patients' initial gaze avoidance in the placebo condition was associated with more severe social anxiety symptoms. This relation was no longer present after testosterone administration. These findings indicate that single dose testosterone administration can alleviate gaze avoidance in SAD.

Chapter 7 investigated the effects of testosterone on social threat processing in participants with SAD and control participants, using temporally fine-grained recordings of event-related brain potentials (ERPs) during an emotional Stroop task with subliminally presented angry, happy and neutral faces. Spatiotemporal clustering analysis on the ERPs showed that testosterone induced two qualitative changes in early face processing (<200 ms after stimulus onset) in participants with and without SAD. An initial testosterone-induced spatial shift reflected a decrease of the typical basic processing (N170/VPP) of neutral faces, which was followed by a specific change for angry faces, suggesting a specific modulation of early threat vigilance. We found no effects in later processing

stages. These findings suggest that testosterone specifically affects early automatic, social information processing.

Integration of findings

Basal testosterone in SAD

Our first prediction was confirmed by the finding that female patients with social phobia had lower salivary testosterone levels than female controls with no lifetime history of depressive or anxiety disorders. Also, higher social phobia scores were associated with lower testosterone levels. Our findings fit previous notions in animal and human research which associate reduced testosterone with social submissiveness and increased levels of social fear behavior, such as social withdrawal (Bedgood et al., 2014; Mehta & Josephs, 2010; Sapolsky, 1990, 1991; van Honk et al., 1999). They concur with the socio-neuroendocrine models of SAD which propose that this disorder and its persistent social avoidance originates from social subordination stress (Gilbert, 2001; Hermans & van Honk, 2006). Individuals with SAD show increased vigilance for (dominance) threat in social interactions, and engage in subordinate behavior in order to enhance their survival chances by avoiding potential harm from others (Gilbert, 2001; Hermans & van Honk, 2006; Weisman et al., 2011). In this light it is interesting to note that several studies suggest that individuals with SAD perceive themselves as being of low social rank, as behaving submissively (Weisman et al., 2011), and also tend to rate others as more dominant and less friendly, compared to non-anxious individuals (Aderka, Haker, Marom, Hermesh, & Gilboa-Schechtman, 2013; Haker, Aderka, Marom, Hermesh, & Gilboa-Schechtman, 2014).

Testosterone alleviates avoidance in SAD

Second, we predicted that administering testosterone to patients with SAD will alleviate social fear and avoidance, and will promote prosocial behavior. We tested this prediction on two indices of social motivational behavior: approach-avoidance tendencies and gaze avoidance.

Previous testosterone administration studies in healthy and high anxious participants have shown that there is a causal relationship between a surge in testosterone and reduction of fear and sensitivity to threat, and increased social dominance related behavior (Bos et al., 2012; Terburg & van Honk, 2013). However, so far evidence was constrained to processing of perceptual information and gaze behavior. Chapter 3 extends our knowledge by confirming the causal effect of testosterone on automatic social motivational action. This is the first study showing that testosterone directly influences behavioral approach actions that have an immediate effect on the relative distance of the social threat stimulus (Rinck & Becker, 2007). Chapter 5 extends these findings by showing that

testosterone also promotes social threat approach in participants with generalized SAD, characterized by exaggerated threat avoidance. Thus, both in healthy participants, and in participants with SAD, testosterone is able to promote social threat approach, indicating an enhanced motivational tendency toward social dominance (Archer, 2006; Bos et al., 2012; Radke et al., 2015; Terburg & van Honk, 2013).

Chapter 6 shows that administering a single dose of testosterone also promotes social gaze behavior, by increasing the initial fixations to the eyes of facial stimuli in SAD compared to healthy control (HC) participants. In addition, higher social phobia scores were associated with increased gaze avoidance of angry eye contact, stressing the clinical relevance of this measure. This study indicates that a single dose testosterone administration can alleviate gaze avoidance, which is one of the core communicative features of social anxiety and SAD in particular (Horley et al., 2003, 2004; Weeks et al., 2013). These findings support theories on the anxiolytic and social dominance enhancing properties of testosterone, and corroborate our findings on threat approach action tendencies in SAD.

Interestingly, unlike the studies in chapters 3 and 5 which found similar effects of testosterone on Approach-Avoidance tendencies in healthy and SAD participants, our eye-tracking study yielded contrasting findings for the control group. We argued that this finding could be attributed to the influence of environmental and individual differences on the modulatory effect of testosterone on social status promoting behavior (Eisenegger et al., 2011; van Honk, Terburg, et al., 2011). For example, research has shown that a previous winning or losing experience, height of endogenous cortisol levels, extent of social anxiety, or amount of prenatal testosterone exposure influences the effects of testosterone on social behavior (Gleason et al., 2009; Maner, Miller, Schmidt, & Eckel, 2008; Mehta & Josephs, 2010; Honk, Schutter, et al., 2011). The context-dependency of testosterone effects may also contribute to the differential findings for healthy controls in the eye-tracking study and the similar findings on the AAT, in that there are notable differences between the tasks. The AAT used in Chapters 3 and 5 measures the automatic behavioral tendencies towards a social stimulus, reflecting the primary underlying motivational disposition. Participants are confronted with a facial stimulus to which they are subsequently required to respond with a behavioral action. The gaze behavior captured in the eye-tracking task also reflects automatic behavioral tendencies, but in addition has a communicative function. Avoidance of eye contact signals social subordination and prevents an aggressive encounter, whereas seeking (angry) eye-contact is typical behavior for socially dominant individuals. Due to the setup of the eye-tracking task, no behavioral action was required and eye-contact could be avoided in the first place, which may have contributed to a more socially submissive behavioral gaze pattern in SAD compared to the healthy controls. Nevertheless, future studies should elucidate the exact interpretation of effects.

In sum, the findings from Chapters 3, 5, and 6 confirm our second hypothesis and indicate that single dose testosterone administration is able to counteract automatic social avoidance behavior, and to promote approach behavior in SAD. In addition, they are in agreement with literature which suggest that the effects of testosterone depend on individual and environmental differences.

Neuroendocrine mechanisms

Thirdly, we investigated underlying neuroendocrine mechanisms of testosterone on social motivational behavior. Chapter 7 indicates that testosterone differentially affects the processing of threatening (angry) and non-threatening (neutral and happy) face stimuli in very early stages. These findings suggest that testosterone alters early vigilance to social threat. In addition, they indicate that no deviation exists between participants with and without SAD on this level of processing.

These findings may reflect the neural processes underlying our findings of threat-specific effects of testosterone on social motivational behavior in Chapters 3, 5, and 6. fMRI studies have shown that testosterone administration increased amygdala responses during the processing of angry faces (Goetz et al., 2014; Hermans et al., 2008), and a study featuring the Approach-Avoidance Task showed that such increased responses were specifically related to threat approach behavior, while testosterone decreased amygdala responses during threat avoidance (Radke et al., 2015). Based on these findings, it can be argued that our findings of increased threat approach may be caused by the modulation of amygdala responses by testosterone, biasing social motivational tendencies toward approach, and away from avoidance, of social threat. Our findings in Chapter 7 suggest that such testosterone-induced modulations of neural activity may happen already during the earliest stages of angry face processing.

In addition, testosterone reduces connectivity between the PFC and the amygdala (van Wingen et al., 2010a; Volman et al., 2016; Volman, Toni, et al., 2011). fMRI studies probing the relation between endogenous testosterone and social approach action showed that testosterone modulates prefrontal brain activity and prefrontal–amygdala connectivity when people had to control their automatic social approach–avoidance tendencies (Volman et al., 2016; Volman, Toni, et al., 2011), indicating that prefrontal control of the amygdala has been reduced when testosterone was higher. Neuroimaging studies in SAD have shown increased activation of the amygdala and alterations in frontal-amygdala coupling in response to threatening facial expressions (Cremers & Roelofs, 2016; Fouche et al., 2013). These findings suggest that the amygdala is not properly regulated during threatening situations in SAD. Although much is still unknown about the neural correlates of Approach-Avoidance behavior in SAD, it is likely that testosterone biases the amygdala towards social approach action in a similar fashion as in healthy persons.

Possible endocrine mechanisms underlying these neural patterns may lie in the upregulation of dopamine levels by testosterone in the PFC (Aubele & Kritzer, 2011; van Honk, Terburg, et al., 2011). In addition, testosterone and its metabolites bring about anxiolytic actions in brain areas involved in the fight-flight response, via steroid receptors and γ -aminobutyric acid (GABA-A) receptors in the amygdala, hypothalamus, and PAG (among other areas; McHenry et al., 2014), as well as inhibition of the HPA-axis (Hermans et al., 2007; Viau, 2002). Also, a testosterone-induced increase of the neuropeptide vasopressin in the amygdala might enhance the inclination towards dominance and social aggression (Bos et al., 2012; Terburg & van Honk, 2013).

Importantly, testosterone reduces punishment sensitivity and promotes reward seeking behavior. Testosterone enhances dopamine levels in the ventral striatum (de Souza Silva et al., 2009; Hermans et al., 2010), which is associated with prediction of the value of rewards (Cools, 2008). In Chapter 2 we show that increased striatal dopamine transmission is associated with augmented approach-avoidance behavior in pursuit of social reward (i.e., approaching happy and avoiding angry faces). It is likely that dopamine modulates the effects of testosterone in stimulating approach motivation (Depue & Morrone-Strupinsky, 2005; Johnson, Leedom, & Muhtadie, 2012). The specific effects of testosterone on approach behavior towards social threat faces are typically related to an enhancement of the motivation to obtain higher social status (Öhman, 1986). An encounter with an angry facial expression may lead to a possibly rewarding outcome, such as increased social status and its benefits. In SAD striatal activity has been shown to be deviating (Freitas-Ferrari et al., 2010) and there are indications for a social-motivational imbalance in SAD. Results suggest that patients show a reduced motivation to obtain social reward and relative increased motivation to avoid social punishment (Cremers et al., 2014). It is conceivable that administering testosterone to these individuals dampens punishment sensitivity and increases motivation to pursue social reward.

Taken together, the neuroendocrine mechanisms underlying our findings of a testosterone-induced increase in social approach behavior may lie in changes in social threat processing by the amygdala and connected frontal-striatal circuits. Testosterone biases the amygdala to social threat approach, reduces prefrontal control, and increases reward sensitivity. Our EEG findings suggest that actions of testosterone happen already at very early processing stages, and that deviations underlying psychopathology may arise at later stages.

Strengths, limitations, and future perspectives

Strengths

The general strengths of the presented studies will be discussed in this paragraph.

First, in the testosterone-administration studies, we used a within-subjects design, which is a powerful method to detect changes related to the experimental manipulation because the same subjects are tested in both placebo and testosterone conditions. In addition, testosterone administration was double-blind and randomized, which minimizes the possibility of expectations - fueled by the widespread ideas on testosterone - of participant and experimenter influencing the results.

Second, the used testosterone administration method is well-established in healthy women. Its pharmacodynamic profile has been determined using a reliable automatic and non-habitual measure (Tuiten et al., 2000a). In addition, over a decade of research applying this method has convincingly shown psychophysiological and behavioral effects (see for a review Bos et al., 2012).

Third, we measured social avoidance with two different indexes of social motivational behavior. The AAT is a well-established, objective measure of social motivational tendencies, gaze avoidance is considered a core characteristic of SAD.

Fourth, for our neurocognitive tasks we carefully selected a target stimulus that has proven to be potent in eliciting avoidance in individuals with SAD (i.e., angry facial expressions; (Horley et al., 2004; Roelofs, van Peer et al., 2009; van Peer et al., 2009). In addition, an angry looking face with direct gaze is a signal of social dominance (Öhman, 1986), and testosterone, as regulator of social motivational behavior, has shown to specifically modulate automatic behavioral responses (Terburg et al., 2012) as well as neural responses to particularly this stimulus (Goetz et al., 2014; Hermans et al., 2010, 2008).

Finally, we selected our participants based on strict criteria, of which the two most important were exclusion of participants using psychotropic medication, which affects brain activity, and exclusion of individuals with a current comorbid diagnosis of major depressive disorder, which affects social threat processing (Bar-Haim et al., 2007) and the neuromuscular response (Sabbe et al., 1999). A consequence is that our selected SAD sample is possibly not an adequate representation of SAD in the general population, which might limit the generalizability of our results.

Limitations

Several limitations should be considered with regard of the presented findings. These are discussed in detail in the empirical chapters; the following paragraph will present the most important limitations in general.

First, the testosterone administration studies in chapters 3, 5, 6, and 7 only featured female participants. This is a consequence of the fact that the administration of testosterone cyclodextrin has only been validated in women (Tuiten et al., 2000). Future studies should test our hypotheses in samples containing male participants. We expect similar results since the effects of testosterone on social motivation are comparable across sexes (Hermans et al., 2007; van Honk et al., 1999, 2001; van Honk & Schutter, 2007). This hy-

pothesis is validated by a recent testosterone administration study in healthy men which replicated earlier findings in healthy women on brain activity in response to angry facial expressions (Goetz et al., 2014; Hermans et al., 2008a), however it has not yet been tested how this translates to social motivational behavior.

Second, as is customary with the used testosterone administration paradigm, we aimed to control for steroid hormone level fluctuations associated with the menstrual cycle by including women on single-phase contraceptives, and naturally cycling women who were tested in the pre-ovulatory phase (e.g., Hermans et al., 2010; van Honk, Schutter, et al., 2011). Although the effects of the testosterone administration studies remained after statistically controlling for contraceptive use, future studies ideally deal with hormone fluctuations by only testing participants who are on single-phase contraceptives, or by directly assessing estradiol levels, especially given evidence that this hormone likely mediates testosterone-effects on social dominance (McHenry et al., 2014; Stanton & Schultheiss, 2007; Terburg & van Honk, 2013; Ziomkiewicz et al., 2015).

Third, due to the combination of strict inclusion criteria, a time-consuming testing schedule, and the particulars of SAD, it was difficult to recruit participants. To be able to complete the study we had to loosen our inclusion criteria, which resulted in an age difference between the SAD and the HC groups. We were able to control for this issue by age-matching our groups before analyses (chapters 6 and 7). This resulted in smaller participant groups than initially aimed for, but they still fall within the range of previously reported samples of 15 up to 23 participants with SAD (eye-tracking studies, see for a review (Armstrong & Olatunji, 2012); AAT-studies, (e.g., Roelofs, van Peer et al., 2009; van Peer et al., 2009). In addition, previously conducted investigations featuring testosterone administration to groups consisting of 12 up to 20 healthy participants in a randomized, placebo-controlled, within-subjects design reported significant multi-way interaction effects for a variety of paradigms (see for a review Bos et al., 2012). Although our sample is probably not too small for genuine positive effects, future studies should replicate our findings in larger samples.

Finally, there is as yet no evidence that the neurocognitive tasks presented in this thesis reflect actual behavior in real life social settings. Although validation with an actual behavioral approach test is still lacking for the social AAT, for the spider phobia AAT it has been shown that the magnitude of avoidance on the spider AAT predicts the distance kept in an actual spider approach task (Klein, Becker, & Rinck, 2011; Rinck & Becker, 2007). There are several studies that have shown that avoidance of angry faces on the social AAT is related to social anxiety (Heuer et al., 2007; Roelofs et al., 2010) and, on the contrary, that aggressive samples show social threat approach on the same task (von Borries et al., 2012). Importantly, Chapter 5 showed that the magnitude of the AAT effect score for angry vs neutral faces was correlated to symptom severity in patients with SAD; this observation validates the currently used social AAT version in SAD. Furthermore, avoidance of eye

contact has been clinically observed to be a typical characteristic of SAD (Stein & Stein, 2008; Weeks et al., 2013). The used eye-tracking task was newly developed for this study, based on previous research (Horley et al., 2003, 2004). In chapter 6 we see the clinical relevance of first fixations on the eye-region confirmed by the correlation between this measure and the severity of symptoms in the SAD sample in the placebo condition.

Suggestions for future research

In addition to the above mentioned suggestions for future investigation, this paragraph proposes several ideas for future research which might help answer outstanding questions.

To date there is still much unclear about the neuroendocrine mechanisms underlying deviations in social motivational behavior in SAD. Research featuring neuroimaging techniques should shed more light on this matter. Future studies could combine the AAT with fMRI, endogenous testosterone measurements (e.g., Volman, Toni, et al., 2011), and/or testosterone administration to find out how testosterone modulates social approach-avoidance behavior in SAD. In addition, it would be interesting to try to probe the functioning of the emotion-network (e.g., fear reduction by GABA-ergic mechanisms) and the reward-network (dopaminergic mechanisms, e.g. whether testosterone indeed facilitates dopaminergic projections from the amygdala to the striatum) in SAD, by using single-photon emission computed tomography (SPECT) or positron emission tomography (PET) scanning (e.g., Schneier et al., 2009; van der Wee et al., 2008), in combination with testosterone administration. This would be particularly interesting in combination with genotyping for androgen and dopaminergic receptor genes. Furthermore, we presented a first EEG study on the effects of administered testosterone on early threat processing and future research featuring EEG or magnetoencephalography (MEG) should further elucidate the temporal dynamics of these processes in both healthy individuals and those with SAD.

As noted in the previous paragraph, it is not yet known how the behavioral effects captured by our neurocognitive tasks translate to behavior in actual social settings. It would be an interesting approach to combine testosterone administration with virtual reality paradigms (e.g., Morina, Brinkman, Hartanto, Kampmann, & Emmelkamp, 2015), or with a controlled social interaction environment, to assess (gaze) avoidance in social interactions.

It is getting more and more clear that the effects of testosterone on social motivational behavior are modulated by individual differences and social context. Future research should address the influence of social anxiety (e.g., Cremers & Roelofs, 2016), endogenous cortisol and testosterone levels (e.g., Mehta & Josephs, 2010b), the role of oxytocin and other hormones (e.g., van Honk, Bos, Terburg, Heany, & Stein, 2015), sex

differences (e.g., Welker et al., 2015), and prenatal testosterone exposure (e.g., 2D:4D ratio; van Honk, Schutter, et al., 2011), among others.

Finally, prospective developmental studies are needed to investigate whether reduced testosterone in women with SAD plays a (causal) developmental role or is rather a consequence of reduced social interaction resulting from social avoidance.

Clinical perspectives

The research studies presented in this thesis primarily aim to elucidate neuroendocrine mechanisms of social motivational behavior in the first place. Nevertheless, given our promising findings showing that testosterone promotes actual social approach action tendencies in SAD during a social challenge, it would be of theoretical and clinical importance to test whether testosterone administration could benefit the treatment of SAD.

Exposure therapy is part of first-line treatment of SAD, and aims at fear extinction by repeated or prolonged exposure to feared social situations, which should lead to a reduction in fear and avoidance behavior. Although exposure therapy has proven effective, nonresponse rates in large clinical trials have been 50% or higher (Hofmann & Bögels, 2006), and most individuals do not achieve remission. In an attempt to enhance exposure therapy efficacy, research has explored the augmentation effects of pharmacological agents thought to enhance the underlying mechanisms of action (e.g., extinction learning) of exposure therapy. This approach has potential as was shown by studies featuring a variety of pharmacological agents (e.g., Hofmann et al., 2014). Despite initially promising findings, the working mechanisms are still not entirely clear and studies yield mixed findings. It is possible that these cognitive enhancers do not target the most optimal mechanism for enhancement of exposure therapy, and an alternative approach targeting social motivational mechanisms directly might provide a more effective solution. Considering the alleviating effects of testosterone on actual social avoidance behavior in SAD, and considering that exposure therapy is also aimed at reduction of avoidance behavior, it would be of relevance to test whether single-dose testosterone administration - applied only a few times to enhance efficacy of the first few exposure sessions - can enhance therapy efficacy for SAD. Nevertheless, it should be noted that there is still much unclear about the working mechanisms of testosterone and of pharmacological add-ons for exposure therapy. It is possible that, besides the potentially beneficial effects of testosterone on dopamine transmission and glucocorticoid mechanisms, its effects on the GABA system might not only work anxiolytic but could also potentially interfere with extinction learning, something worth investigating in future research (Singewald et al., 2015).

Another interesting approach would be to explore whether the automatic avoidance tendencies in SAD could be diminished by approach-avoidance training on the AAT. Research on this topic across various disciplines has shown positive results (Becker

et al., 2016). Two studies featuring socially anxious participants showed that, after being required to approach positive social stimuli on the AAT, they subsequently showed more approach behavior during social interactions, elicited more positive reactions by their interaction partners (Taylor & Amir, 2012), and reported better mood and less anxiety after a social challenge (Rinck et al., 2013). Single-dose testosterone administration might aid the training process by biasing the brain towards social approach, although this effect is likely specific for social threat faces.

Conclusion

This thesis aimed to elucidate the role of testosterone in the neuroendocrine mechanisms underlying social fear and avoidance in SAD. Despite the social approach-enhancing and anxiolytic effects of testosterone, its role in fear and avoidance in SAD thus far was scarcely investigated.

Our findings indicate that baseline testosterone is lower in women with SAD and related to social anxiety symptoms. This is in line with animal and human research which associates reduced testosterone with social submissiveness and increased levels of social fear behavior, and confirms socio-neuroendocrine theories of SAD. Furthermore, in agreement with our prediction, the findings show that single-dose testosterone administration is able to counteract persistent social avoidance tendencies in SAD, thereby confirming and extending earlier findings on the social dominance-enhancing theories of testosterone. In addition, the notion that testosterone affects automatic motivational processes is supported by the finding that testosterone affects early threat processing.

Future research should look further into the neuroendocrine mechanisms underlying these behavioral effects, and consider exploring whether single-dose testosterone administration could benefit the treatment of SAD.



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Nederlandse Samenvatting

Durf te naderen: Effecten van testosteron op vermijdingsgedrag bij sociale angst.



Inleiding

Het hormoon testosteron speelt een belangrijke rol bij de regulatie van sociaal gedrag, samen met onder andere cortisol. Waar testosteron een angstremmende werking heeft en sociale toenadering bevordert, wordt cortisol juist geassocieerd met stress en sociale vermijding. Een disbalans in het functioneren van testosteron en cortisol kan leiden tot storingen in de regulatie van sociaal toenaderings- en vermijdingsgedrag. Dit is het geval bij de sociale angststoornis (SAD), waarbij verhoogde cortisolresponsen samenhangen met hardnekkige sociale vermijding. Over de rol van testosteron bij sociale angst was nog nauwelijks iets bekend en daar probeerde dit proefschrift meer duidelijkheid in te scheppen.

SAD is de meest voorkomende angststoornis en wordt gekarakteriseerd door een intense angst voor sociale situaties waarin het individu de kans loopt kritisch beoordeeld te worden door anderen. Daardoor ontstaat de neiging om sociale interacties te vermijden. Dit vermijdingsgedrag speelt een belangrijke rol bij het instandhouden van de stoornis. Vermijding van oogcontact is karakteristiek voor mensen met SAD en wordt geassocieerd met typisch sociaal onderdanig gedrag dat als doel heeft om sociale dreiging te verminderen. Een andere factor die in verband gebracht wordt met de hardnekkigheid van de stoornis is een veranderde verwerking van sociale informatie, waarbij het erop lijkt dat mensen met SAD een onbewuste voorkeur hebben voor negatieve informatie in hun omgeving, wat ertoe leidt dat de situatie als meer negatief beoordeeld wordt dan deze in werkelijkheid is.

Een goede regulatie van sociale toenadering en vermijding is van cruciaal belang voor succesvol functioneren. In reactie op een sociale stimulus, bijvoorbeeld een boos kijkend gezicht (sociale dreiging), zal de amygdala in het brein razendsnel een evaluatie maken van de situatie en automatisch toenaderings- of vermijdingsgedrag in gang zetten via neurotransmitters en hormonen. De amygdala wordt hierbij gereguleerd door de prefrontale cortex, die het mogelijk maakt om deze automatische neigingen bij te sturen. De prefrontale cortex reguleert ook het ventrale striatum, belangrijk in beloning en de regulatie van sociaal gedrag, welke ook verbonden is met de amygdala. Wanneer deze complexe neurale netwerken niet in balans zijn is er kans op psychopathologie, wat het geval is bij SAD. Verschillende onderzoeken hebben laten zien dat bovengenoemde hersengebieden afwijkend reageren op sociale dreiging in SAD, wat er waarschijnlijk op wijst dat de amygdala een verhoogde verwerking heeft van deze dreiging, en dat de prefrontale cortex niet goed in staat is om de amygdala te reguleren. Recente onderzoeken laten ook zien dat de het ventrale striatum anders functioneert, wat er op kan wijzen dat mensen met SAD minder gevoelig zijn voor sociale beloning en juist meer gevoelig voor straf.

Testosteron beïnvloedt de werking van de amygdala en andere hersengebieden die betrokken zijn bij de regulatie van sociaal gedrag. Door testosteron wordt de amygdala meer geneigd tot het aansturen van toenaderingsgedrag en vermindert de remming door de prefrontale cortex. Er treden ook interacties op met andere hormonen en neurotransmitters, waarbij de dopamine-verhogende effecten van testosteron in het ventrale striatum kunnen leiden tot verhoogde gevoeligheid voor sociale beloning en toename in motivationeel gedrag. Onderzoek met testosterontoediening bij gezonde en angstige vrouwen heeft laten zien dat testosteron angstmakend werkt, gevoeligheid voor straf vermindert, de gevoeligheid voor beloning juist verhoogt en leidt tot toenaderingsgedrag dat gericht is op het verhogen van de sociale status.

SAD wordt gekenmerkt door verhoogde cortisolresponsen, hardnekkige sociale vermijding, verhoogde gevoeligheid voor dreiging en verlaagde gevoeligheid voor beloning, een gedragspatroon dat geassocieerd wordt met sociale onderdanigheid. Aan de andere kant zorgt testosteron juist voor een verhoging van sociale dominantie, wat inhoudt dat het angstmakend werkt, de gevoeligheid voor sociale beloning versterkt en toenaderingsgedrag bevordert. Aangezien testosteron en cortisol antagonistisch werken is het heel interessant om uit te zoeken welke rol testosteron speelt bij SAD. Onze hypothesen waren 1) testosteronspiegels zijn verlaagd in personen met SAD, vergeleken met gezonde personen 2) toediening van testosteron bij personen met SAD zal de sociale angst en vermijding verminderen. Daarbij hebben we gekeken naar neuro-endocriene mechanismen. We hebben dit onderzocht door te kijken naar testosteron in speeksel in een grote groep deelnemers en in een aantal andere studies waarin testosteron werd toegediend en het effect daarvan op sociale toenadering en vermijding werd gemeten.

Methoden

In hoofdstuk 4 werden basale testosteronspiegels bepaald in speeksel, deze zijn een afspiegeling van de actieve hoeveelheid testosteron in het bloed en zijn globaal gerelateerd aan psychologische eigenschappen en gedrag.

De toediening van testosteron in hoofdstuk 3, 5, 6 en 7 maakt het mogelijk om een causaal verband vast te stellen tussen een tijdelijke verhoging in testosteronspiegels en sociaal motivationeel gedrag en hersenactiviteit. Testosteron werd via de mond ingenomen en werd daar opgenomen in de bloedbaan. De farmacodynamiek van deze methode is alleen gevalideerd voor vrouwen, vandaar dat er alleen vrouwen deelnamen bij de toedieningsexperimenten die beschreven zijn in dit proefschrift.

De automatische neiging om iets toe te naderen of te vermijden kan objectief gemeten worden met een sociale approach-avoidance taak (AAT), welke ingezet is in hoofdstuk 2, 3 en 5. Deelnemers laten gezichten met een boze, lachende of neutrale

uitdrukking naar zich toe of van zich af bewegen op een computerscherm doordat ze een joystick naar zich toe trekken of van zich afduwen. Het toenaderen van lachende gezichten en het vermijden van boze gezichten is makkelijker, want dit stemt overeen met de automatische neigingen, en gaat dus sneller dan het toenaderen van boze en het vermijden van lachende gezichten. Mensen met sociale angst zijn over het algemeen sneller in het vermijden van boze gezichtsuitdrukkingen vergeleken met neutrale.

In hoofdstuk 7 werd vermijding van oogcontact gemeten door middel van een eye-tracker. Deze gebruikt infrarood licht dat reflecteert op de oogbal en geregistreerd wordt door een camera, veranderingen in reflecties geven informatie over waar het oog naar kijkt. Oogbewegingen kunnen onderverdeeld worden in fixaties, wanneer de blik op een bepaald punt rust voor 100ms, en saccades, de beweging van het oog tussen twee fixaties in.

Als laatste hebben we in hoofdstuk 8 elektro-encefalografie (EEG) gebruikt om vroege aandachtsprocessen te meten. Cognitieve processen in het brein veroorzaken elektrische potentiaalverschillen welke gemeten kunnen worden op de hoofdhuid. De analysetechniek die gebruikt is in dit proefschrift maakt het mogelijk om verschillende neurale bronnen en de sterkte van cognitieve processen te onderscheiden. De hersenactiviteit van deelnemers met en zonder SAD werd gemeten terwijl zij zo snel mogelijk moesten aangeven welke kleur een ovaal op een computerscherm had. Onbewust namen zij daarbij gezichten waar die heel kort voor de ovalen werden getoond. Deze gezichten hadden een boze, lachende of neutrale uitdrukking welke onbewust de reactiesnelheid van de deelnemers beïnvloedde.

Discussie

Onze eerste hypothese werd bevestigd door de bevindingen uit hoofdstuk 4: testosteronspiegels in speeksel zijn lager bij vrouwen met SAD (en bij depressie, gegeneraliseerde angststoornis en agorafobie) ten opzichte van vrouwen zonder voorgeschiedenis van angst en depressie. Daarbij bleken lagere testosteronspiegels samen te hangen met ernstiger sociale angst. De bevindingen in deze vrouwen bevestigen onze hypothese dat SAD samenhangt met lagere testosteronspiegels en zijn in lijn met eerder onderzoek in dieren en mensen dat lage testosteronspiegels verbindt met sociale onderdanigheid en vermijding. Daarbij past het in socio-neuroendocriene modellen van SAD die ponereren dat deze stoornis en bijbehorende hardnekkige vermijding ontstaan zijn vanuit sociale subordinatiestress. De groep mannen liet geen verlaagde testosteronspiegels zien in relatie tot SAD, dit kan komen door genetische factoren en door de twee keer zo kleine groepsgrootte, maar dit moet nog beter onderzocht worden.

De tweede hypothese werd getoetst in de hoofdstukken 3, 5 en 6, waarbij testosteroontoediening werd gecombineerd met twee verschillende maten voor sociaal toenaderings- en vermijdingsgedrag: de AAT (3, 5) en kijkgedrag (6).

Hoofdstuk 3 laat zien dat testosteron daadwerkelijk in staat is om automatische motivationele neigingen te beïnvloeden. Na inname van testosteron lieten gezonde vrouwen een duidelijke afname zien in de vermijding van boze gezichten, terwijl er geen verschil was in gedrag ten opzichte van lachende gezichten. Deze resultaten laten zien dat testosteron sociaal toenaderings- en vermijdingsgedrag kan beïnvloeden en de vermijding van sociale dreiging kan verminderen. Daarbij zijn ze in lijn met theorieën die dit hormoon verbinden met sociale dominantie, wat inhoudt dat het de motivatie om toe te naderen versterkt en angstremmend werkt. Hoofdstuk 5 trekt deze bevindingen door naar SAD en laat zien dat testosteron ook in staat is om de automatische neiging tot vermijding, die zo karakteristiek is voor deze stoornis, te beïnvloeden: na testosteroontoediening bleken de vrouwen een toename te laten zien in het toenaderen van boze gezichten, maar geen verschil in hun reactie op lachende gezichten.

In hoofdstuk 6 werd gekeken naar een andere maat van sociale vermijding. Het spontane kijkgedrag van vrouwen met SAD werd geregistreerd met een eye-tracker terwijl ze naar boze, lachende en neutraal kijkende gezichten keken. Testosteron verhoogde het aantal eerste fixaties naar de ogen van deze gezichten bij de deelnemers met SAD ten opzichte van de gezonde controle deelnemers. Daarbij lieten de deelnemers met SAD meer vermijding van oogcontact zien wanneer ze meer sociale angst klachten hadden, wat de klinische relevantie van deze maat benadrukt. Deze resultaten suggereren dat testosteron inderdaad de vermijding van oogcontact kan verminderen bij SAD, door de angstremmende en sociale dominantie-verhogende werking.

De gezonde deelnemers lieten een ander kijkpatroon zien dan de deelnemers met SAD: na testosteroontoediening hadden ze de neiging om juist minder naar de ogen van de getoonde gezichten te kijken. Dit effect zou verklaard kunnen worden vanuit eerder waargenomen effecten van testosteron. Afhankelijk van de sociale context kan testosteron mensen minder sociaal maken en een egocentrische neiging om dominant te zijn bevorderen. De precieze oorzaak van het verschil in resultaten tussen de gezonde en SAD groepen is onduidelijk en er ligt een taak voor toekomstig onderzoek om dit te ontrafelen.

Bijelkaar genomen bevestigen de hoofdstukken 3, 5 en 6 onze tweede hypothese en laten zien dat testosteron inderdaad in staat is om sociale vermijding te verminderen en sociale toenadering te bevorderen bij vrouwen met SAD. Toekomstig onderzoek bij mannen zal moeten uitwijzen of dit ook zo uitwerkt voor deze groep. Op basis van eerder onderzoek valt te verwachten dat de effecten van testosteron op sociaal toenaderings- en vermijdingsgedrag bij mannen vergelijkbaar zullen zijn.

Als derde zijn de onderliggende neuro-endocriene mechanismen van bovenstaande processen onderzocht. Hoofdstuk 7 laat zien dat testosteron specifiek vroege (<200ms)

verwerkingsprocessen beïnvloedt en dat dit hetzelfde is voor vrouwen met en vrouwen zonder SAD. Daarbij wordt de verwerking van sociaal dreigende gezichten (boze) en niet-dreigende (neutraal en lachend) gezichten verschillend beïnvloed door testosteron. De resultaten laten zien dat een initiële verhoogde verwerking van sociale dreiging vermindert wordt door testosteron, wat waarschijnlijk te verklaren valt vanuit de angstremmende werking van dit hormoon. Deze processen vormen mogelijk de basis van de hierboven beschreven effecten van testosteron op sociaal toenaderings- en vermijdingsgedrag en laten zien dat deze modulaties door testosteron al optreden gedurende de vroegste verwerkingsprocessen.

Een ander belangrijk werkingsmechanisme van testosteron is het verhogen van beloningsgevoeligheid, onder andere middels een toename van dopamine spiegels in het ventrale striatum. In hoofdstuk 2 laten we zien dat meer dopamine in het striatum samenhangt met verhoogde toenaderings- en vermijdingsreacties. Dit gedrag wordt geassocieerd met een hogere gevoeligheid voor sociale beloning en laat zien dat de striatale dopamine transmissie inderdaad een rol speelt in de regulatie van sociaal toenaderings- en vermijdingsgedrag.

Samengenomen ziet het er naar uit dat de neuro-endocrine mechanismen achter onze bevindingen van testosteron op sociaal vermijdingsgedrag liggen in veranderingen in de verwerking van sociale dreiging door de amygdala en frontale-striatale netwerken. Testosteron zorgt voor een verhoogde aansturing van toenaderingsgedrag ten opzichte van sociale dreiging door de amygdala, vermindert prefrontale controle en verhoogt de gevoeligheid voor beloning. Deze werkingsmechanismen zijn met name interessant voor SAD, waarbij de automatische neiging tot sociale vermijding versterkt is, en de gevoeligheid voor sociale beloning vermindert. Onze EEG bevindingen suggereren dat testosteron al inwerkt op heel vroege verwerkingsprocessen en dat afwijkingen in verwerking die samenhangen met psychopathologie pas later optreden. Toekomstig onderzoek zal moeten uitwijzen hoe neuro-endocrine processen precies sociaal toenaderings- en vermijdingsgedrag beïnvloeden in SAD, en waar de verschillen optreden met gezonde personen.

De bevindingen in dit proefschrift kunnen, behalve bijdragen aan kennis en inzicht, mogelijk ook van belang zijn voor de behandeling van SAD. Wanneer SAD niet behandeld wordt, kan dit leiden tot ernstige beperkingen in sociaal functioneren. Exposure therapie is een belangrijk onderdeel van de behandeling en is erop gericht sociale angst en vermijding van sociale situaties te verminderen. Deze therapie helpt helaas niet alle patiënten, dus er wordt gezocht naar methoden om de effectiviteit te verbeteren. Aangezien testosteron sociale vermijding vermindert en toenadering-bevorderend werkt, zou het interessant zijn om te onderzoeken of toediening van testosteron voorafgaand aan een exposure-sessie de therapie effectiever maakt. Een andere benadering die onderzocht

zou kunnen worden is het met testosteron verhogen van de effectiviteit van het trainen van toenaderingsgedrag op de AAT als middel om de vermijding in SAD te verminderen.

Conclusie

Het doel van dit proefschrift was het verwerven van meer kennis over de rol van testosteron bij sociale angst. Onze bevindingen laten zien dat testosteronspiegels bij vrouwen met SAD verlaagd zijn en gerelateerd aan sociale angstklachten. Bovendien lieten we zien dat toediening van testosteron aan vrouwen met SAD in staat is om vermijding te verminderen en sociale toenadering te bevorderen. Deze bevindingen passen in de socio-neuroendocriene modellen van SAD en bevestigen de rol van testosteron in sociaal motivationeel gedrag. Daarbij ondersteunt de vinding dat testosteron vroege aandachtsprocessen beïnvloedt de theorie dat dit hormoon inwerkt op automatische motivationele processen. De bevindingen kunnen mogelijk bijdragen aan een effectievere behandeling van SAD.



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Curriculum Vitae



Dorien Enter was born on the 12th of July 1980 in Hoogeveen, The Netherlands. In 1998 she started studying Biology at Utrecht University. In her fourth year, she did a first internship in Neurotoxicology, featuring molecular genetics and electrophysiological techniques, but decided that this kind of research was, though interesting, not her cup of tea. After a road trip in the USA, she started a second internship at the Faculty of Social Sciences, section of Psychonomics. She was involved in a study by dr. Dennis Schutter, which applied Transcranial Magnetic Stimulation to investigate the role of the prefrontal cortex in psychophysiological affective reactivity. This internship fostered her enthusiasm for scientific research, and was followed by a research assistantship and a master's thesis on the neurobiology of music experience.



In 2008 she was very happy to be able to start the PhD project featured in this thesis at the department of Clinical and Health Psychology, Leiden University, under the supervision of prof. dr. Karin Roelofs and prof. dr. Philip Spinhoven. This project moved with the lab of Karin Roelofs to the Behavioral Science Institute at the Radboud University Nijmegen in 2011. Besides research, Dorien also loves dancing, painting, reading, hiking, and exploring the world.



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