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ADORA2A Gene Variation, Caffeine, and Emotional Processing: A Multi-level Interaction on Startle Reflex

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There is converging evidence for genetic, biochemical, and neuropsychological factors to increase the risk for anxiety and anxiety disorders. The pathogenesis of anxiety disorders is assumed to be influenced by a complex interaction of these individual risk factors on several levels, affecting intermediate phenotypes of anxiety such as the startle reflex. Thus, in the present double-blind, placebo-controlled study we attempted to paradigmatically investigate a multi-level pathogenetic model of anxiety by testing the effect of 300 mg caffeine citrate as an antagonist at the adenosine A2A receptor *vs* placebo on the emotion-potentiated (unpleasant, neutral, and pleasant International Affective Picture System pictures) startle reflex in 110 healthy individuals (male = 56, female = 54) stratified for the adenosine A2A receptor (*ADORA2A*) 1976T > C polymorphism (rs5751876). In addition to the expected main effect of picture category (highest startle amplitude for unpleasant, lowest for pleasant pictures) groups across all *ADORA2A* 1976T > C genotype and intervention (caffeine *vs* placebo) groups, an interaction effect of genotype, intervention, and picture category was discerned: In *ADORA2A* 1976TT risk genotype carriers, highest startle magnitudes were observed after caffeine administration in response to unpleasant pictures, with this effect arising particularly from the female subgroup. Our data point to a complex, multi-level, and potentially gender-specific pathogenetic model of anxiety, with genetic and biochemical factors interactively increasing the risk of maladaptive emotional processing and thereby possibly also anxiety disorders. The present findings may eventually aid in improving primary and secondary prevention by sharpening the risk profiles of anxiety-prone individuals.
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INTRODUCTION

Anxiety disorders such as panic disorder, generalized anxiety disorder, social phobia, and specific phobias are common diseases with a 12-month prevalence of about 12–14% (cf. Wittchen and Jacobi, 2005; Wittchen *et al*, 2011). The pathogenesis of pathological anxiety and anxiety disorders, respectively, has been suggested to be multifactorial, with a complex interaction of genetic risk factors, biochemical alterations, for example, of the adenosinergic system, and neuropsychological factors such as perception and processing of anxiety-relevant emotional stimuli. Association of each of these single risk factors with anxiety or anxiety disorders has repeatedly been demonstrated in previous studies:

(1) Family and twin studies report a strong genetic contribution to the development of anxiety disorders, with an estimated heritability between 28 and 48% (Hettema *et al*, 2001). Association studies of anxiety disorders and panic disorder have yielded evidence for several genes to increase the disease risk (for review see Jacob *et al*, 2010). Particularly, the adenosine A2A receptor gene (*ADORA2A*) has been suggested to have a pivotal role in the pathogenesis of anxiety and anxiety disorders in that the T allele of a silent polymorphism in exon-2 of the *ADORA2A* gene located on chromosome 22q11.23 (SNP rs5751876, 1976T > C, formerly 1083T > C, Tyr/Tyr) has been found to be associated with panic disorder mainly in Caucasian populations (Deckert *et al*, 1998; Hamilton *et al*, 2004; Lam *et al*, 2005; but Yamada *et al*, 2001). Additionally, the *ADORA2A* 1976T

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allele was associated with sympathetic psychophysiological indicators of anxiety-related arousal in blood-injury phobia (Hohoff *et al*, 2009). *ADORA2A* gene variation has furthermore been found to influence anxiety levels also in other psychiatric phenotypes such as autism spectrum disorder (Freitag *et al*, 2010) and in healthy individuals (Hohoff *et al*, 2010). These findings are in line with animal studies, where *ADORA2A*-knockout mice show increased anxiety levels (Ledent *et al*, 1997), suggesting a genetically driven reduced adenosine A2A receptor-mediated neurotransmission as a risk factor for anxiety (cf. Deckert, 1998).

(2) Caffeine exerts its arousal-promoting and potentially also anxiogenic effect through antagonism at adenosine A2A receptors (Huang *et al*, 2005). Consistently, caffeine has been shown to act as a potent anxiogenic challenge substance in the rodent model (eg, Bhattacharya *et al*, 1997) as well as in patients with anxiety disorders, first-degree relatives of patients with panic disorders, and healthy subjects (Boulenger *et al*, 1984; Bruce *et al*, 1992; Charney *et al*, 1985; Lee *et al*, 1998; Nardi *et al*, 2007, 2008, 2009; Nickell and Uhde, 1994). Also, an increase in acoustic startle reflex amplitude and a delayed habituation of acoustic startle blink amplitude as physiological correlates of anxiety have been observed after caffeine administration (Andrews *et al*, 1998; Blumenthal *et al*, 2005; Schicatano and Blumenthal, 1995). Reciprocally, caffeine abstinence has been reported to be beneficial as an adjunct in the treatment of anxiety disorders (eg, Bruce and Lader, 1989; Smith, 1988). In several twin studies, caffeine response has been shown to be considerably heritable (eg, Kendler and Prescott, 1999). Indeed, first molecular genetic studies investigating the impact of *ADORA2A* gene variation on anxiety after caffeine intervention demonstrated higher self-reported anxiety levels upon caffeine administration dependent on the *ADORA2A* 1976T risk allele (Alsene *et al*, 2003; Childs *et al*, 2008; Rogers *et al*, 2010). The *ADORA2A* 1976 genotype has furthermore been shown to drive subjective and objective responses to caffeine during sleep (Retey *et al*, 2007) as well as to amphetamines (Hohoff *et al*, 2005; for review see Yang *et al*, 2010).

(3) Converging evidence points toward a crucial role of unflexible and maladaptive processing of emotional stimuli in the pathogenesis of anxiety and anxiety disorders: anxiety-related emotional stimuli are rated as more threatening by patients than by control individuals and seem to be processed differently within the limbic-medial prefrontal circuit, partly driven by genetic factors (eg, Domschke *et al*, 2006, 2008b; Domschke and Dannlowski, 2010; Etkin, 2010; Lang and Cuthbert, 1984; Mathews, 1993; Mühlberger *et al*, 2006, 2007; Pauli *et al*, 1996; Wiedemann *et al*, 1999).

While there is support for each of those individual risk factors in anxiety as well as first evidence for two-way interactions between *ADORA2A* gene variation and caffeine consumption (eg, Alsene *et al*, 2003; Childs *et al*, 2008; Rogers *et al*, 2010), the complex interplay between all of these factors, including emotionally relevant stimuli, remains to be elucidated. Also, previous studies have mainly focused on subjective measures of anxiety as their primary outcome measure. It has been proposed, though, that intermediate phenotypes (synonymously used with the term endophenotypes), which are more narrowly and precisely defined than the complex overall phenotype (cf. Flint and Munafo, 2007; Gottesman and Gould, 2003), might be more apt to objectively capture the effect of risk factors in complex genetic phenotypes such as anxiety and anxiety disorders. The emotion-potentiated startle reflex with its characteristic amplification by unpleasant emotional stimuli (for review see Filion *et al*, 1998; Grillon and Baas, 2003; Grillon, 2008) has repeatedly been suggested as a promising objective intermediate phenotype of anxiety and anxiety disorders (eg, Butler *et al*, 1990; Filion *et al*, 1998; Grillon, 2002, 2008; Grillon *et al*, 1994, 1998; Grillon and Baas, 2003).

Thus, given evidence from previous studies for (1) a pivotal role of the *ADORA2A* gene in the pathogenesis of anxiety and anxiety disorders; (2) an anxiogenic effect of caffeine, which seems to be moderated by variation in the *ADORA2A* gene; and in addition (3) a maladaptive emotional reactivity in the development of pathological anxiety, the present study aims at investigating the multilevel interaction of these three risk factors on a genetic level (*ADORA2A* 1976T > C genotype), a biochemical level (placebo-controlled caffeine intervention), and the neuropsychological level of emotional processing (emotional stimuli) on the startle reflex as an objective intermediate phenotype of anxiety disorders. Our *a priori* hypothesis was that all three individual risk factors would interact synergistically in increasing startle response.

MATERIALS AND METHODS

Sample

A sample of 126 (male = 60, female = 66; mean age: 26.01 years, SD: 5.75) unrelated healthy subjects was consecutively recruited at the Department of Psychiatry, University of Muenster, and Department of Psychiatry, University of Wuerzburg, Germany, between 2009 and 2010. In order to minimize the risk of ethnic stratification, Caucasian descent was ascertained by Caucasian background of both parents. Current or prior diagnosis of DSM-IV axis-I disorders were excluded by using the Mini-International Neuropsychiatric Interview (M.I.N.I.; Sheehan *et al*, 1998). Additionally, anxiety sensitivity (AS), the general tendency to fear anxiety-related symptoms, was recorded by the German version of the Anxiety Sensitivity Index (ASI, German version; Alpers and Pauli, 2002; Reiss *et al*, 1986). To exclude any neurological or other somatic disorders, subjects underwent a physical and neurological examination in a screening session 1 week before the experiment, where additionally heart activity (electrocardiogram) and basic blood parameters were checked. Further exclusion criteria comprised caffeine or lactose intolerance, high and frequent caffeine consumption (more than three cups of coffee per day), illegal drug consumption (assessed by a urine drug screening for amphetamine, barbiturates, benzodiazepines, cocaine, ecstasy, methamphetamine, methadone, opiates, tricyclic antidepressants, tetrahydrocannabinol), alcohol consumption of more than 140 g per week (equivalent to about 15–20 U of alcohol), daily smoking of more than 20 cigarettes a day (smoking reduces the half-life of caffeine; Hart *et al*, 1976), daily use of any medication (except for hormonal contraception), pregnancy (assessed by a rapid urine pregnancy test) or breast feeding, less than a high school education, age under 18 and over 50 years, and left handedness. The subjects were asked to

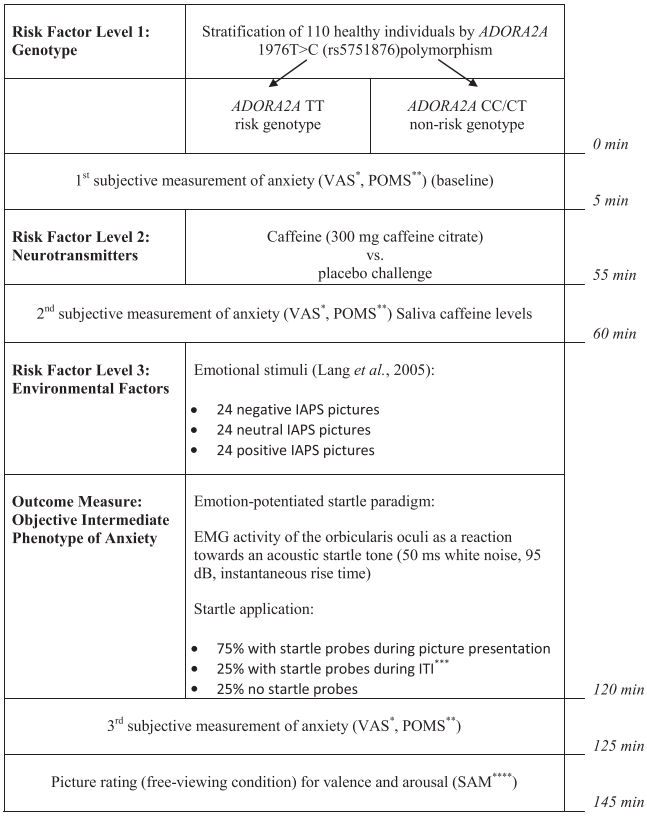

Figure 1 Study design. EMG, electromyogram; ITI, inter-trial interval; POMS, Profile of Mood States; SAM, Self-Assessment Manikin; VAS, Visual Analogue Scale.

refrain from caffeine or tea consumption for 1 week prior to caffeine intervention and not to smoke, consume alcohol (assessed by a breath test), or take any medication for at least 24 h prior to the investigation. The protocol was approved by the ethics committees of the University of Muenster, and the University of Wuerzburg, Germany, and written informed consent from all subjects was obtained during the screening session. The design of the study is illustrated in Figure 1.

Genotyping

Subjects were genotyped for the *ADORA2A* 1976T > C (rs5751876) polymorphism according to published protocols (see Alsene *et al*, 2003; Deckert *et al*, 1998). According to previous findings, subjects were stratified into risk (TT) and non-risk (CT/CC) genotype carriers (cf. Alsene *et al*, 2003; Childs *et al*, 2008; Deckert *et al*, 1998; Rogers *et al*, 2010).

Caffeine Intervention

The study used a one-session, double-blind, placebo-controlled, between-subject design. All experimental sessions were conducted from 0815 to 1130 hours. After a negative drug and pregnancy urine test all electrodes were fixed and checked for impedances below 5 kΩ. Caffeine intervention was performed by oral administration of 300 mg caffeine citrate (Fagron, Barsbuettel, Germany; equivalent to 150 mg freebase caffeine), which has been shown to be close to the threshold for producing anxiogenic effects and as such might be the optimal dose to detect subtle genotype effects (cf. Alsene *et al*, 2003; Childs *et al*, 2008; Rogers *et al*, 2010). Caffeine citrate was administered in white opaque gelatin capsules; placebo capsules contained mannitol and aerosil (99.5 : 0.5) according to the German Drug Law (Arzneimittelgesetz; AMG). At about 0900 hours—after the first self-report anxiety measurement—subjects were administered a placebo or caffeine capsule with a glass of water. To assure correct implementation of the caffeine/placebo intervention, caffeine levels were determined by saliva test (0.5 ml) 55 min after caffeine administration. All subjects with a concentration differing more than one and a half interquartile ranges from the respective median (placebo or verum distribution) were excluded from further analysis.

Saliva samples were immediately frozen and analyzed not until all subjects were tested. Saliva caffeine levels were analyzed by means of reversed-phase, high-performance liquid chromatography.

Emotionally Relevant Stimuli

Twenty-four emotionally threatening unpleasant images taken from the International Affective Picture System (IAPS; Lang *et al*, 2005) were selected as anxiety-relevant emotional cues along with 24 neutral and 24 pleasant IAPS pictures (unpleasant IAPS pictures: 3000, 3053, 3170, 3102, 9410, 3080, 6313, 3120, 3130, 3071, 3100, 3010, 3060, 3064, 3140, 3110, 3150, 9300, 2800, 3030, 6540, 9252, 9250, 9040; neutral IAPS pictures: 2200, 2880, 5510, 5531, 7002, 7004, 7006, 7009, 7010, 7025, 7034, 7050, 7080, 7090, 7100, 7130, 7175, 7185, 7217, 7224, 7950, 2215, 5535, 7031; pleasant IAPS pictures: 1710, 2091, 2160, 2216, 2340, 2345, 4608, 4626, 4641, 8120, 5623, 5831, 5833, 8041, 8370, 8200, 8210, 8461, 8496, 5814; for men: 4220, 4290, 4607, 4680; for women: 4550, 4658, 4687, 5631). Ninety-five percent of all pictures were exactly the same for both genders, whereas different erotic pictures were chosen for men and women to ensure comparable valence and arousal levels.

Objective Outcome Measure: Emotion-Potentiated Startle Paradigm

At the assumed maximum plasma level of caffeine and the described time to peak increases in self-report ratings of anxiety, respectively (60 min after administration; Alsene *et al*, 2003; Childs *et al*, 2008; Rogers *et al*, 2010), the emotion-potentiated startle experiment was started (at 1000 hours). In order to get subjects used to the startle stimulus (50 ms of 95-dB white noise with an instantaneous rise-time presented through Bose Around-Ear Headphones) and to prevent outlier startle responses during the critical trials, eight startle stimuli at random intervals of 1–12 s were presented. The startle experiment *per se* consisted of three blocks of 24 anxiety-relevant, neutral, or pleasant IAPS pictures, respectively, as described above, and 3-min breaks between the blocks. An experimental block contained eight pictures of each of the three categories in random order, with the constraint that no two of the same type (unpleasant, neutral, or pleasant) were presented successively. During the experiment, in a dimly lit room subjects sat in a recliner, which was separated by a room divider from the experimenter. Visual stimuli were presented for 8 s

(inter-trial interval (ITI): mean = 21 s, range = 16.5–25.5 s) on a 19″ LCD computer screen approximately 1 m away from the subject. Startle probes were administered 2.5, 4, or 5.5 s after picture onset during picture presentation, and 10 as well as 12 s after picture offset during the ITI. In each block as well as in the overall experiment, 75% of all trials contained startle probes during picture presentation (evenly distributed across each picture category), 12.5% of all trials contained startle probes during the ITI, and 12.5% of the trials did not contain any startle probe.

Electromyogram activity of the M. orbicularis oculi, which is responsible for eyelid closure, was measured as a commonly used variable for recording startle reactions in humans (Blumenthal *et al*, 2005). For this purpose, two transparent pediatric 13-mm electrodes were placed under the left eye. The reference electrode was placed on the forehead, 2 cm beneath the hairline, and the ground electrode was placed on the processus mastoideus behind the left ear (sintered Ag/AgCl electrodes). Electromyogram activity was recorded by using V-Amp 16 (Brain Products GmbH, Gilching, Germany), a 16-channel DC amplifier system using the BrainVision Recorder Software (V-Amp Edition 1.10; Brain Products GmbH). Sampling rate was 1000 Hz and an online notch filter of 50 Hz was applied. Stimuli were presented by using the software package Presentation (v13.0; Neurobehavioral Systems, Albany, CA). BrainVision Analyzer 2 (Brain Products GmbH) was used as an offline analyzing software with which the signals were rectified, filtered (low cut-off, 28 Hz; high cut-off, 500 Hz; notch, 50 Hz), and smoothed (using a time constant of 50 ms). Startle magnitude was quantified as the difference between the highest peak 21–200 ms after and the average during 50 ms before startle probe presentation. Startle data were checked for zero responses and artifacts in each subject. Startle reactions with no detectable responses ($<5\,\mu V$) were scored as 0. Artifacts were defined as spontaneous eye blinks during baseline or within 20 ms after startle probe onset, and were scored as missing values. Subjects were excluded from data analysis when having too many zero responses (more than 2.5 SDs above mean number of zero responses) or less than three valid startle responses in one picture category. All startle magnitudes were T-transformed within subjects in order to assure comparability of data (for methodical overview Blumenthal *et al*, 2005; Mühlberger *et al*, 2008; Pauli *et al*, 2010).

After the experiment, electrodes were removed and subjects were given the possibility to clean their faces from the electrode paste and have a short break. Finally, each subject had to rate all pictures in a free-viewing condition by valence (1 = highly pleasant, 9 = highly unpleasant) and arousal (1 = excited, 9 = calm) by using Self-Assessment-Manikin (SAM) scales (Lang, 1980). At about 1130 hours subjects were discharged by a physician and paid an allowance of 100€.

Subjective Outcome Measures: VAS and POMS

Subjects were asked to rate their anxiety level by placing a mark on a visual analogue scale (VAS) consisting of a 100 mm horizontal line labeled 'not at all anxious' (score of 0) and 'extremely anxious' (score of 100). Additionally, the 35-item German version of the Profile of Mood States (POMS) was administered, on which subjects report their current mood on a seven-point scale from 'not at all' (score of 0) to 'extremely' (score of 6) (Biehl *et al*, 1986; McNair *et al*, 1992). Four categories have been distinguished within the German version of the POMS: (1) 'Depression–Anxiety,' (2) 'Fatigue,' (3) 'Vigor,' and (4) 'Hostility' (cf. Albani *et al*, 2005). In the present study, the subscale 'Depression–Anxiety' was used as a subjective measure of anxiety. VAS and POMS measurements were taken at three points in the course of the study: (1) before the respective intervention (caffeine/placebo); (2) at maximum caffeine plasma level and the described time to peak increases in self-report anxiety, respectively, (60 min; Alsene *et al*, 2003); and (3) after emotion-potentiated startle.

Statistical Analysis

Sample characteristics were evaluated by χ^2 tests for genotype (*ADORA2A* 1976TT risk vs. 1976CC/CT non-risk genotypes) and gender with intervention (caffeine versus placebo) as between-subjects-factor as well as by one-way ANOVAs for caffeine consumption, age and anxiety sensitivity with intervention, genotype, and gender as between-subjects-factors.

VAS and POMS ratings were analyzed by ANOVAs for repeated measures, with genotype and intervention (caffeine *vs* placebo) as between-subject factors, and measurement time (three measurement times: before substance intake, 1 h after substance intake, and after the startle experiment) as a within-subject factor. Baseline startle response (assessed in the ITIs) was analyzed by ANOVA for repeated measures, with measurement time (the 12 ITI startle responses were divided into four measurement times (T1–T4) each being the mean of three consecutive startle responses) as a within-subject factor and genotype and intervention as between-subject factors. Picture ratings, which were conducted at the end of the startle experiment (see Figure 1), and picture viewing times—defined as time periods between picture onset and consecutive ratings—were analyzed by ANOVA for repeated measures, with genotype and intervention as between-subject factors, and picture category (unpleasant, neutral, and pleasant) as a within-subject factor. Pairwise comparisons of picture valence or time of measurement were assessed by *post-hoc t*-tests.

According to *a priori* hypotheses, the main multi-level analysis of emotion-potentiated startle response was performed by ANOVA for repeated measures, with genotype and intervention as between-subject factors, and picture category (unpleasant, neutral, and pleasant) as a within-subject factor. Picture categories were further analyzed by *post-hoc* univariate ANOVAs.

Further explorative analyses were performed by using gender and AS (median split) as additional factors for all above mentioned ANOVAs.

Alpha level was set at 5% using Greenhouse–Geisser corrections where appropriate.

RESULTS

Sample Characteristics

Eight subjects of initially 126 recruited subjects showed too many zero startle responses (>35; mean zero responses per

subject in the whole group: 5.7, SD: 11.5; see Materials and Methods) and were therefore excluded from further analyses. Eight additional subjects were excluded because of unexpected saliva caffeine concentrations (placebo group: 7 subjects, mean concentration: 72.2 mg/l, SD: 31.9; verum group: 1 subject, mean concentration: 173.4 mg/l; see Materials and Methods) not consistent with caffeine abstinence prior to the experiment.

The remaining sample of 110 subjects was equally distributed regarding genotype (*ADORA2A* 1976TT *vs* 1976CC/CT) and gender across intervention groups (caffeine *vs* placebo; both $\chi^2(1) < 0.17$, $p > 0.68$; see Table 1).

One-way ANOVA of mean caffeine consumption (calculated in mg/day, with one cup of coffee corresponding to 100 mg of caffeine) revealed no differences between genotypes, intervention groups, or gender (all $F(1, 109) < 2.01$, $p > 0.16$). Comparing caffeine consumers ($n = 83$) with caffeine non-consumers ($n = 26$) a marginally significant association between caffeine consumption and genotype was identified ($\chi^2(1) = 3.84$, $p = 0.05$), with a 2.45 higher odds of being an *ADORA2A* 1976TT risk genotype carrier in caffeine non-consumers than in caffeine consumers, and a trend for an association between caffeine consumption and gender ($\chi^2(1) = 3.42$, $p = 0.06$), with the odds of being a non-consumer being 2.35 times higher in women than in men. No association was seen between caffeine consumption and intervention condition ($\chi^2(1) = 0.08$, $p = 0.77$).

Factorial ANOVA revealed that neither genotype nor intervention condition or gender groups differed in age (all $F(1, 102) < 2.64$, $p > 0.11$) or AS (AS; $F(1, 102) < 2.13$, $p > 0.15$). Mean AS was 13.4 (SD: 6.6; range: 3–35; median: 13), which is lower than the expected mean AS of about 18 in a non-clinical population (Peterson and Reiss, 1992).

Picture Ratings and Viewing Times

Valence ratings corresponded to *a priori* categories ($F(2, 212) = 3000.06$, $p < 0.001$; linear trend, $F(1, 99) = 3714.12$, $p < 0.001$; pleasant > neutral > unpleasant: all $t(109) > |33.02|$, $p < 0.001$). There were no direct or interaction effects of genotype or intervention on valence ratings (all $F(2, 212) < 2.86$, $p > 0.08$).

Arousal ratings were affected by picture valence ($F(2, 212) = 1146.89$, $p < 0.001$), with highest ratings for unpleasant pictures, followed by relatively high ratings for pleasant pictures and low ratings for neutral pictures (all $t(109) > |18.85|$, $p < 0.001$). There were no direct or interaction effects of genotype or intervention on arousal ratings (all $F(2, 212) < 1.18$, $p > 0.30$).

Analysis of picture viewing times revealed no significant effect of picture category or additional interactions of genotype or intervention (all $F(2, 210) < 0.77$, $p > 0.46$) (One additional subject had to be excluded from this analysis because of technical problems during recording of picture viewing times.). However, there was a main effect of intervention ($F(2, 210) = 5.28$, $p = 0.02$), with higher overall viewing times under caffeine as compared with placebo ($t(107) = -2.35$, $p = 0.02$).

Table 1 Sample Characteristics

Intervention	*ADORA2A* 1976T > C genotype	Gender		Total
		Men	Women	
Placebo	Risk (TT)	*n* = 13	*n* = 13	*n* = 26
		Age = 28.0 *(8.68)*	Age = 24.1 *(3.25)*	Age = 26.1 *(6.72)*
		ASI = 14.2 *(7.89)*	ASI = 15.6 *(6.85)*	ASI = 14.9 *(7.27)*
		cc = 151.7 mg/day *(141.76)*	cc = 111.5 mg/day *(96.08)*	cc = 131.6 mg/day *(120.39)*
	Non-risk (CC/CT)	*n* = 15	*n* = 12	*n* = 27
		Age = 25.7 *(4.77)*	Age = 27.3 *(7.68)*	Age = 26.4 *(6.16)*
		ASI = 13.9 *(5.93)*	ASI = 12.6 *(7.35)*	ASI = 13.3 *(6.50)*
		cc = 140.0 mg/day *(82.81)*	cc = 91.7 mg/day *(70.17)*	cc = 118.5 mg/day *(79.84)*
Verum	Risk (TT)	*n* = 14	*n* = 13	*n* = 27
(300 mg caffeine citrate)		Age = 27.2 *(3.26)*	Age = 25.3 *(7.30)*	Age = 26.3 *(5.55)*
		ASI = 13.5 *(5.93)*	ASI = 14.2 *(4.36)*	ASI = 13.9 *(5.15)*
		cc = 113.2 mg/day *(103.34)*	cc = 111.5 mg/day *(129.35)*	cc = 112.4 mg/day *(114.29)*
	Non-risk (CC/CT)	*n* = 14	*n* = 16	*n* = 30
		Age = 28.8 *(6.17)*	Age = 25.3 *(2.73)*	Age = 27.2 *(4.84)*
		ASI = 12.4 *(6.57)*	ASI = 11.0 *(7.49)*	ASI = 11.7 *(6.99)*
		cc = 137.4 mg/day *(108.02)*	cc = 112.5 mg/day *(95.74)*	cc = 123.7 mg/day *(100.35)*
Total		*n* = 56	*n* = 54	*n* = 110
		Age = 27.4 *(5.93)*	Age = 25.6 *(5.48)*	Age = 26.5 *(5.76)*
		ASI = 13.5 *(6.44)*	ASI = 13.2 *(6.72)*	ASI = 13.4 *(6.55)*
		cc = 135.3 mg/day *(107.73)*	cc = 107.4 mg/day *(97.81)*	cc = 121.5 mg/day *(103.41)*

Abbreviations: ASI, anxiety sensitivity index; cc, caffeine consumption.
Standard deviation in parentheses.

Habituation and Baseline Startle Response

Analysis of the startle response during the inter-trial interval revealed a significant effect of measurement time on startle magnitude ($F(3,306)=51.31$, $p<0.001$): Mean baseline startle magnitudes declined across the first three measurement times (all $t(105)>4.72$, $p<0.001$), with no difference between the third and the fourth measurement time ($t(105)=0.78$, $p=0.43$) (Four additional subjects had to be excluded from this analysis because of consecutive zero responses, leading to a missing mean in one of the four measurement times (see Materials and Methods)). There was no genotype or intervention effect, or a genotype × intervention interaction effect, on baseline startle response times (all $F(3,306)<1.35$, $p>0.25$).

Startle Modulation: Influence of Picture Category

ANOVA revealed a significant main effect of picture category on startle response ($F(2,212)=23.51$, $p<0.001$), which was due to increasing startle magnitudes from pleasant to neutral to unpleasant pictures (linear trend, $F(1,106)=44.99$, $p<0.001$; each $t(109)>2.81$, $p<0.007$; see Figure 2).

Genotype Effects on Startle Modulation

There was no significant main effect of genotype on mean startle magnitudes ($F(1,106)=0.65$; $p=0.42$), and no significant interaction effect of genotype (*ADORA2A* 1976TT *vs* 1976CC/CT) and picture category could be discerned ($F(2,212)=0.003$, $p=0.99$).

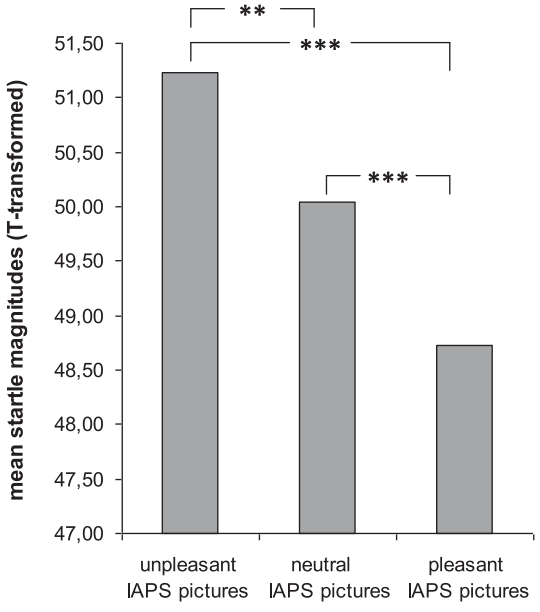

Figure 2 Mean startle magnitude modulated by picture category. Mean startle magnitude was significantly modulated by picture category ($F(2,212)=23.51$, $p<0.001$), with decreasing magnitudes from unpleasant to neutral to pleasant pictures (linear trend, $F(1,106)=44.99$, $p<0.001$; each $t(109)>2.81$, $p<0.007$). **, significant at a significance level of $p\leqslant 0.01$; ***, significant at a significance level of $p\leqslant 0.001$.

Effects of Intervention (Caffeine *vs* Placebo) on Startle Modulation

There was no significant main effect of intervention (caffeine *vs* placebo) on mean startle magnitudes ($F(1,106)=0.43$; $p=0.51$). Also, no significant interaction effect of intervention (caffeine *vs* placebo) and picture category was observed ($F(2,212)=0.04$, $p=0.95$).

Genotype × Intervention Effects on Startle Modulation

A significant interaction between genotype, intervention, and picture category was discerned ($F(2,212)=3.84$, $p=0.02$; see Figure 3). *Post-hoc* separate analyses for each genotype revealed that under placebo *ADORA2A* TT risk genotype carriers did not show significant differences in startle magnitude between unpleasant and neutral pictures ($t(25)=0.24$, $p=0.82$), but between neutral and pleasant ($t(25)=2.83$, $p=0.01$), as well as between unpleasant and pleasant pictures ($t(25)=3.07$, $p=0.005$), whereas in the caffeine condition, subjects carrying the *ADORA2A* TT risk genotype showed significant differences in startle magnitude between unpleasant and neutral pictures ($t(26)=2.38$, $p=0.03$) as well as between unpleasant and pleasant pictures ($t(26)=3.77$, $p=0.001$), but not between neutral and pleasant pictures ($t(26)=1.18$, $p=0.25$).

Vice versa, under placebo *ADORA2A* CC/CT non-risk genotype carriers showed significant differences between unpleasant and neutral ($t(26)=3.19$, $p=0.004$) as well as

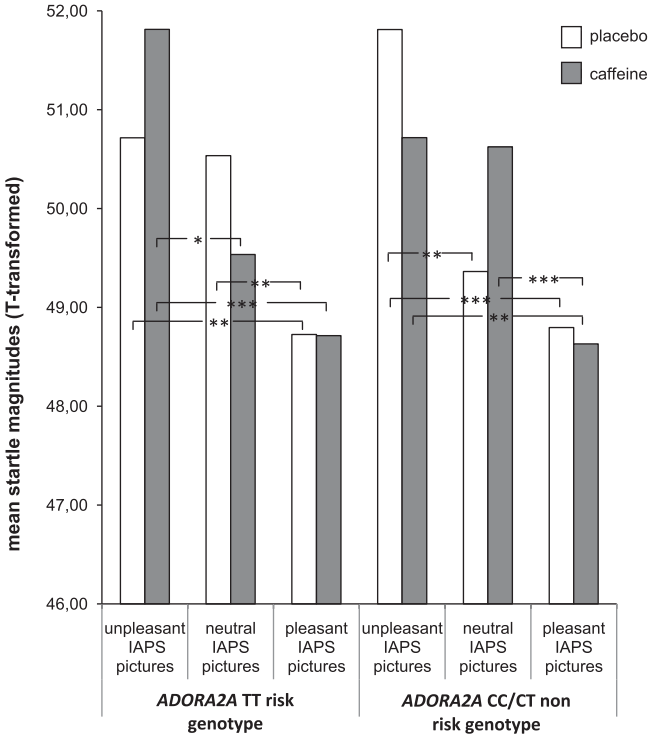

Figure 3 Multifactorial startle modulation by genotype (*ADORA2A* 1976T>C), intervention (caffeine *vs* placebo), and picture category. A significant interaction between genotype, intervention, and picture category was discerned ($F(2,212)=3.84$, $p=0.02$). *, significant at a significance level of $p\leqslant 0.05$; **, significant at a significance level of $p\leqslant 0.01$; ***, significant at a significance level of $p\leqslant 0.001$.

between unpleasant and pleasant pictures ($t(26) = 4.04$, $p < 0.001$), but not between neutral and pleasant pictures ($t(26) = 1.31$, $p = 0.20$), whereas in the caffeine condition *ADORA2A* CC/CT subjects showed no significant differences between unpleasant and neutral pictures ($t(29) = 0.23$, $p = 0.82$), but between neutral and pleasant ($t(29) = 3.69$, $p = 0.001$) as well as between unpleasant and pleasant pictures ($t(26) = 2.70$, $p = 0.01$).

Explorative Analysis of Gender and AS Effects on Startle Modulation

When using gender and AS (median split of the ASI) as additional factors in the analysis of startle modulation, ANOVA revealed significant interaction effects of picture category and gender ($F(2, 188) = 10.30$, $p < 0.001$), and of picture category, gender, and AS ($F(2, 188) = 5.55$, $p = 0.006$). Therefore, further analyses were conducted separately for both gender groups. In both men and women, a significant effect of picture category on startle magnitude emerged (women: $F(2, 92) = 23.66$, $p < 0.001$; men: $F(2, 96) = 9.03$, $p = 0.001$). In contrast to the overall effect of picture category, no significant differences between unpleasant and neutral pictures were discerned in men ($t(55) = -0.25$; $p = 0.80$), and no significant differences between neutral and pleasant pictures in women ($t(53) = 1.75$; $p = 0.09$). In women, but not in men, ANOVA revealed the above mentioned genotype × intervention × picture category effect (women: $F(2, 92) = 4.60$, $p = 0.01$; men: $F(2, 96) = 1.04$, $p > 0.34$), with *post-hoc* univariate ANOVAs in women showing a significant interaction of genotype and intervention for unpleasant pictures ($F(1, 46) = 6.83$, $p = 0.01$; with higher startle magnitudes for TT risk genotype carriers in the caffeine condition and higher startle magnitudes in non-risk CC/CT genotype carriers in the placebo condition) and neutral pictures ($F(1, 46) = 6.26$, $p = 0.03$; with higher startle magnitudes for TT risk genotype carriers in the placebo condition and higher startle magnitudes in CC/CT non-risk genotype carriers in the caffeine condition), but not for pleasant pictures ($F(1, 46) = 0.49$, $p = 0.49$). In men, but not in women, a significant interaction effect of picture category and AS on startle magnitudes was observed ($F(2, 96) = 4.96$, $p = 0.01$): Only men with high AS showed—like women—the above mentioned overall pattern of startle magnitudes (unpleasant > neutral > pleasant; linear trend: $F(1, 28) = 23.67$, $p < 0.001$). Men with low AS, however, showed a different pattern (unpleasant < neutral > pleasant; quadratic trend: $F(1, 26) = 8.26$, $p = 0.008$; see Figure 4).

Subjective Measures of Anxiety

A significant main effect of measurement time on VAS anxiety ratings was observed ($F(2, 212) = 11.41$, $p < 0.001$), with significantly decreasing anxiety levels from point-1 (before capsule intake) to point-2 (1 h after capsule intake; $t(109) = 2.76$, $p = 0.007$) and significantly increasing anxiety levels from point-2 to point-3 (after the startle experiment; $t(109) = -4.51$, $p < 0.001$) (Figure 2). However, there were no significant effects of genotype, intervention, or genotype × intervention on VAS ratings (all $F(2, 212) < 1.28$, $p > 0.28$).

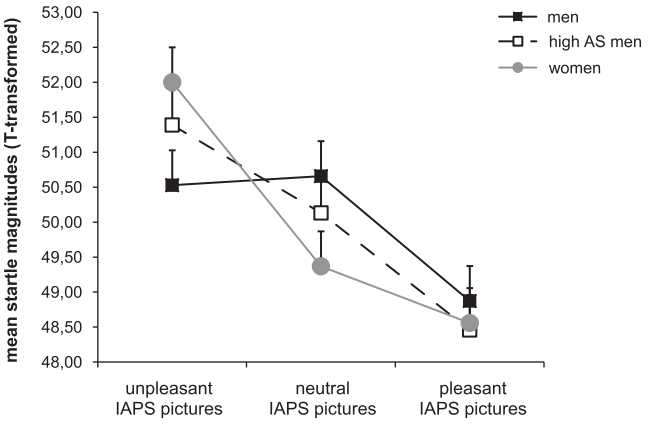

Figure 4 Multifactorial startle modulation by gender, AS, and picture category. AS, anxiety sensitivity. In men, but not in women, there was a significant interaction effect of picture category and AS on startle magnitudes ($F(2, 96) = 4.96$, $p = 0.01$): Only men with high AS showed—like women—the overall pattern of startle magnitudes (unpleasant > neutral > pleasant; linear trend: $F(1, 28) = 23.67$, $p < 0.001$).

Analysis of the POMS subscale 'Depression–Anxiety' revealed a significant main effect of measurement time ($F(2, 212) = 22.52$, $p < 0.001$), with highest scores at the end of the experiment and lowest scores prior to the startle experiment (point-3 > point-2 and point-1; point-2 < point-1; all $t(109) > |2.76|$, $p < 0.008$). There were no significant effects of genotype or intervention (both $F(2, 212) < 1.30$, $p > 0.27$), but a significant interaction effect of genotype × intervention ($F(2, 212) = 5.25$, $p = 0.02$). Further analysis revealed that the above mentioned pattern (point-1 > point-2 < point-3) was valid for the *ADORA2A* non-risk genotype group under both conditions and for the risk genotype group only under verum (all $t(26) > |2.26|$, $p < 0.03$), but not under placebo (all $t(25) < |1.75|$, $p > 0.09$). In risk genotype carriers, increase in POMS 'Depression–Anxiety' ratings from point-2 to point-3 was significant in the verum condition ($t(26) = -5.20$, $p < 0.001$) but not in the placebo condition ($t(25) = -1.10$, $p = 0.28$).

DISCUSSION

In the present study, we observed an interaction of genetic factors (*ADORA2A* 1976TT risk genotype), biochemical factors (caffeine intervention), and anxiety-related emotional stimuli to increase psychophysiological parameters of anxiety: The *ADORA2A* 1976TT risk genotype seems to be generally linked to maladaptive emotional processing in that under the placebo condition risk genotype carriers show equally increased startle magnitudes for unpleasant and neutral pictures as compared with pleasant pictures. This finding pointing to an undifferentiated potentiation of anxiety-related measures in high-risk populations is paralleled by a number of studies showing increased startle or brain activation levels not only during processing of negative but also of neutral stimuli (Armbruster *et al*, 2010; Bernat *et al*, 2006; Bradley, 2001; Rosen and Donley, 2006), particularly in patients with anxiety disorders (Yoon and Zinbarg, 2007), who perceive these stimuli as ambiguous or uncertain. Additionally, according to our *a priori*

hypothesis of a synergistic effect of the *ADORA2A* risk genotype and caffeine on startle response to negative emotional stimuli, in *ADORA2A* 1976TT risk genotype carriers highest startle magnitudes were observed after caffeine administration in response to unpleasant emotional material, with caffeine now eliciting a significant contrast between unpleasant and neutral emotional stimuli. Reciprocally, *ADORA2A* 1976CC/CT low-risk genotype carriers challenged with caffeine show equally high startle magnitudes for unpleasant and neutral pictures, whereas under placebo—as expected in an unmodulated emotion-potentiated startle paradigm in healthy probands—a gradual increase in startle response from pleasant to neutral to unpleasant emotional material was observed. Thus, subjects carrying either the *ADORA2A* anxiety 1976TT risk genotype or receiving caffeine, respectively, as single risk-increasing factors react to unpleasant and neutral stimuli in an equally aroused manner as reflected by comparable startle magnitudes, whereas in a multi-level risk model caffeine in synergy with genotype further increases startle reaction specifically for unpleasant emotional stimuli.

The present study extends previous genetic and pharmacological findings of (1) reports of association of the *ADORA2A* 1976T allele with panic disorder (Deckert *et al*, 1998; Hamilton *et al*, 2004; Hohoff *et al*, 2010) and (2) reports of caffeine exerting an anxiogenic effect (eg, Boulenger *et al*, 1984; Charney *et al*, 1985) partially conferred by the *ADORA2A* 1976TT risk genotype (Alsene *et al*, 2003; Childs *et al*, 2008; Rogers *et al*, 2010) by including emotional stimuli as a third risk factor and by integrating these individual risk factors into a multi-level risk model of anxiety. Also, besides subjective measures of anxiety, in the present study startle reflex potentiation during unpleasant stimuli, which has previously been linked to fearfulness and behavioral inhibition as dimensional phenotypes of anxiety disorders, and therefore has been suggested as a promising intermediate phenotype of anxiety (Cook *et al*, 1992; Grillon and Baas, 2003; Hawk and Kowmas, 2003; Koch, 1999), was used as an objective outcome measure. Based on this experimental model of anxiety, the present results for the first time suggest a multi-level interaction effect between three risk factors on a genetic level (*ADORA2A* 1976TT genotype), a biochemical level (caffeine), and the neuropsychological level of emotional processing (anxiety-related emotional stimuli) on the startle reflex as an objective intermediate phenotype of anxiety disorders.

The observed female-predominant influence of the *ADORA2A* 1976TT genotype on increased startle response dependent on caffeine intervention and anxiety-related emotional stimulation is in accordance with a higher prevalence and also a higher heritability of AS or anxiety disorders, respectively, in female patients (Jang *et al*, 1999; Weissman *et al*, 1997) as well as with previous genetic findings restricted to female patients with anxiety and affective disorders (eg, Deckert *et al*, 1999; Domschke *et al*, 2004, 2007, 2008a, in press). Also, there are first reports of a potentially gender-differential effect of caffeine in animal models as well as in humans, with, however, still inconsistent conclusions (eg, Botella and Parra, 2003; Fisher and Guillet, 1997; Noschang *et al*, 2009). Thus, a potential gender specificity of the presently investigated multi-level risk model of anxiety warrants further investigation in future studies preferably involving additional potential mediators, particularly, as we observed AS to apparently mediate startle response to emotional stimuli in a gender-differential manner.

We did not discern a significant influence of caffeine (150 mg freebase) on VAS or POMS measures of anxiety as opposed to previous studies (Alsene *et al*, 2003; Childs *et al*, 2008). There might be several reasons for that: First, the presently applied 35-item German version of the POMS scale is not fully comparable to the American version (65 items; Albani *et al*, 2005; McNair *et al*, 1992). A lower than average mean AS in the present sample (cf. Peterson and Reiss, 1992) might further account for low anxiety ratings on VAS or POMS, respectively. Also, the presently administered dose of caffeine might have been too low to elicit increased self-report anxiety, as Childs *et al* (2008) observed increases in VAS anxiety ratings only after administration of 450 mg caffeine, but not at the equivalent of 150 mg freebase caffeine, and other studies have shown that only high doses of caffeine increase anxiety (cf. Evans and Griffiths, 1991; Griffiths and Woodson, 1988). Finally, the lack of a cross-over design in the present study might have prevented detection of effects on self-report data.

The present results have to be interpreted in the light of some considerations and limitations: The sample size is modest, however, within the range of comparable previous studies applying the startle reflex paradigm or measures of neuronal activation as an intermediate phenotype approach (eg, Giakoumaki *et al*, 2008; Mattay *et al*, 2003; Pauli *et al*, 2010). Furthermore, in the present study caffeine non-users as well as light-to-intermediate caffeine users were enrolled, who were asked to refrain from caffeine consumption for 1 week prior to the investigation, which was controlled only at the experimental day, thus not excluding shorter abstinence periods. A potentially confounding effect of withdrawal and/or tolerance therefore cannot be completely excluded. Also, a potential selection bias has to be taken into consideration given that *ADORA2A* 1976T allele carriers have been reported to be more likely to be lighter caffeine consumers in the first place (Cornelis *et al*, 2007), which tended to be true also in the present sample. Furthermore, only a single dose of caffeine was administered so that no conclusions can be drawn on the effects of a chronic caffeine medication. Almost all of the female probands had been on hormonal contraceptives during participation in the present study. The actual hormonal status, however, was not considered in the present study, which might have introduced a potential confounder based on case reports suggesting oral contraceptives to potentially induce panic attacks (Deci *et al*, 1992; Ushiroyama *et al*, 1992) and animal as well as human studies providing evidence for gonadal hormones and menstrual cycle to modulate AS (Nillni *et al*, 2011; Toufexis *et al*, 2006). Furthermore, the functional relevance of the silent *ADORA2A* 1976T > C polymorphism is still unknown and therefore the mechanism by which this variant might confer susceptibility to anxiety remains to be elucidated. However, in analogy to synonymous mutations, that is, mutations not changing the amino-acid sequence in the human dopamine D2 receptor (*DRD2*) gene (Duan *et al*, 2003), it may have drastic functional effects by altering mRNA stability or translation. Alternatively, the associated polymorphism

might not constitute the actual causative variant, but rather reflect association of other polymorphisms in linkage disequilibrium with this locus such as the *ADORA2A* 2592T/− (rs35320474) polymorphism (cf. Alsene *et al*, 2003). Finally, owing to the limited sample size the present results have not been controlled for influence of other relevant genetic risk factors such as the *DRD2* rs1110976 polymorphism (Childs *et al*, 2008). This could have constituted a potentially confounding factor, as a mutual functional relationship between the adenosinergic and the dopaminergic system has been suggested: Adenosine A2A receptors form functional heteromeric complexes with DRD2 receptors interacting at multiple levels to control cell function, with activation of A2A reducing DRD2 signaling (Fuxe *et al*, 2007). Furthermore, in DRD2-knockout mice the behavioral properties of caffeine are altered and DRD2 antagonists attenuate the discriminative stimulus effects of low caffeine doses (Powell *et al*, 1999), and DRD2 receptors in the amygdala have been suggested to have a role in setting up adaptive responses to cope with aversive environmental stimuli (de la Mora *et al*, 2010).

In summary, this study—exemplarily focusing on the adenosinergic system—provides evidence for a multi-level pathogenetic model of anxiety involving genetic factors and biochemical alterations interactively increasing the risk of maladaptive emotional processing as reflected by potentiation of the startle response as an objective intermediate phenotype of anxiety. Provided replication in independent studies, these data could help to define subtypes of anxiety-prone individuals and therefore finally may aid in improving primary and secondary prevention of anxiety disorders.

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DISCLOSURE

All affiliations mentioned below have no relevance to the work covered in the manuscript: AR has received a research grant from Astra Zeneca. KD has received speaker fees from Pfizer, Lilly, and Bristol-Myers Squibb; she is a consultant for Johnson & Johnson and has received funding from Astra Zeneca. In the past 3 years, JD has received speaker's honoraria by Janssen, Bristol-Myers Squibb, Wyeth, Lundbeck, Astra Zeneca, and Pfizer, and grant support from Medice. PZ has received speaker fees from Pfizer, Servier, Lilly, Astra Zeneca, and Bristol-Myers Squibb; he is on the advisory board of Pfizer, is a consultant for Ironwood Pharmaceuticals, and has received funding from Astra-Zeneca. VA is member of advisory boards and/or gave presentations for the following companies: Astra-Zeneca, Janssen-Organon, Lilly, Lundbeck, Servier, Pfizer, and Wyeth. He chaired the committee for the 'Wyeth Research Award Depression and Anxiety'. All other authors have no conflicts of interest to declare, financial or otherwise.

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