



The impact of cue learning, trait anxiety and genetic variation in the serotonin 1_A receptor on contextual fear

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ABSTRACT

In everyday life, aversive events are usually associated with certain predictive cues. Normally, the acquisition of these contingencies enables organisms to appropriately respond to threat. Presence of a threat cue clearly signals 'danger', whereas absence of such cues signals a period of 'safety'. Failure to identify threat cues may lead to chronic states of anxious apprehension in the context in which the threat has been imminent, which may be instrumental in the pathogenesis of anxiety disorders.

In this study, existing data from 150 healthy volunteers in a cue and context virtual reality fear conditioning paradigm were reanalyzed. The aim was to further characterize the impact of cue acquisition and trait anxiety, and of a single nucleotide polymorphism in the serotonin 1_A receptor gene (5-HTR_{1A}, rs6295), on cued fear and contextual anxiety before and after fear contingencies were explicitly introduced. Fear conditioned responding was quantified with fear potentiation of the eyeblink startle reflex and subjective fear ratings.

First, we replicated previous findings that the inability to identify danger cues during acquisition leads to heightened anxious apprehension in the threat context. Second, in subjects who did not identify the danger cue initially, contextual fear was associated with trait anxiety after the contingencies were explicitly instructed. Third, genetic variability within 5-HTR_{1A} (rs6295) was associated with contextual fear independent of awareness or trait anxiety.

These findings confirm that failure to acquire cue contingencies impacts contextual fear responding, in association with trait anxiety. The observed 5-HTR_{1A} effect is in line with models of anxiety, but needs further replication.

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1. Introduction

Anxiety disorders are highly prevalent and cause high cost and suffering (Alonso et al., 2004; Gustavsson et al., 2011). Processes related to the onset and maintenance of fear and anxiety responses can be modeled using the fear conditioning model in carefully controlled laboratory experiments (Craske et al., 2006). In the fear conditioning procedure, an initially neutral stimulus serves as a conditional stimulus (CS) that precedes the presentation of an intrinsically aversive stimulus (unconditioned stimulus, US). After several pairings of the CS and US, the CS will come to elicit a conditioned fear response. Fear conditioning experiments enable the study of general laws that govern behavior across individuals, as well as individual differences in responding to threat. Processes that contribute to the level of fear responding during a fear conditioning experiment have been studied extensively at the neurobiological level and the knowledge base on cognitive and

neurobiological mechanisms is now being extended to how these may vary across individuals (Holmes and Singewald, 2013).

Etiological models for anxiety disorders have suggested that reduced capacity to make use of (learned) safety cues as well as generalization of fear to other stimuli may underlie the development of pathological anxiety (Lissek et al., 2005, 2010). However, most studies focused on single, discrete cues as CSs. Yet, in one form of maladaptive generalization, fear may extend to the context in which the US has been encountered, even when there are more specific predictors of threat (i.e., the CS; e.g. see Baas et al., 2008; Grillon, 2002). During the acquisition of conditioned fear, all cues present during US administration may be associated with the US initially, including contextual ones. However, when a single stimulus is consistently present during US administration, Pavlov (1927) theorized that the anxious responding to the general context will disappear due to inhibition of fear to the context, while fear will be focused on the specifically conditioned stimulus (CS). Indeed, previous studies demonstrated inhibition of contextual fear as a result of the introduction of a predictive cue (Baas, 2013; Fonteyne et al., 2009). However, high levels of initially acquired contextual fear may be expected to at least partially, and at least in certain individuals, block the acquisition of the more specific CS–US relationship (Meulders et al., 2012).

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Initial conditioning to the context, acquisition of the CS–US association, and the inhibition of the context–US association after the CS–US association has been acquired may all be affected by individual difference factors. This means that the expression of contextual fear will vary across individuals in a context in which a US is sometimes presented, but only when a specific conditioned stimulus (CS) is present. Many individual difference factors have been investigated that determine adaptive acquisition, expression, and extinction of conditioned fear responses, among which cue awareness (Hamm and Vaitl, 1996; Grillon, 2002; Baas et al., 2008), personality (Baas, 2013; Glotzbach-Schoon et al., 2013a) and genetic factors (e.g. Glotzbach-Schoon et al., 2013b; Heitland et al., 2012, 2013; Klumpers et al., 2012; Muhlberger et al., 2014; see Lonsdorf and Kalisch, 2011 for an overview). In general, replication studies of such findings are scarce, and therefore an important aim of this paper is to replicate some of our previous findings (Baas et al., 2008; Baas, 2013) in a larger sample.

This paper reports an analysis of several factors that may affect such contextual fear responses, measured with fear-potentiated startle (FPS). The startle reflex provides a direct translational measure for levels of fear responding and allows monitoring of the development of fear conditioned responding to both explicit cues and contexts within one paradigm. Startle reactivity is directly related to activity in subcortical fear circuitry, in both animals and humans (Davis et al., 2010; Klumpers et al., 2010a). There are several mechanisms through which variation in context responding may be achieved. From the many potential factors involved in contextual anxiety, the present study will use a virtual reality design to focus on three in particular. Virtual reality enables the manipulation of relatively realistic contexts while the subjects stay stationary to allow physiological recording (Baas et al., 2004).

First, contextual responding may be affected by how individuals differ in the acquisition of the CS–US contingency. Previous studies have demonstrated that participants who fail to identify the CS–US relationship display a lack of differentiation in responding between the presence and absence of the CS, both at the physiological level (fear-potentiated startle, skin conductance recordings) and at the subjective level (subjective fearfulness ratings; Baas, 2013; Baas et al., 2008; Grillon, 2002). In these studies, the lack of differentiation in responding to the absence versus presence of the predictor of threat (CS+) was associated with chronically elevated subjective fear (Baas et al., 2008) and increased FPS (Grillon, 2002) in the conditioning context. We demonstrated in previous work that without explicit instructions, many individuals fail to acquire the CS–US relationship and will remain unaware of this association, even after many blocks of acquisition. Consistent with the safety-signal hypothesis by Seligman and Binik (1977), cue-unaware participants displayed continuously strong fear responding to the conditioning context throughout the experiment (Baas, 2013; Baas et al., 2008). However, whether individuals who fail to acquire the CS–US contingency initially will persistently show higher contextual fear, even when this relationship has become clear, is still unknown. Therefore, in contrast to previous studies, in this study the relationship between the CS and US is instructed explicitly after an initial uninstructed acquisition phase. The first aim of the present study is thus to test in a larger sample whether individuals who fail to acquire the threat CS–US contingency initially show sustained contextual anxiety, and whether this effect persists after initially unaware participants are made aware of the relationship by means of explicit instructions. Considering the strong effects of awareness on cue and contextual fear responding in the previous studies, the expectation was that initially unaware subjects would no longer show increased contextual fear after the instructions made them aware.

Second, individuals may vary in their ability to reduce contextual fear after the introduction of a predictive cue. In a previous study, we found that high levels of trait anxiety predicted relatively high levels of fear in the conditioning context after first conditioning to the context alone, followed by the instruction that shock would from then on only be administered in the presence of the CS (Baas, 2013). This suggests

that high trait anxiety may interfere with modulating levels of contextual fear adaptively. In this study, this situation is mimicked by the explicit instructions after the uninstructed acquisition phase. However, subjects who had spontaneously acquired the CS–US association can be expected to have acquired conditioned fear to the CS specifically, and may have developed less fear to the context to begin with. Therefore, we expected to replicate the correlation between trait anxiety and the level of fear responding in the threat context after instructions specifically in the group who was initially unaware of the CS–US contingency.

Third, in experimental studies, treatment with selective serotonin reuptake inhibitors (Grillon et al., 2007, 2009a) and serotonin depletion (Robinson et al., 2012) were shown to specifically affect contextual anxiety, but not cued fear. PET studies demonstrated altered serotonin 1_A binding in panic disorder (Neumeister et al., 2004) and posttraumatic stress disorder (Sullivan et al., 2013). Preclinical findings suggest an involvement of the serotonin 1_A receptor (5-HTR $_{1A}$) in the bed nucleus of the stria terminalis (BNST) in inhibition of contextual fear responses (Hammack et al., 2009). Developmental models in animals suggest that genetic deletion of the serotonin 1_A receptor leads to altered fear circuits in the brain (Gross et al., 2000). More specifically, increased responding to contextual cues was demonstrated in these knock out animals, interpreted as ‘inappropriate generalization of fearful behavior to a context containing both fearful and neutral stimuli’ (Klemenhagen et al., 2006). The impact of genetic variation in 5-HTR $_{1A}$ in human experimental models may be investigated through a supposedly functional polymorphism in the 5-HTR $_{1A}$ gene (rs6295, also referred to as C(-1019)G; see Albert, 2012). The G-allele of rs6295 has been associated with panic disorder (Rothe et al., 2004), comorbid generalized anxiety and major depression (Molina et al., 2011) and with panic symptoms (Huang et al., 2004; Choi et al., 2010). However, effects on mood and anxiety disorders were not demonstrated in another study (Hettema et al., 2008). Also, G-carriers of this polymorphism were shown to have higher neuroticism and related personality traits (Strobel et al., 2003). In contrast, increased amygdala activity (Fakra et al., 2009) and risk for anxiety disorders in patients with temporal lobe epilepsy (Schenkel et al., 2012) were reported in C-carriers of this 5-HTR $_{1A}$ polymorphism. Based on all these findings, variability in the regulation of contextual anxiety through different functionality of the serotonin 1_A receptor may contribute to individual differences in contextual responding, as discussed above. The third aim of this study is thus to investigate genetic variability in rs6295 as a possible contributor to interindividual variability in contextual anxiety. Despite compelling preclinical evidence, there are no previous reports of the impact of polymorphisms in the 5-HTR $_{1A}$ gene in humans on fear conditioning. Based on the available preclinical studies, the hypothesis was tested that genetic variation in the 5-HTR $_{1A}$ gene would specifically affect contextual fear responding. Due to conflicting clinical findings, we did not make a specific prediction on which genetic variant would be associated with increased contextual fear.

Taken together, the present study employs a cue and context fear conditioning paradigm in a virtual environment in a sample of 150 young healthy volunteers. Aims were to study the regulation of contextual anxiety in relation to (1) acquisition of the threat CS–US contingency, (2) individual differences in trait anxiety and (3) genetic variability in the serotonin 1_A receptor gene (rs6295), measured with fear-potentiated startle (FPS) and subjective fearfulness ratings.

2. Methods

2.1. Participants

The data analyzed in this study come from a sample reported on previously (Heitland et al., 2012, 2013). The sample included 150 subjects recruited via advertisements at Utrecht University comprising 90 females and 60 males (mean age = 21.6, SD = 2.4) free of any current or previous psychiatric or neurological disorder, drug or alcohol dependence, current psychoactive medication, hearing problems and

color blindness. 148 out of 150 subjects reported Caucasian (Western European) descent, and 2 reported Asian ancestry. Compensation for participation in the experiment was € 30. Incomplete data led to exclusion of 5 subjects from the final sample (insufficient quality of isolated DNA, $n = 2$; failure to genotype 5-HTR_{1A}, $n = 3$). The final sample included 145 subjects between 18 and 28 years of age (86 females, 59 males; mean age = 21.5, SD = 2.3). Data from 2 females and 1 male are included in the analysis of subjective data but excluded from the startle analysis due to incomplete recordings of startle data in (part of) the extinction phase. The ethical institutional review board of the University Medical Centre Utrecht approved this study, and all subjects gave written informed consent. All study procedures have been conducted according to the Declaration of Helsinki.

2.2. Experimental paradigm

The study comprised a fear-potentiated startle (FPS) conditioning paradigm in a virtual reality environment (see Heitland et al., 2012, 2013). In this paradigm, fear conditioned responding to both a threat cue and a threat context is acquired. Two virtual environments (an apartment in a downtown area and a house in a suburban area) were used as contexts (see Baas, 2013 for illustrations). Assignment of the context where shocks were administered (CXT+) was counterbalanced across participants, whereas the other context was never associated with shock administration (CXT−). See Fig. 1 for a schematic of the study design. Within both contexts, an 8 second duration increase in background illumination (light-on) was presented, which served as cue that signaled when shocks could be administered in the threat context. Light-on presentations were not followed by shock in the safe context. The experiment consisted of blocks of 5 min 25 s duration, during which movie clips from visits to the virtual environments were played. Each block began in a virtual metro station from which visits to both contexts started, and which was revisited in the transition between contexts.

The experiment started with uninstructed acquisition to assess the uninstructed development of conditioned responding (uninstructed acquisition phase), followed by explicit instructions (verbal and written) to ensure awareness of the contingency between threat context, threat cue and shock reinforcements in all participants. Prior to the uninstructed phase, subjects were instructed to pay attention to the videos, as they might be able to predict the shocks. This phase consisted of six blocks. After instructions, five more blocks assessed instructed fear conditioning (expression phase), followed by four blocks of immediate extinction

without shock reinforcements and no further instructions. During the acquisition and expression phases, some blocks contained a relatively high reinforcement rate of 75% (3 out of 4 CS presentations in the shock context were followed by a shock) to facilitate acquisition during uninstructed acquisition (blocks 1, 3, 4) and the first block after instructions (block 7; training blocks). The other blocks during acquisition and expression contained a relatively low reinforcement rate (37.5%; only with the last CS presentation per visit) to allow the assessment of context conditioned responses without selective contamination of the physiological responses in the threat context due to shock sensitization (Baas, 2013; Baas et al., 2008). Therefore, only startle data from test blocks (blocks 2, 5, 6 and 8–15) are reported.

Throughout the experiment, each block contained four light-on presentations in each of the contexts. Startle probes were presented during three out of these four CS presentations. To assess levels of contextual fear, three startle probes were presented in the absence of the light cue in each context. These are further referred to as the context dark condition. During transitions between contexts startle probes were presented with identical inter-startle intervals to maintain startle habituation (Baas, 2013; Baas et al., 2008).

2.3. Shock administration & workup

Electrical shocks were administered on the inner left wrist approximately over the medial nerve with a constant current generator (Digitimer DS7A, Digitimer Ltd., Letchworth Garden City, United Kingdom) with tin cup electrodes. A shock workup procedure was performed at the beginning of the test session to determine individual shock intensities as described in previous papers (Baas, 2013; Baas et al., 2008; Klumpers et al., 2010b). Intensities were adjusted per subject so that they corresponded to a level of 4 out of 5, representing 'quite annoying/painful'.

2.4. Startle probe presentation, data recording and processing

The eyeblink startle reflex was recorded via electromyography of the right orbicularis oculi muscle using a BioSemi ActiveTwo system (BioSemi Instrumentation, Amsterdam, The Netherlands). Startle probes were 50-ms, 105 dB white noise bursts with instantaneous rise time, delivered through headphones (Sennheiser Electronic HD202, Wennebostel, Germany). Startle data was processed in Brain Vision Analyzer software (Brain Products, Gilching, Germany) according to published guidelines (Blumenthal et al., 2005) and previous studies

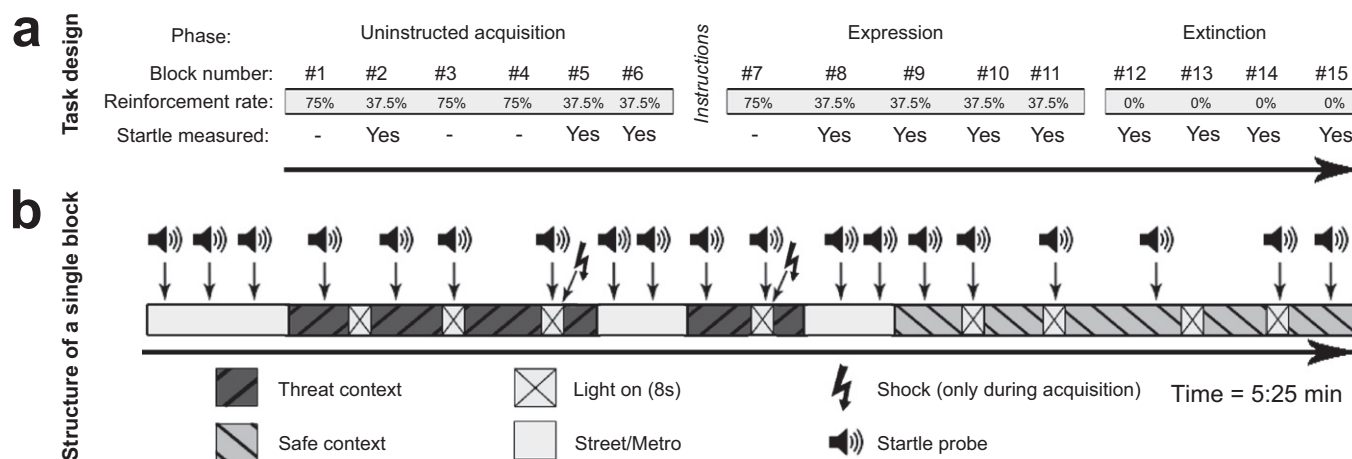


Fig. 1. Schematic illustration of the study set-up. Panel a (top) lists the experimental phases and the blocks presented therein. Panel b (bottom) represents the structure of an example block. Between blocks the order of contexts, and whether the single or double visit came first was counterbalanced.

(Baas, 2013; Baas et al., 2008; Heitland et al., 2012, 2013; Klumbers et al., 2010b, 2012). After segmentation of trials, artifacts were rejected and null responses identified as described previously [see Bocker et al., 2004 for procedural details and criteria]. Participants were only included in the final analysis if at least one artifact-free startle trial for each condition and each phase was recorded ($n = 1$ excluded). After preprocessing, startle data were Z-transformed per subject based on individual trial amplitudes from all startles recorded during the experiment to remove between-subjects variance in baseline startle amplitude. All statistical analyses involving startle data were conducted on Z-scores following previous studies.

2.5. Subjective measures

Before the start of the test session, subjects filled out Spielberger's Trait Anxiety Inventory [Dutch translation, (Spielberger, 1972)] and the neuroticism subscale of the NEO-PI-R questionnaire [Dutch translation, (Costa and McCrae, 1992)]. During the virtual reality fear conditioning paradigm, subjects rated their subjective fearfulness between blocks for all conditions. A visual analog scale (VAS) was displayed on the computer screen together with screenshots from the pre-recorded videos representative for each condition with the question 'How fearful do you feel in this situation?' and anchors: 'Not at all fearful of shock' [0] and 'Very fearful of shock' [100]. After each block, two trials per condition with different screenshots were presented, and an average rating was computed per condition and block. Further analysis of the data was similar to the analysis of the startle data, but VAS data were not Z-transformed as the theoretical range of the scores was the same for every subject. In addition to these fearfulness ratings, shock contingency awareness was assessed by forced choice ratings of shock expectancy between blocks as described earlier (Baas, 2013; Baas et al., 2008), but because there were relatively few blocks of the uninstructed acquisition phase, criteria of consistent responding across multiple blocks were less reliable. Therefore an open question posed prior to the explicit instructions was used to determine awareness of the cue contingency. In this question, subjects were asked to verbalize what relationship between events in the virtual environment and the shock administration they had noticed. Only when subjects spontaneously brought up that the shock administration was contingent upon the light going on and off, they were qualified as cue aware for further analyses.

2.6. Genotyping

DNA was harvested by collecting buccal swabs frozen immediately at -40°C for later genotyping. Genomic DNA was extracted and purified using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The 5-HTR_{1A} SNP (rs6295) was genotyped using a Taqman SNP Genotyping assay (ASSAY ID: C_11904666_10; Applied Biosystems, Foster City, CA). Subjects were classified through endpoint analysis performed on an ABI Prism 7000 (Applied Biosystems, Foster City, CA.) as either C/C homozygotes, C/G heterozygotes or G/G homozygotes. Genotyping

was performed in duplicate for ~80% of the sample without deviations. As commonly done, rs6295 major allele homozygotes (G/G) were grouped against heterozygotes (C/G) and minor allele homozygotes (C/C). Genotype frequencies and statistics of the genetic rs6295 polymorphism are reported in Table 1.

2.7. Statistical analyses

For clarity of presentation, planned comparisons concerning the context fear levels are reported separate from more exploratory analyses of the cue conditions. As in earlier publications using this paradigm (Heitland et al., 2012, 2013), contextual fear was defined as potentiation to the threat context in the absence of the light cue (context FPS: dark/CXT + vs. dark/CXT –). Cued fear was defined as potentiation to the threat cue within the threat context (cue FPS: light/CXT + vs. dark/CXT +). To assess the impact of trait anxiety on contextual fear with trait anxiety (aim 2), the absolute level of responding in the threat context in the dark (dark/CXT +) in the expression phase after explicit instructions was correlated with STAI-trait levels, following an earlier publication on this topic (Baas, 2013).

To assess effects of cue awareness (aim 1) and 5-HTR_{1A} genotype (aim 3), separate repeated-measures ANOVAs for context and cue FPS were conducted across phases (uninstructed acquisition, expression and extinction). 5-HTR_{1A} genotype was included as between subjects' factors with two levels (G/G homozygotes, C-carriers). In addition, to test the hypotheses on the effects of CS-US acquisition and trait anxiety on contextual fear, cue awareness in the uninstructed acquisition phase (aware, unaware) was included as between-subjects variable of interest. In these analyses, no effects of STAI-trait were expected, but this measure was included as a continuous covariate to allow assessment of and control for potential unexpected effects of this variable. Sex and age were added as covariates for all statistical comparisons that involved the genetic polymorphisms under study as commonly done in behavioral genetic research. Also, 5-HTTLPR (L/L vs S-carriers) and CRHR1 (rs878886; C/C versus G-carriers) were included as covariates because of our earlier publication on effects of these polymorphism on the uninstructed acquisition of fear in this data set (Heitland et al., 2013), and CNR1 (rs2180619) because of our earlier publication on its effect on extinction of fear (Heitland et al., 2012). Of note, statistical test outcomes (significant or non-significant) reported in the following were identical with and without inclusion of all these covariates. Follow-up tests were not moderated by any of the covariates of violations of the sphericity assumption, and Greenhouse–Geisser corrected p-values are reported. There were no interactions between cue awareness and 5-HTR_{1A} genotype on any of the outcome measures (F-values for FPS and VAS, cue and context all <2.2 , $p > 0.14$). Therefore results for these factors are reported separately. In addition, in most analyses, effects of cue awareness or 5-HTR_{1A} genotype were not significantly associated with any of the covariates included to control for their potential effects in the overall repeated measures ANOVA. The only significant finding was a main effect of age on total FP-VAS

Table 1

Descriptive statistics, genotype frequencies, personality scores and experimental parameters are shown for the whole sample and per 5HTR_{1A} (rs6295) genotype and cue awareness group.

	Total		C/C		C/G		G/G		Cue aware		Cue unaware	
Ns	145		25		83		37		76		74	
% females	59%		52%		61%		59%		53%		68%	
% cue-aware	51%		56%		51%		49%		100%		0%	
STAI-trait	36.0	7.9	36.2	8.9	36.1	8.0	35.7	7.2	35.2	7.0	37.0	9.0
NEO-N	131.6	21.8	132.5	20.9	132.4	23.4	129.6	19.3	129.3	20.2	133.7	23.2
Habituation startle	86.6	58.4	88.4	73.0	92.0	56.5	73.4	51.0	86.2	65.8	83.9	49.7
Shock intensity	1.7	0.9	1.7	0.9	1.6	0.8	2.0	1.0	1.7	0.8	1.7	0.9

Hardy–Weinberg equilibrium was not violated. There were no significant differences between groups. The only trend level difference was on shock intensity, between 5HTR_{1A} C-carriers and G/G $F(1,143) = 3.8$, $p = .053$.

($F(1,133) = 4.6, p = .034$), and all other tests were not significant ($F(1,131) < 3.0, p > .084$, including STAI, $F(1,131) < 3.2, p > .076$).

3. Results

3.1. Descriptive statistics and cue awareness

Gender distribution, personality scores and experimental parameters (shock intensities, baseline startle amplitude), for the entire sample as well as for the 5-HTT_{1A} genotype and cue awareness groups are summarized in Table 1. Cue awareness and 5-HTT_{1A} genotype did not significantly affect shock intensities, baseline startle amplitude and raw startle data across conditions (light-on/CXT+, dark/CXT+, dark/CXT–; all p -values $> .05$), though the difference in level of shock intensity was trend level significant and was added as covariate to the subsequent analyses. Moreover, 5-HTT_{1A} genotype was neither associated with trait anxiety nor with NEO-neuroticism. According to our criteria, 74 subjects were classified as aware of the CS–US contingency at the end of uninstructed acquisition, 76 were unaware.

3.2. Effects of initial cue awareness: fear-potentiated startle

3.2.1. Context FPS

There was a main effect of cue awareness on context FPS ($F(1,131) = 4.8, p = .030$), reflecting stronger contextual fear in the initially cue-unaware subjects (mean context FPS (Z-score) across phases = 0.44, SD = 0.29) than the cue-aware subjects (mean context FPS (Z-score) across phases = 0.32, SD = 0.34). There was no interaction between phase and cue awareness ($F(2,262) = 0.6, n.s.$), indicating

that the effects of initial cue awareness on contextual fear remained stable across the experiment. See Fig. 2, top row, dark gray bars. This is of interest, as this indicates that even though all subjects received explicit instructions regarding the CS–US association before the expression phase began, the effect of initial learning on contextual fear is not significantly affected by these instructions. There was also no main effect of the factor phase indicating that on average, context FPS remained at a similar level across phases of the experiment, reflecting the fast learning of the context association in the beginning of the experiment.

3.2.2. Cue FPS

There was a significant effect of phase on cue FPS ($F(2,262) = 3.9, p = .023$), which interacted with cue awareness (Fig. 2, top row, light gray bars, $F(2,258) = 10.5, p < .001$). Tested per phase, the effect of cue awareness on cue FPS was significant in the uninstructed acquisition phase only ($t(148) = 5.6, p < .001$) but not in the other two phases (t -values $< 1, n.s.$).

3.3. Effects of initial cue awareness: fear-potentiated VAS fearfulness (FP-VAS)

3.3.1. Context FP-VAS

In contrast to the startle data, in the context analysis, phase interacted with cue awareness ($F(1,129) = 6.2, p = .006$). This means that in the subjective fearfulness measures, the effect of awareness on contextual fear was reduced after instructions (tested per phase: uninstructed acquisition $t(148) = 5.0, p < .001$; expression $t(148) = 1.7, p = .095$; extinction $t(147) = 1.1, n.s.$). In addition to the interaction

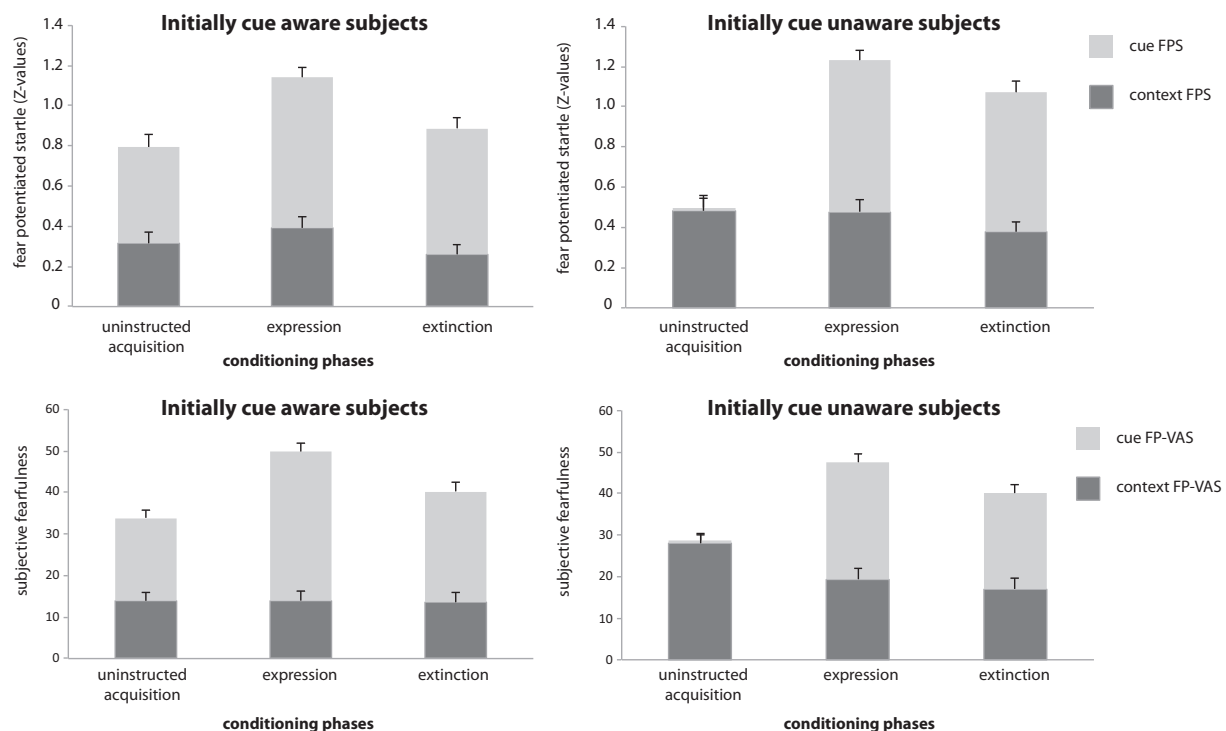


Fig. 2. Contextual (shock context dark–safe context dark; dark gray bars) and cued (shock context light–shock context dark; light gray bars) fear is displayed across the three phases of the experiment, as a function of initial cue-awareness. Context potentiation and cue potentiation are displayed with stacked bars to also visualize total (context [dark gray] + cue [light gray]) fear-potentiation. In both fear-potentiated startle (FPS) and fear-potentiated visual analog scale fearfulness ratings (FP-VAS), initial cue-awareness greatly affected cue potentiation in the uninstructed acquisition phase. Initially cue-aware subjects displayed strong cued fear (left column, left-most bars; VAS: $n = 74$; startle = 73) and unaware subjects showed no cued fear (right column, left-most bars; VAS: $n = 71$; startle = 69). Context FPS and FP-VAS were affected by cue awareness to a lesser but significant extent: in both measures there was a main effect of cue awareness on contextual fear, with stronger context fear in unaware participants (right column). The effects of initial cue-awareness on cue FPS and FP-VAS disappear after explicit instructions (no significant differences between groups in the expression and extinction phases). However, the effect of awareness on context FPS does not significantly diminish after instructions (no significant interaction between cue-awareness and phase, see text for details). Error bars represent 1 SEM. Note: cued FPS and FP-VAS are close to zero in the uninstructed acquisition phase in the initially cue-unaware subjects, therefore only an additional error bar shows visibly in these graphs (right-hand column).

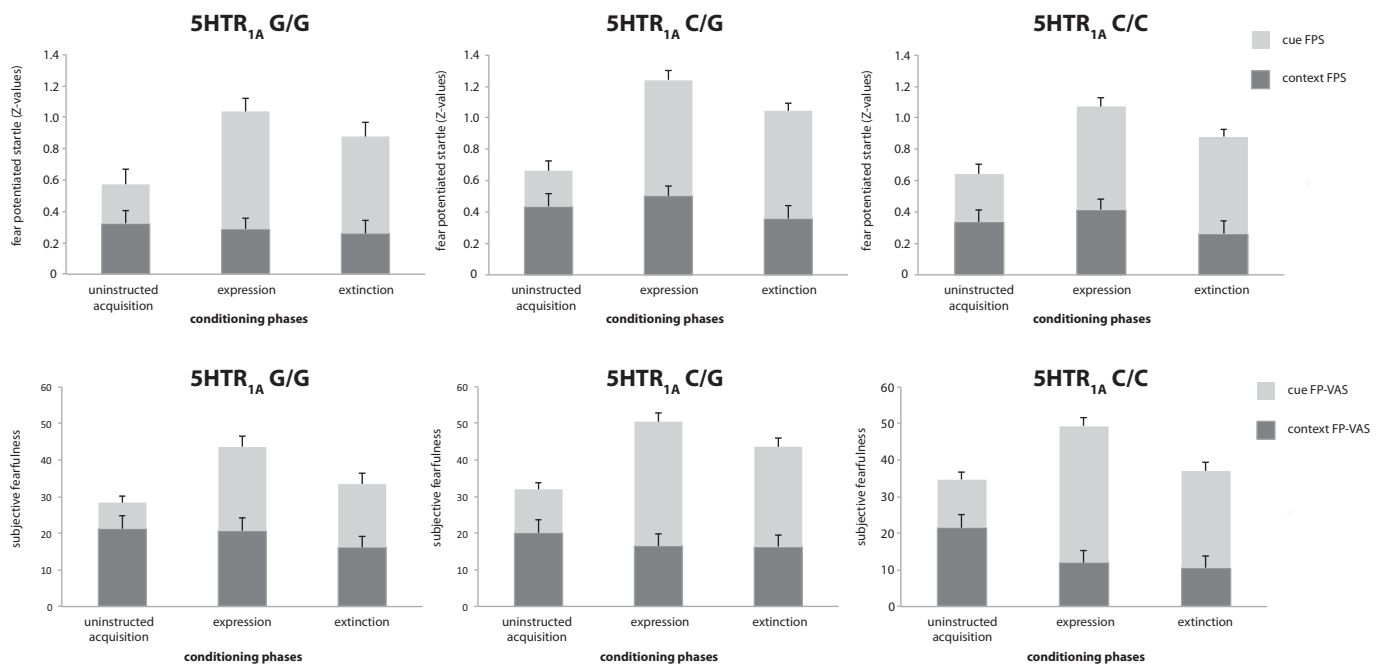


Fig. 3. Contextual (shock context dark–safe context dark; dark gray bars) and cued (shock context light–shock context dark; light gray bars) fear is shown across the three phases of the experiment, as a function of variation in the 5-HTR1A polymorphism (rs6295). Context potentiation and cue potentiation are displayed with stacked bars to also visualize total (context [dark gray] + cue [light gray]) fear potentiation. Error bars represent 1 SEM. Statistical differences were found in context FPS (top row, dark gray bars) with C-carriers (middle and right hand columns, top panels, total $n = 107$) displaying stronger contextual fear responses than the G/G group (left hand column, top panel, $n = 35$). In addition, for the subjective fearfulness data (bottom row) the C-carriers ($n = 108$) showed stronger cue fear-potentiated scores on the visual analog scale (FP-VAS) compared to the G/G group ($n = 37$).

with phase, the main effect of cue awareness was also significant ($F(1,133) = 8.3$, $p = .005$).

3.3.2. Cue FP-VAS

As with cue FPS, the analyses of cue FP-VAS indicated a main effect of phase ($F(2,266) = 5.1$, $p = .010$) and an interaction between cue awareness and phase ($F(2,262) = 6.4$, $p = .003$), with a similar pattern in which the effect of cue awareness is significant in the uninstructed acquisition phase and not (or only trend level) significant across the following phases: uninstructed acquisition $t(148) = 8.4$, $p < .001$; expression $t(148) = 1.9$, $p = .055$; and extinction $t(147) < 1$, n.s. In addition to the interaction with phase, there was a main effect of cue awareness ($F(1,133) = 8.1$, $p = .005$).

3.4. Effects of 5-HTR1A genotype: fear-potentiated startle

3.4.1. Context FPS

The repeated measures ANOVA on context FPS across the different phases of the experiment resulted in a main effect of 5-HTR1A genotype (G/G versus C-carriers; $F(1,131) = 5.3$, $p = .023$), reflecting larger context potentiation for the C-carriers across the experiment. This difference is illustrated with the dark gray bars in Fig. 3, top row.¹ There was no interaction between phase and 5-HTR1A genotype, indicating that the effect of genotype on contextual fear remained stable across the experiment.

3.4.2. Cue-FPS

The ANOVA for cue FPS did not reveal a main effect of 5-HTR1A genotype ($F(1,129) = 0.05$, n.s.), nor an interaction with phase ($F(2,262) < 1.0$, n.s.).

3.5. Effects of 5-HTR1A genotype: fear-potentiated VAS fearfulness

3.5.1. Context FP-VAS

In contrast to the startle data, the repeated measures ANOVA on context FP-VAS did not result in a significant main effect of 5-HTR1A genotype ($F(1,133) = 0.4$, n.s.), nor an interaction with phase ($F(2,266) = 1.2$, n.s.).

3.5.2. Cue FP-VAS

Unexpectedly, this analysis revealed a main effect of 5-HTR1A genotype on cue FP-VAS ($F(1,131) = 9.1$, $p = .003$), indicating stronger cue FP-VAS in the C-carriers (Fig. 3, bottom row). The interaction between 5-HTR1A genotype and phase was not significant, but bordered on significance ($F(2,266) = 2.8$, $p = .075$).

3.6. Analyses of total potentiation of startle and subjective fearfulness

The a-priori analysis was based on the specific hypotheses for contextual fear, and hence performed on cue and context separately. However, the unexpected differences in how context-potentiated startle and cue-potentiated VAS were modulated by 5-HTR1A genotype, combined with an apparent correspondence in how total fear potentiation is increased in C-carriers (see Fig. 3) led to a follow-up analysis on total FPS/FP-VAS. Total potentiation was defined as light/CXT + vs. dark/CXT–, which is equal to the summation of the effects of context and cue (see also Heitland et al., 2012 for a similar approach). Total fear-potentiation of startle was not significantly affected by 5-HTR1A genotype ($F(1,131) = 1.6$, n.s.). The effect of 5-HTR1A on total potentiation of VAS fearfulness was marginally significant ($F(1,133) = 3.7$, $p = .056$).

3.7. Effects of trait anxiety on conditioned responding – correlational analyses

In order to test whether the effect of STAI-trait on fear responding in the context can be replicated in subjects who had not spontaneously acquired the CS–US association, the sample was split up for cue awareness

¹ From Fig. 3, it appears that the C/G group displays stronger context potentiation than the C/C homozygote group, but these differences were not statistically significant ($p > .2$).

in the uninstructed phase, and analyses focused on the expression phase that followed the explicit instructions.

The correlation between normalized startle responding during the shock context (dark) in the expression phase with the Spielberger trait anxiety was analyzed. Startle during the shock context in the expression phase was only significantly correlated with STAI in those who had not become aware of the CS–US contingency in the uninstructed acquisition phase (unaware: $r(73) = .24$, $p = .041$). As expected, the effect of this factor could not be demonstrated in subjects who became aware of the cue association previously ($r(76) = .02$, n.s.). The positive correlation indicates that the higher the STAI trait anxiety score, the higher the fear responses in the shock context. See Fig. 4 for an illustration. Only startle responding in the shock context was correlated with STAI, but VAS fearfulness was not (unaware: $r(74) = .09$, n.s.; aware: $r(76) = .16$, n.s.).

4. Discussion

The present study employed a virtual reality cue and context conditioning paradigm to investigate the impact of cue awareness, trait anxiety and 5-HTT_{1A} genotype on contextual fear responses. The analyses presented in this paper demonstrate effects of factors related both to cue awareness and individual differences that may underlie enhanced sensitivity to contextual threat. These individual difference factors, comprising trait anxiety and genetic variability within the 5-HTT_{1A}, impact different aspects of conditioned contextual fear responding.

The first aim of this study was to investigate the effects of cue acquisition on contextual fear. In line with previous findings with this cue and context fear conditioning paradigm (Baas, 2013; Baas et al., 2008), there were very strong effects of initial cue-awareness on fear responding. In both FPS and FP-VAS, the group that did not become aware of the CS–US contingency in the uninstructed conditioning phase showed a complete absence of cue potentiation, and at the same time, a stronger context potentiation. In the FPS data, the stronger context potentiation lasted throughout the experiment, as evidenced in a main effect of cue-awareness on context potentiation, without interaction with phase. The subjective fearfulness ratings indicated greater contextual fear in the initially unaware group during the uninstructed phase that did not persist after explicit instructions. This indicates that on a conscious, subjective level, the group who was initially unaware of the CS–US contingency succeeded in regulation their fear-responses according to the instructions. However, at the level of more basic defense mechanisms indexed by fear-potentiated startle measures, this increased responsivity to the context remained. The lack of cued startle potentiation in the subjects who remained unaware of the cue contingency is in contrast with previous studies in which skin conductance recording was

shown to track awareness, but fear-potentiated startle was apparent also in the absence of awareness (Hamm and Vaitl, 1996; Weike et al., 2005, 2007; Sevenster et al., 2014). However, these studies all involved discrete cue conditioning, whereas the absence of cue potentiation in cue-unaware participants in the current study replicates previous findings with this cue/context conditioning paradigm (Baas, 2013; Baas et al., 2008). Perhaps such an implicit learning mechanism reflected in startle potentiation to a CS without conscious awareness of the CS–US contingency breaks down when the conditioned stimuli are embedded in a more complex, contextual contingency. Taken together, the present study supports prior findings (Baas, 2013; Baas et al., 2008), demonstrating that initial cue awareness is an important factor in the regulation of anxious apprehension in the context where the threat is encountered.

The second aim of this study was to investigate whether trait anxiety predicts contextual fear. As stated earlier, patients suffering from panic disorder (Grillon et al., 2008) and post-traumatic stress disorder (Grillon et al., 2009b) show stronger contextual fear in an unpredictable shock context than healthy control subjects. Furthermore, we demonstrated in a previous study (Baas, 2013) that subjects vary in the ability to reduce contextual fear after the introduction of a predictive cue. In this prior study, a significant positive correlation was found between trait anxiety on the one hand, and on the other hand startle responding and subjective fearfulness to the shock context after a predictive cue was explicitly introduced. We interpreted this as an inability to utilize the periods of relative safety (during the absence of the threat cue) to regulate contextual anxiety. Indeed, subjects high on trait anxiety failed to regulate their contextual anxiety even though they were made aware of the danger CS–US contingency. In the current study, this situation is mimicked by subjects who did not become cue aware during uninstructed acquisition, but were then explicitly instructed. In agreement with the earlier findings (Baas, 2013), in the initially unaware subjects trait anxiety was correlated with startle responding to the threat context after they were made aware of the CS–US contingency through instructions. This finding is in line with observed hypoactivity in brain areas associated with down-regulation of fear (the ventro-medial prefrontal cortex) in neuroimaging studies in patients with anxiety disorders like panic disorder and post-traumatic stress disorder (PTSD, Killgore et al., 2014; Rougemont-Bücking et al., 2011). The inhibition of contextual fear after learning about more specific predictors of threat (safety signal; Seligman and Binik, 1977) depends on adaptive regulation in a relatively ambiguous situation that is likely to involve the same regions (Rougemont-Bücking et al., 2011). In the current sample, the relationship between context inhibition and trait anxiety was exclusive to startle responding, and not present in subjective fearfulness data. Taken together, the current pattern of results suggests that high trait anxiety might prevent subjects to utilize safety signals to inhibit fear

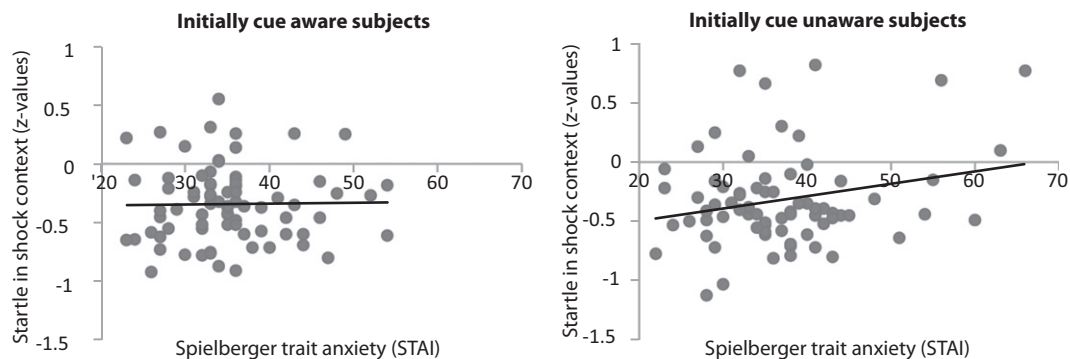


Fig. 4. Correlation between Spielberger trait anxiety (STAI) and the level of startle responding in the shock context in the dark, i.e. in the absence of the light cue. Contextual fear is not affected by trait anxiety in the group of subjects who spontaneously acquired the CS–US association in the uninstructed acquisition phase (cue aware subjects, left-hand panel, $r(76) = .02$, n.s.), but these variables were associated in the subjects who failed to spontaneously acquire this association ($r(73) = .24$, $p = .041$, right-hand panel).

responding at a physiological level of defense, even though they may be consciously aware of their presence and meaning.

The third aim of this study was to investigate involvement of the serotonin 1_A receptor (5-HTR_{1A}) in the inhibition of contextual fear responses, as suggested by preclinical data (e.g., see Hammack et al., 2009). The data reviewed by Hammack et al. (2009) demonstrates that feedback projections from the dorsal raphe nucleus to the bed nucleus of the stria terminalis (BNST) are critical in the inhibition of contextual fear responses. Due to the paucity of applicable pharmacological tools that selectively target 5-HTR_{1A} in humans, several studies have focused on innate variability in the 5-HTR_{1A} gene. A supposedly functional single nucleotide polymorphism in the promoter region of the 5-HTR_{1A} gene (Albert, 2012), rs6295, has among other findings been associated with panic disorder (Rothe et al., 2004) and panic symptoms (Huang et al., 2004). In the fear-potentiated startle data, enhanced contextual fear was observed in C-carriers of the 5-HTR_{1A} gene. In the subjective fearfulness ratings, not fearful responding to the context, but to the cue was significantly affected by this polymorphism. This was in contrast to our expectations based on the preclinical model that contextual fear would be specifically affected by the 5-HTR_{1A} polymorphism. The same group (C-carriers) that showed stronger context potentiation in the startle data, displayed stronger cue potentiation of fearfulness ratings.

Dissociations in findings between physiological (startle) and subjective (shock expectancy or fearfulness ratings) measures have been found in many previous studies (Grillon et al., 2006; Heitland et al., 2012, 2013; Kindt et al., 2009). However, it is usually the case in studies looking at a neurobiological manipulation (e.g., the administration of a pharmacological substance), or genetics, that physiological measures are more sensitive than subjective measures. This is not unexpected, given that the physiological measures are more closely related to the neuronal substrate of the fear circuitry affected by these factors (Meyer-Lindenberg, 2012). The impact of the 5-HTR_{1A} polymorphism seems to affect different experimental conditions in the physiological and subjective measures in the present study. Upon closer inspection of the data in Fig. 2, besides the differences between the patterns across genotypes for the physiological (FPS, upper row) and subjective (FP-VAS, bottom row) measures, a similarity pops out. In both measures, the total startle potentiation seems increased for the C-carriers (middle and right-hand panels combined) versus the G/G homozygotes (left hand panels), but genotype effects on total startle potentiation were not significant. In sum, fear conditioned responding seems to be affected by the polymorphism under study. The exact nature of these effects remains to be determined.

The effect of the 5-HTR_{1A} polymorphism (rs6295) on contextual startle responding can be interpreted as a functional difference in how contextual fear is regulated at the level of the BNST (Hammack et al., 2009). In an extensively tested framework on how fear-potentiated startle is regulated by fear versus anxiety responses, the BNST is strongly implicated in contextual responses specifically (Davis et al., 2010), which are modeled in our context condition. As proposed by Hammack et al. (2009), exposure to stressful circumstances may downregulate 5-HT_{1A} receptor functioning and with that, reduce the negative feedback that limits activation of the BNST. Via that route, variability in this gene may cause variability in individuals' adaptivity in response to stressful circumstances. Many studies have also implicated the hippocampus in context conditioning (Alvarez et al., 2008; Hasler et al., 2007; Marschner et al., 2008). However, recent preclinical studies suggest that involvement of the hippocampus in context conditioning is not via 5-HTR_{1A} (Chang and Liang, 2012), which may be consistent with the interpretation that the hippocampus is not so much involved in the expression of fear, but rather with processing of contextual information (Barot et al., 2009).

A final point of discussion is the direction of the 5-HTR_{1A} effect. Several studies have found that increased risk of anxiety disorders (see references in the Introduction section) is associated with the G/G

genotype. In the present study C-carriers display the largest context-FPS and the largest cue-FP-VAS. This may appear contradictory, but C-carriers demonstrated higher amygdala reactivity in an imaging study (Fakra et al., 2009) and increased risk for anxiety disorders in patients with temporal lobe epilepsy was found in C-carriers (Schenkel et al., 2012). As has been argued for other polymorphisms, speaking of an allele that confers just 'risk' may not accurately describe the different effects the 5-HTR_{1A} polymorphism under study (Beste et al., 2011). Either way, further knowledge with regard to human 5-HTR_{1A} functioning and genetics, and its neuronal and behavioral associations is needed before definite conclusions can be drawn. As of now, this single association should therefore be viewed with great caution until it is replicated. The relatively small sample for genetic analysis and the lack of a replication sample are clear limitations of the current study, in particular with respect to the genetic analyses.

In sum, using fear conditioning to contexts in which explicit and discrete cues signal danger, we demonstrated that cue learning, trait anxiety and genetic variability in 5-HTR_{1A} are involved in the regulation of contextual anxiety. Future studies in healthy subjects and patients in which contextual fear responses are assessed in combination with contingency awareness, genetics and personality analyses are needed to further delineate the role of individual difference factors in regulation of contextual fear and their relevance to clinical anxiety.

Disclosure

The authors declare no conflict of interest.

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