Psychopharmacology (2012) 222:533–541
DOI 10.1007/s00213-012-2678-0

ORIGINAL INVESTIGATION

Modification of caffeine effects on the affect-modulated startle by neuropeptide S receptor gene variation

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Received: 20 December 2011 / Accepted: 22 February 2012 / Published online: 8 March 2012
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Abstract
Rationale/objectives Both the neuropeptide S (NPS) system and antagonism at the adenosine A2A receptor (e.g., by caffeine) were found to play a crucial role in the mediation of arousal and anxiety/panic in animal and human studies. Furthermore, a complex interaction of the neuropeptide S and the adenosinergic system has been suggested with administration of the adenosine A2A receptor antagonist caffeine downregulating NPS levels (Lage et al., 2006) and attenuating the stimulatory effects of NPS in rodents (Boeck et al., 2010).
Methods Thus, in the present study, the impact of the functional neuropeptide S receptor (NPSR) A/T (Asn^{107}Ile; rs324981) variant on affect-modulated (neutral, unpleasant, and pleasant IAPS pictures) startle response depending on the administration of 300 mg caffeine citrate was investigated in a sample of 124 (m=58, f=66) healthy probands using a double-blind, placebo-controlled design.
Results ANOVA revealed a significant interaction between *NPSR* genotype, challenge condition, and picture valence. Comparing startle magnitudes upon stimulation with neutral or emotional pictures between the placebo and caffeine condition, in AA/AT non-risk genotype carriers no significant difference was discerned, while TT risk genotype carriers showed a significantly increased startle magnitude in response to neutral stimuli (p=.02) and a significantly decreased startle magnitude in response to unpleasant stimuli (p=.02) in the caffeine condition as compared to the placebo condition.
Conclusions In summary, the present findings — extending previous evidence from rodent studies — for the first time provide support for a complex, non-linear interaction of the neuropeptide S and adenosinergic systems affecting the affect-modulated startle response as an intermediate phenotype of anxiety in humans.

Katharina Domschke and Benedikt Klauke contributed equally to this work and therefore both considered as first authors.

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Keywords NPS · NPSR · Caffeine · Adenosine 2A receptor · Emotion · Acoustic startle reflex

Introduction

The neuropeptide S (NPS) system has been suggested to play a crucial role in the mediation of arousal and anxiety. In rodent models, NPS or neuropeptide S receptor (NPSR) agonists reliably elicit anxiolytic effects, paralleled by a robustly increased arousal as expressed by elevated locomotor activity and increased wakefulness (Leonard et al. 2008; Rizzi et al. 2008; Wegener et al. 2011; Xu et al. 2004; for review, see Pape et al. 2010; Reinscheid and Xu (2005)), while *NPSR* knock-out mice exhibit increased anxiety-like behavior and accordingly reduced behavioral arousal as mirrored by decreased exploratory activity along with an increase in rest time (Duangdao et al. 2009). In humans, contrary to most animal data proposing an anxiolytic effect of NPS, the more active T allele of the functional A/T (Asn^{107}Ile; rs324981) variant in the *NPSR* (Reinscheid et al. 2005) has consistently been reported to be associated with panic disorder in several independent samples (Domschke et al. 2011; Donner et al. 2010; Okamura et al. 2007). Furthermore, *NPSR* T allele carriers showed significantly elevated anxiety sensitivity and increased autonomic arousal during a behavioral avoidance test (Domschke et al. 2011). Thus, *NPSR* gene variation seems to influence anxiety and anxiety-related disorders with, however, apparently differential effects on anxiety and arousal across animal and human studies.

Specification to unravel the influence of genetic factors on complex traits or diseases can be reached by investigation of so-called endophenotypes on an intermediate level between genetic factors and categorical disease phenotypes (Meyer-Lindenberg and Weinberger 2006). The acoustic startle reflex has been proposed to constitute a neurobiologically founded defensive response potentially intermediately related to anxiety-related states (Grillon 2008; Lang et al. 1990). In mice, the acoustic startle reflex has been shown to be influenced by the neuropeptide S system, with, however, contradictory results: both intra-amygdalar injection of NPS (Fendt et al. 2010) as well as genetic *NPSR* deficiency (Fendt et al. 2011; Zhu et al. 2010) were found to be associated with a decreased acoustic startle response, while another study did not detect any influence of *NPSR* on the acoustic startle response (Duangdao et al. 2009). Prepulse inhibition of the startle reflex is apparently not affected by the neuropeptide S system (Duangdao et al. 2009; Fendt et al. 2011). In addition to the basic startle reflex, the startle reflex modulated by emotionally salient stimuli ("affect-modulated startle reflex") has been suggested as a valuable tool to study emotional and motivational processes in emotion/motivation-related psychopathological states such as anxiety disorders (cf. Grillon and Baas, 2003; Hamm et al. 1997), with evidence for exaggerated startle potentiation in response to negative emotional stimuli in anxiety and anxiety disorders (e.g., Butler et al., 1990; Grillon et al., 1994; Grillon et al., 1998; Melzig et al., 2009; for review, see Grillon (2002)). To date, a possible influence of neuropeptide S on the affect-modulated startle has not been subject to investigation yet. However, as the *NPSR* T allele has been found to influence emotion processing by conferring decreased cortical versus increased amygdala activity during processing of anxiety-relevant emotional stimuli as well as increased dorsomedial prefrontal cortex activity during a classic aversive conditioning paradigm in healthy probands and patients with panic disorder, respectively (Dannlowski et al. 2011; Domschke et al. 2011; Raczka et al. 2010), the affect-modulated startle might be an ideal paradigm to further delineate the role of *NPSR* gene variation in the mediation of anxiety- and emotion processing-related biophysiological traits.

Additional specification of the genetic underpinnings of complex genetic phenotypes such as anxiety or anxiety disorders can be reached by integratively analyzing several interacting neurotransmitter systems rather than considering single systems in an isolated way. The neuropeptide S system has been shown to be tightly linked with, e.g., glutamatergic, serotonergic, and noradrenergic transmission (Jüngling et al. 2008; Okamura et al. 2011; Raiteri et al. 2009). In addition, studies in rodents point to a complex, primarily synergistic or additive interaction of the neuropeptide S and the adenosinergic system: Acute administration of caffeine, which is an antagonist at the adenosine A2A receptor (Huang et al. 2005) and a potent anxiogenic and arousal-increasing substance (Charney et al. 1985; see Yang et al. 2010), has been observed to induce a marked decrease in mRNA levels of NPS and at the same time to upregulate NPSR expression levels in the brainstem (Lage et al. 2006). On a behavioral level, treatment with caffeine and A2A antagonists has been reported to prevent the increase in locomotion evoked by NPS (Boeck et al. 2010). Also, adenosine depletion by inhibition of ecto-nucleotidases blocked the hyperlocomotor effects of NPS (Pacheco et al. 2011). In turn, NPS injections have been observed to reduce cumulative burying behavior, which is increased by caffeine (Vitale et al. 2008), while a NPSR receptor antagonist did not affect the hyperlocomotor effect of caffeine in mice (Ruzza et al. 2010). In summary, as the neuropeptide S system seems to closely interact with the adenosine system and as caffeine has previously been shown to influence anxiety-related measures as well as the affect-modulated startle reflex (Alsene et al. 2003; Childs et al. 2008; Domschke et al., 2012; Rogers et al. 2010), investigation

of the interactive effects of *NPSR* gene variation and caffeine on startle response to emotional stimuli might aid in further elucidating the complex interplay between the neuropeptide S and adenosine systems in the human model.

Thus, given evidence from animal and human studies for the neuropeptide S system to (1) partly mediate the acoustic startle reflex, (2) influence emotional processing, and (3) interact with the adenosinergic system, the present study — based on our previous study setting of a double-blind, placebo-controlled caffeine administration prior to measurement of the affect-modulated startle response (Domschke et al. 2012) — for the first time aims at investigating the impact of *NPSR* A/T (Asn^{107}Ile; rs324981) genotype on the startle reflex and its modulation by emotional stimuli and caffeine in human probands.

Methods

Sample

A sample of 124 (male=58, female=66; mean age, 26.22 years; SD, 5.88) unrelated healthy subjects was consecutively recruited at the Department of Psychiatry in the University of Muenster and University of Wuerzburg, Germany, respectively, between 2009 and 2010 from a large pool of 1.033 subjects described elsewhere (see Klauke et al. 2011; Klauke et al. 2012). Briefly, in the larger overall sample, Caucasian descent was ascertained by the Caucasian background of both parents. Exclusion criteria comprised severe somatic, neurological, or psychiatric disorders, illegal drug consumption, alcohol consumption of more than 140 g per week (equivalent to about 15–20 units of alcohol), daily smoking of more than 20 cigarettes, daily use of any medication (except for hormonal contraception), pregnancy, and age under 18 and over 50 years. These criteria were checked in a screening telephone call. During the actual recruitment session, illegal drug consumption was assessed by a urine drug screening. Current or prior diagnosis of DSM-IV axis I disorders was excluded using the Mini-International Neuropsychiatric Interview (M.I.N.I.; Sheehan et al. 1998). Approximately 5 % of the subjects were excluded based on evidence for a potential axis I disorder (in most cases, anxiety disorders) in the M.I.N.I.; 1 % of the probands was excluded because of a positive drug screening. A blood sample (20 ml EDTA blood) was taken for genetic analyses. From this larger pool of probands, a subsample of 124 subjects was recruited for the presently applied affect-modulated startle experiment as described in detail by Domschke et al. (2012). Additional exclusion criteria were caffeine or lactose intolerance, high and frequent caffeine consumption (more than three cups of coffee per day), lower than high school education, or breast feeding. Pregnancy was assessed by a rapid urine pregnancy test and again illegal drug consumption was assessed by a urine drug screening. Neurological or other somatic disorders were excluded by a thorough physical examination and medical history. No subjects had to be excluded based on these criteria. The subjects were asked to refrain from caffeine or tea consumption for 1 week prior to caffeine administration and not to smoke, consume alcohol, or take any medication for at least 24 h prior to the investigation. The protocol was approved by the ethics committee of the University of Muenster and University of Wuerzburg, Germany, respectively, and written informed consent from all subjects was obtained.

Genotyping

The sample was genotyped for the *NPSR* rs324981 A/T (Asn^{107}Ile) polymorphism according to published protocols (Domschke et al. 2011). Briefly, DNA isolated from venous blood samples was amplified by PCR using the primers F: 5′-GAAGGAAAAAAATTAAAAATGAACCTCCCCAG-GATTTCAT and R: 5′-TTCTACCCAGGAGAAAGCGGG-CAGTTTGATGCA. Standard PCR was carried out in a 20-µl volume containing 45–60 ng of genomic DNA, 10 pmol of each primer, 200 µM dNTPs, 0.4 U Taq™ DNA Polymerase (Eppendorf AG, Hamburg, Germany), 50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris–HCl (pH 8.4). After a 5-min denaturation, 35 cycles were carried out consisting of 30 s at 94°C, 30 s at 66°C, and 60 s at 72°C, followed by a final extension time of 10 min at 72°C. Amplicons were digested with TasI (Fermentas) (1 U), separated for 2 h on a 15 % polyacrylamide gel and visualized by silver staining. Genotypes were determined by investigators blinded for phenotypes and independently by two investigators. Hardy–Weinberg criteria, assessed with the online available program DeFinetti (T.F. Wienker and T.M. Strom, unpublished data, http://ihg.gsf.de/cgi-bin/hw/hwa1.pl), were fulfilled for *NPSR* genotype distribution in the present sample (T/T, 20.2 %; A/T, 50.8 %; A/A, 29.0 %; p=0.85). Given previous association findings of a silent polymorphism in exon 2 of the *ADORA2A* gene (rs5751876, 1976 T > C, formerly 1083 T > C, Tyr/Tyr) with anxiety-related phenotypes (Deckert et al. 1998; Freitag et al. 2010; Hamilton et al. 2004; Hohoff et al. 2009; Hohoff et al. 2010) and subjective as well as psychophysiological anxiety responses to caffeine (Alsene et al. 2003; Childs et al. 2008; Rogers et al. 2010; Domschke et al. 2012), the present results were controlled for *ADORA2A* 1976 T > C genotype distribution (C/C, 23.3 %; C/T, 36.2 %; T/T, 40.5 %; for genotyping conditions, see Deckert et al. (1998)).

Caffeine challenge

The study utilized a one-session, double-blind, placebo-controlled between-subject design as described in detail before (Domschke et al. 2012). Briefly, caffeine administration was performed by oral administration of a capsule containing 300 mg caffeine citrate (Fagron, Barsbuettel, Germany; equivalent to 150 mg freebase caffeine) 60 min before starting the startle paradigm. This dose has been shown to correspond to about two cups of coffee and 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). Placebo capsules contained mannitol and aerosil. Caffeine levels were determined by saliva test.

Emotionally relevant environmental stimuli

Twenty-four emotionally threatening unpleasant images taken from the International Affective Picture System (IAPS; Lang et al. 2005) were selected as anxiety-relevant environmental cues along with 24 neutral and 24 pleasant IAPS pictures. Ninety-five percent of all pictures were exactly the same for both genders, while different erotic pictures were chosen for men and women to ensure comparable valence and arousal levels.

The pictures were matched concerning the arousal level of unpleasant and pleasant pictures ($t(46)=1.48$, $p=.15$), but differences were observed concerning the extremity of valence ($t(46)=-08.59$, $p<.001$; cf. Libkuman et al. 2007), with more extreme values for unpleasant pictures.

Objective outcome measure: affect-modulated startle paradigm

At the assumed maximum plasma level of caffeine and the described time-to-peak increases in subjective ratings of anxiety, respectively (60 min after administration; Alsene et al. 2003; Childs et al. 2008; Rogers et al. 2010), the affect-modulated startle experiment was started. The startle stimulus (50 ms of 95 dB white noise with an instantaneous rise time) was presented via Bose® Around-Ear Headphones. After eight pre-test startle stimuli, the startle experiment per se consisted of three blocks of 24 anxiety-relevant, neutral, or pleasant IAPS pictures, respectively, 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 sequentially. Visual stimuli were presented for 8 s (intertrial interval (ITI): mean=21 s, range=16.5-25.5 s) on a 19-in. 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. 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 musculus orbicularis oculi was recorded by V-Amp 16 (Brain Products GmbH, Gilching, Germany). Sampling rate was 1,000 Hz, and an online notch filter of 50 Hz was applied. BrainVision Analyzer 2 (Brain Products GmbH, Gilching, Germany) was used as offline analyzing software, with which the signals were rectified, filtered (Low Cutoff 28 Hz, High Cutoff 500 Hz, Notch 50 Hz), and smoothened (using a time constant of 50 ms). Startle magnitude was quantified as the difference between the highest peak 21 to 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 μV) were scored as zero. Artifacts were defined as spontaneous eye blinks during baseline or within 20 ms after startle probe onset and scored as missing values. All startle responses were T-transformed within individual subjects in order to assure comparability of the data and to reduce interindividual variability (for methodical overview: Blumenthal et al. 2005; Mühlberger et al. 2008; Pauli et al. 2010; cf. Domschke et al. 2012). Eight of the initially 124 participants examined showed too many zero startle responses (more than 2.5 standard deviations above mean number of zero responses) or unexpected saliva caffeine concentrations not consistent with caffeine abstinence prior to the experiment and were therefore excluded from further analyses.

Statistical analysis

Sample characteristics regarding sample distributions, caffeine consumption, or age were evaluated by χ^2 tests or one-way ANOVAs with genotype (*NPSR* TT vs. AA/AT genotypes), challenge condition (caffeine versus placebo), or gender as between-subject factors. Influences of genotype, challenge condition, and gender on baseline startle (ITI startle response) were investigated by one-way ANOVA. Habituation effects (assessed in the ITIs) were 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 within-subject factor and genotype as between-subject factor. The main multi-level analysis of affect-modulated startle response was performed using ANOVA for repeated measures with genotype, challenge condition, and gender as between-subject factors and picture category (unpleasant, neutral, and pleasant) as within-subject factor. Post-hoc tests were performed using

ANOVAs for repeated measures as detailed earlier with stratification for genotype. Pairwise comparisons between picture categories were performed by means of post-hoc *t*-tests. Alpha level was set at 5 % using Greenhouse–Geisser corrections where appropriate.

Results

Sample characteristics

The final sample of 116 subjects was almost equally distributed regarding gender and genotype ratio (*NPSR* TT vs. AA/AT genotypes) across challenge condition groups (caffeine vs. placebo; both $\chi^2(1)<.13$, $p>.72$; see Table 1). *ADORA2A* 1976 T > C genotype distribution did not differ between challenge conditions ($\chi^2(1)=.106$, $p=.75$) or *NPSR* TT vs. AA/AT genotype groups ($\chi^2(1)=.497$, $p=.48$).

One-way ANOVAs revealed no differences regarding mean caffeine consumption (calculated in mg/day with one cup of coffee corresponding to 100 mg of caffeine) across genotype groups, challenge conditions, gender (all $F(1,115)<.55$, $p>.46$), or regarding age across the three between-subject factors (all $F(1,115)<1.19$, $p>.17$).

Baseline startle, habituation, and NPSR

No influences of *NPSR* genotype ($F(1,108)=0.02$, $p=.90$), challenge condition ($F(1,108)=0.00$, $p=.997$), or gender ($F(1,108)=0.30$, $p=.59$) on baseline ITI startle were observed. No significant interaction effects were observed (data not shown).

Analysis of the ITI startle response revealed a significant effect of measurement time on startle magnitude ($F(3,330)=32.34$, $p<0.001$): mean baseline startle magnitudes declined between the first and second ($t(114)=5.22$, $p<.001$) and between the second and third measurement time ($t(115)=5.09$, $p<.001$), but no difference between the third and fourth measurement time ($t(112)=1.29$, $p=.20$) was observed. There was no *NPSR* genotype effect on baseline startle response times ($F(3,330)=0.68$, $p=.56$).

Startle modulation by *NPSR* genotype, challenge condition (caffeine placebo), and affect

Investigating the influence of emotionally valent pictures, genotype, challenge condition, and gender on startle magnitudes (affect-modulated startle), ANOVA revealed a significant main effect of picture category with increasing startle magnitudes from pleasant to neutral to unpleasant pictures ($F(2,216)=14.71$, $p<.001$). In addition, a significant interaction between picture valence, *NPSR* genotype, and challenge condition ($F(2,216)=3.61$, $p=.03$) was identified. No significant two-way interactions between picture valence and *NPSR* genotype or challenge condition, respectively, and no further significant three-way (picture valence × challenge condition × gender; picture valence × *NPSR* genotype × gender) or four-way interaction (picture valence × challenge condition × *NPSR* genotype × gender) was observed.

Re-running the ANOVA with *ADORA2A* 1976 T > C genotype as an additional between-subject factor, the results remained. A significant main effect of picture category ($F(2,200)=13.22$, $p<.001$) and a significant interaction between picture valence, *NPSR* genotype, and challenge condition ($F(2,200)=3.91$, $p=.02$), but neither a significant interaction between *ADORA2A* 1976 T > C genotype and picture valence ($F(2,200)=0.81$, $p=.49$) nor any significant interactions of *ADORA2A* 1976 T > C genotype, picture valence, and any of the other between-subject factors (*NPSR*, gender, challenge condition) were observed (data not shown) so that confounding of the results by *ADORA2A* 1976 T > C genotype can be excluded.

Post-hoc analyses within *NPSR* genotype groups showed the reported order of startle magnitude depending on picture valence (unpleasant pictures > neutral pictures > pleasant pictures) for the AA/AT non-risk genotype group under placebo and under caffeine, but for the *NPSR* TT risk group under placebo only: in other words, all groups showed significant linear trends for an increased startle response after unpleasant picture presentation compared to neutral pictures ($p<.005$), except for *NPSR* TT genotype carriers under caffeine ($F(1,11)=1.70$, $p=.22$): here, *NPSR* TT genotype carriers displayed an inverse U-shaped curve, with an increased startle response from pleasant to neutral and a decreased startle magnitude from neutral to unpleasant pictures (see Fig. 1). Accordingly, stratification for genotype revealed a significant interaction effect between picture valence and challenge condition for the *NPSR* TT risk group ($F(2,38)=5.53$, $p<.001$), but not for the AA/AT non-risk genotype group ($F(2,178)=.36$, $p=.67$).

Comparing challenge conditions, we found no difference between the placebo and the caffeine condition in AA/AT non-risk genotype carriers ($t(91)=-0.72$, $p=.48$), while TT risk genotype carriers showed a significant difference in startle magnitudes (caffeine condition<placebo condition) in response to unpleasant stimuli ($t(21)=2.51$, $p=.02$; see Fig. 1). After neutral stimuli, differences were not apparent in AA/AT non-risk genotype carriers ($t(91)=-0.47$, $p=.64$), while TT risk genotype carriers showed a significantly increased startle magnitude in the caffeine condition as compared to the placebo condition ($t(21)=-2.65$, $p=.02$). Comparing challenge conditions, startle magnitude after pleasant picture stimulation did not differ across challenge conditions, neither for AA/AT non-risk genotype carriers

Table 1 Sample characteristics

Challenge condition	*NPSR* genotype	Gender		Total
		Men	Women	
Placebo	Risk (TT)	*N*=7 Age=24.1 (3.98) cc=110.2 mg/d (86.69)	*N*=4 Age=26.5 (3.70) cc=200.0 mg/d (141.42)	*N*=11 Age=25.0 (3.87) cc=142.9 mg/d (112.08)
	Non-risk (AA/AT)	*N*=21 Age=27.0 (7.51) cc=150.0 mg/d (123.49)	*N*=23 Age=25.0 (5.95) cc=102.2 mg/d (81.85)	*N*=44 Age=26.0 (6.73) cc=125.0 mg/d (105.38)
Verum (300 mg caffeine citrate)	Risk (TT)	*N*=5 Age=26.8 (6.69) cc=70.0 mg/d (44.72)	*N*=7 Age=25.4 (7.46) cc=150.0 mg/d (104.08)	*N*=12 Age=26.0 (6.86) cc=116.7 mg/d (91.29)
	Non-risk (AA/AT)	*N*=24 Age=27.5 (4.55) cc=130.0 mg/d (109.41)	*N*=25 Age=25.5 (5.41) cc=104.0 mg/d (105.99)	*N*=49 Age=26.5 (5.06) cc=116.7 mg/d (107.35)
Total		*N*=57 Age=26.9 (5.89) cc=129.7 mg/d (108.77)	*N*=59 Age=25.4 (5.67) cc=115.3 mg/d (100.54)	*N*=116 Age=26.1 (5.80) cc=122.3 mg/d (104.46)

All statistical comparisons of age and cc across genotypes, challenge conditions, and gender were not significant

N sample size, *Age* mean (standard deviation) (years), *cc* caffeine consumption (standard deviation) (mg/d)

($t(91)=.33$, $p=.74$) nor for *NPSR* TT risk allele carriers ($t(21)=-0.15$, $p=.88$).

Post-hoc analyses within challenge conditions revealed no differences between *NPSR* TT and *NPSR* AA/AT genotype carriers after unpleasant picture presentation in the placebo condition ($t(53)=.87$, $p=.39$), while after caffeine administration *NPSR* TT risk as compared to AA/AT non-risk genotype carriers exhibited a trend towards a decreased startle response after unpleasant picture presentation ($t(59)=-1.93$, $p=.058$). Comparing *NPSR* TT and *NPSR* AA/AT genotypes, no significant differences in startle magnitude were observed after neutral picture presentation in the placebo condition ($t(53)=-0.57$, $p=.57$). Under caffeine, carriers of the more active *NPSR* TT genotype displayed a significantly increased startle response after neutral picture presentation as compared to *NPSR* AA/AT genotype carriers ($t(27.800)=2.91$, $p=.01$; see Fig. 1). For pleasant pictures, post-hoc analyses within challenge conditions revealed no differences between *NPSR* TT and *NPSR* AA/AT genotype carriers, neither in the placebo ($t(53)=-0.19$, $p=.85$) nor in the caffeine condition ($t(59)=.17$, $p=.87$).

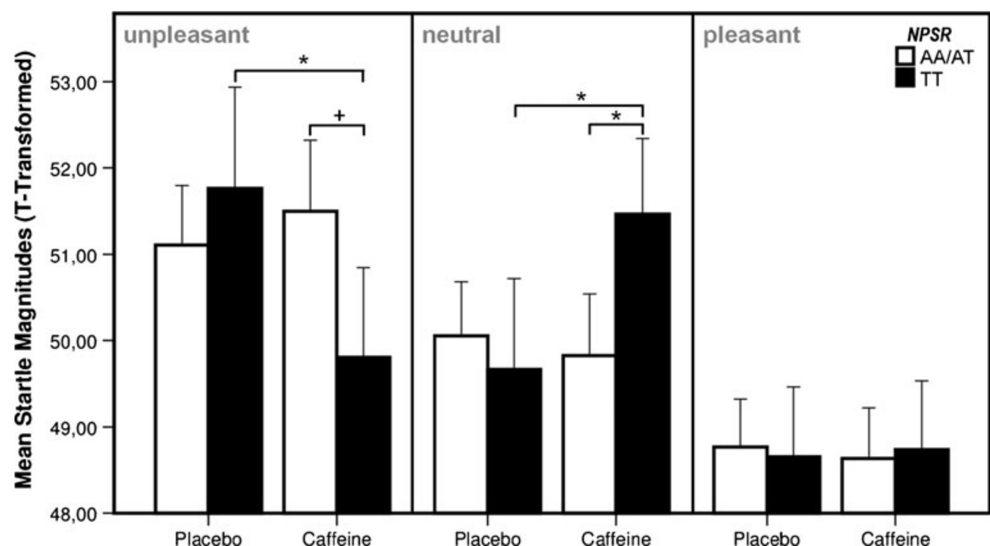

Fig. 1 Mean startle magnitudes after unpleasant, neutral, and pleasant picture presentation for the placebo and the caffeine challenge condition stratified for *NPSR* genotype. *Error bars* represent two standard errors. + trend at significance level of $p \leq .06$; * significant at significance level of $p \leq .05$

Discussion

In the present study, no overall influence of the neuropeptide S receptor (*NPSR)* A/T (Asn^{107}Ile; rs324981) polymorphism on startle response was discerned in healthy probands under placebo. However, in carriers of the more active *NPSR* TT genotype — previously found to be associated with anxiety and panic disorder (Domschke et al. 2011; Donner et al. 2010; Okamura et al. 2007) — administration of caffeine synergistically resulted in an increased startle magnitude in the neutral emotional condition. After presentation of unpleasant emotional stimuli, however, *NPSR* TT genotype carriers showed a blunted startle magnitude in response to caffeine.

The fact that in the placebo condition of the present study no overall effect of *NPSR* A/T genotype on startle response was detected in a way is in line with the inconsistent murine literature with a report of intra-amygdalar injection of NPS to be associated with a decreased acoustic startle response (Fendt et al. 2010), a report of *NPSR* deficiency to lead to decreased startle magnitudes (Fendt et al. 2011; Zhu et al. 2010) and reports of no influence at all of the neuropeptide S system on startle magnitudes or startle habituation (Duangdao et al. 2009) or prepulse inhibition of the startle reflex (Duangdao et al. 2009; Fendt et al. 2011).

The described rodent studies earlier, however, are not fully comparable to the present study employing the startle paradigm in the more complex human model (see Grillon and Baas (2003); Lang et al. 1990), where subtle genetic effects possibly only become visible in a multi-level approach, e.g., involving alterations in related neurotransmitter systems such as the adenosinergic system.

In interaction with the adenosine A2A antagonist caffeine, we observed a significant attenuation of startle response to unpleasant and a significantly increased startle magnitude in the neutral emotional condition in carriers of the more active *NPSR* TT genotype. A possible explanation for this pattern of startle modulation by *NPSR* genotype in interaction with caffeine might be that affect-modulated startle response reflects not only a valence but also an arousal effect (cf. Dillon and LaBar 2005; Pauli et al. 2002). Besides its impact on anxiety-related behavior, NPS is crucially involved in the mediation of arousal. Animal models have shown that NPS or neuropeptide S receptor agonists — corresponding to the more active *NPSR* T allele in humans — elicit a robustly increased arousal. As caffeine and A2A antagonists have been reported to prevent hyperarousal evoked by NPS (Boeck et al. 2010; Pacheco et al. 2011) potentially by decreasing brainstem NPS expression (Lage et al., 2006), startle magnitudes in response to high-arousing pictures (unpleasant, pleasant) might be blunted particularly in high NPS tonus *NPSR* TT genotype carriers. The observed relative increase in startle response in the neutral picture condition — representing minimal emotional arousal — in carriers of the *NPSR* TT genotype after caffeine administration might therefore not be due to an arousal effect but rather a consequence of a general maladaptive emotional processing in *NPSR* TT genotype carriers (Dannlowski et al., 2011; Domschke et al., 2011). In anxiety-prone populations, increased startle or brain activation levels have been demonstrated for processing of neutral stimuli (Armbruster et al. 2010; Bernat et al. 2006), particularly in patients with anxiety disorders (Yoon and Zinbarg 2007), who perceive these stimuli as ambiguous or uncertain and therefore potentially anxiety relevant. Thus, in the present study, the valence effect as expected in the affect-modulated startle paradigm (startle response unpleasant > neutral > pleasant) and as presently observed in *NPSR* non-risk genotype carriers as well as in *NPSR* TT risk allele carriers in the placebo condition might have been altered by an arousal effect based on a complex interaction of the more active *NPSR* TT genotype and caffeine. However, these interpretations have to considered as highly speculative, and the exact mechanism of an interaction between the neuropeptide S and the adenosinergic system with respect to anxiety-related phenotypes in humans remains to be further elucidated in future studies specifically designed to differentiate attention, arousal, and valence effects.

The present results have to be interpreted with caution considering several limitations: the present sample size, particularly of the *NPSR* TT risk genotype group, 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 (e.g., Giakoumaki et al. 2008; Pauli et al. 2010). Also, although the present results have been controlled for a potential impact of the *ADORA2A* 1976 T > C (rs5751876) variant, which has previously been found to be associated with anxiety-related phenotypes (Deckert et al. 1998; Freitag et al. 2010; Hamilton et al. 2004; Hohoff et al. 2009; Hohoff et al. 2010) and subjective as well as psychophysiological anxiety responses to caffeine (Alsene et al. 2003; Childs et al. 2008; Rogers et al. 2010; Domschke et al. 2012), an influence of further anxiety-relevant genetic background, e.g., the dopamine D2 receptor (*DRD2*) (see Childs et al. 2008), which has been suggested to play a role in setting up adaptive responses to cope with aversive environmental stimuli (de la Mora et al. 2010), cannot be excluded. Finally, IAPS pictures used in the present study were matched regarding arousal level of unpleasant and pleasant pictures, but valence was higher for unpleasant as compared to pleasant pictures, which could have influenced our results.

In summary, the present findings — extending previous evidence from rodent studies — for the first time suggest a complex interaction of the neuropeptide S and the

adenosinergic system in the mediation of the affect-modulated startle response as a well-established intermediate phenotype of anxiety in humans.

Acknowledgements This study was supported by grants from Deutsche Forschungsgemeinschaft (SFB-TRR-58 project B1 to PP and AM, C2 to KD and JD, project C1 to PZ, and project Z2 to JD, AR and PP). We gratefully acknowledge the skillful technical support by Max Hilscher, Heike Ewald, Thilo Rattay, and Julian Conrad. All authors had full control of all primary data and they agree to allow the journal to review their data if requested. The experiments comply with the current laws of the country in which they were performed.

Conflict of interest All authors have no conflicts of interest to declare, financial or otherwise, that may have a direct bearing on the subject matter of this article.

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