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The PAC1 receptor risk genotype does not influence fear acquisition, extinction, or generalization in women with no trauma/low trauma

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ABSTRACT

Women are known to have twice as much lifetime prevalence of post-traumatic stress disorder (PTSD) as men do. It has been reported that the risk genotype (CC) of a single nucleotide polymorphism (SNP) (rs2267735) in the pituitary adenylate cyclase-activating polypeptide (PACAP-PAC1R) system is associated with PTSD risk and altered fear conditioning and fear extinction in women. Surprisingly, no previous work has studied the effect of this SNP on fear conditioning, extinction, or generalization in non-traumatized/low trauma load women. Here, two separate groups of women underwent either a two-day fear conditioning and fear extinction paradigm, or a one-day fear conditioning and fear generalization paradigm. Results showed no significant differences between genotypes in conditioned stimulus discrimination, during fear acquisition, extinction, or generalization. These findings suggest that the previously reported fear processing impairments in traumatized CC women are not a consequence of this genotype alone, but likely dependent on the interaction between this genetic risk and the exposure to traumatic stressors.

1. Introduction

Fear memory allows to preserve well-being and to adapt to the environment in threatening situations. Fear processes can exhibit maladaptive characteristics, like exaggerated or generalized responses in safe environments, which are key symptoms of fear-based disorders. Previous research has found that patients suffering from fear-based disorders, including post-traumatic stress disorder (PTSD), present alterations in fear conditioning (FC) and fear extinction (FE). Studies show that overexpression of conditioned fear, impairments in within-session FE, and reduced between-session FE recall are among these alterations (Andero & Ressler, 2012; Milad et al., 2009; Norrholm et al., 2011; Orr et al., 2000; Rougemont-Bücking et al., 2011; Zuj et al., 2016).

Moreover, women show twice as much risk as men of suffering panic attacks and social phobias, and are more likely to present comorbidities and a longer disease course (Kessler et al., 2015; McLean et al., 2011). The PTSD lifetime prevalence in men is 5 %-6 %, but in women it doubles to 10 %-12 % (Olff, 2017). Among the causes for these differences in prevalence it has been reported that women are usually victims of collective and domestic violence more often than men, and overall,

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are more exposed to traumatic events than men, which naturally increases the prevalence of PTSD (Robles-García et al., 2020). Not only social factors, but also hormonal fluctuations in women are believed to make them more physiologically vulnerable to PTSD by increasing HPA axis reactivity (Christiansen & Berke, 2020). Furthermore, epigenetic effects of estradiol may also partake in PTSD heritability, which has been found to be larger in women than in men (Duncan et al., 2017; Guffanti et al., 2013). Interestingly, important sex differences have been widely described in fear processing in both rodents and humans (Velasco et al., 2023).

Among the different affectations present in PTSD, overgeneralization to non-threatening or irrelevant stimuli is often seen (PTSD) (Dunsmoor & Paz, 2015; Laufer, Israeli, & Paz, 2016). Fear generalization is defined as the process by which conditioned fear responses transfer to novel stimuli perceptually similar to the CS+ (Torrents-Rodas et al., 2013). In a prior study a machine learning model ranked fear generalization-related features as the most critical predictors for a firefighter sample (Li et al., 2022). Moreover, during a fear generalization task, PTSD patients showed higher risk ratings to generalized stimuli than healthy controls and trauma-exposed resilient individuals (Zhu et al., 2022). As shown in different studies and a meta-analysis, this overgeneralization is observed in patients with distinct backgrounds, including combat and childhood-related trauma among others (Kaczkurkin et al., 2017; Lis et al., 2020; Morey et al., 2015).

FC and FE paradigms are used to study healthy and pathological fear processing (Maren & Holmes, 2016). After acquisition, fear memories undergo long-term storage through synaptic plastic changes and systems consolidation (Ressler et al., 2002). Hence, fear memories can be modulated during acquisition, consolidation, or expression. During FC, fear acquisition is evidenced by the progressive increase of conditioned responses to a reinforced stimulus (CS+) in comparison to a non-reinforced stimulus (CS-). Fear expression is assessed by the presentation of a few unreinforced CS+ , either immediately after acquisition or after an incubation period (consolidation window). Fear extinction refers to the gradual decrease of conditioned responses upon the presentation of unreinforced CS+ (Myers & Davis, 2007).

The PACAP-PAC1R (pituitary adenylate cyclase-activating polypeptide and its type I receptor), encoded by *ADCYAP1-ADCYAP1R1*, has a regulatory role in neuroendocrine stress responses and fear memory (Lebois & Ressler, 2016). Previous findings in humans show that the risk genotype (CC) within the SNP rs2267735 of *ADCYAP1R1* is associated with PTSD severity and amygdalo-hippocampal activation to threats, in traumatized women but not men (Ressler et al., 2011; Stevens et al., 2014). An *in vitro* functional characterization of the SNP suggested that the risk for PTSD may be conferred by its interactions with estradiol dynamics (Mercer et al., 2016). We recently demonstrated that the risk genotype (CC) within the SNP rs2267735 of *ADCYAP1R1* was associated with differences in fear extinction in a cohort of traumatized women (Velasco et al., 2022). However, no studies to date have explored whether this risk genotype (CC) modulates fear memory in non-traumatized/low trauma load adults. Due to the lack of studies focusing on non-clinic samples, we do not have information about whether this polymorphism can also impact fear learning in non-traumatized women, and therefore, pose a genetic risk factor for developing PTSD. This study allows for the distinction between genetic vulnerability and gene-trauma interactions.

Here we performed two different fear studies analyzing the risk genotype (CC) within the SNP rs2267735 of *ADCYAP1R1* in young women with no-trauma/low trauma load. In the first study, participants completed a FC and delayed-FE task (FC-FE study). Based on previous results, we hypothesized that CC women would show impaired fear extinction compared to women with the G allele. In the second study, a different group of participants underwent a fear acquisition and fear generalization task (ACQ-GEN study). Given the close relationship between fear generalization and PTSD, and the association between this disorder and the rs2267735 risk genotype, we expect women with the CC risk genotype to exhibit enhanced fear generalization.

2. Experimental procedures

2.1. Overview

Data from two different fear-learning studies (FC-FE and ACQ-GEN) that used two different samples are included in this report (Florido et al., 2024; Torrents-Rodas et al., 2013). Both studies were approved by the Ethics Committee on Animal and Human Research from the Universitat Autònoma de Barcelona and conducted in accordance with the Declaration of Helsinki (The World Medical Association, 2008) and had identical recruitment (by advertisement) and assessment methods. Exclusion criteria (age >45 to avoid menopausal/premenopausal effects (McKinlay, 1996), current drug abuse, current psychiatric or medical disorders, pregnancy, visual or auditory impairments and current use of medications that could interfere with the study objectives, as per a semi-structured interview) were also the same for both studies. Low trauma load/no trauma condition was assessed by the self-reported status of the participants and from the lack of trauma- and stressor-related disorders. All participants included in our analyses were women and signed informed consent forms. In the FC-FE study, a two-day experimental protocol was used: fear acquisition (day 1) and fear extinction (day 2, 24 h after). In the ACQ-GEN study, a fear generalization paradigm developed by Lissek and colleagues was used, consisting of: pre-acquisition, fear acquisition, and fear generalization. For this experiment, all phases were performed on the same day (Lissek et al., 2008) (see Fig. 1 for a schematic of these experiments). In both studies, fear was assessed using skin conductance responses (SCR), fear-potentiated startle (FPS), and subjective risk ratings. Skin conductance was selected for its wide use, which allows for comparison of our results with most previous research (e.g., Jovanovic et al., 2013; Merz et al., 2016; Pohlack et al., 2015; Seligowski et al., 2019). Moreover, fear-potentiated startle is often used due to its high translational value, and its sensitivity to individual differences (Lonsdorf et al., 2017). Risk ratings were employed to obtain not only objective psychophysiological measures of fear learning, but also perceived assessments. Overall, findings support the use of multiple fear measures in human fear conditioning studies (Constantinou et al., 2021). Fear acquisition was different between the studies because of the complementary experimental goals (see below for details). All participants completed the trait section from the Spanish Version of the State-Trait Anxiety Inventory (STAI-T) (Spielberger et al., 1982) and provided a saliva sample. Participants were asked to abstain from alcohol, tobacco, and any other drug for 24 h and from caffeinated drinks for 12 h before the experiment. Participants were paid 20 € (FC-FE) or 15 € (ACQ-GEN). Results obtained with the FC-FE study sample have been previously included in a different publication from our lab. In this previous study a similar approach to this paper was followed, focusing on the effect of a different SNP in fear acquisition and extinction (Florido et al., 2024). Here, we include only women, hence the sample differences. The sample from the ACQ-GEN study was obtained from Torrents-Rodas et al. (2013). Both sexes were included in the original study, where certain cutouts for STAI-T scores were also applied to divide participants in three groups. Here, we include only women, regardless of STAI-T scores. See supplementary table 1 for sample comparison.

We used Lonsdorf et al. (2010) to calculate the sample size as it uses a differential FC (Day1) and delayed extinction paradigm (Day2). The calculation of the sample size was based on the estimated effect size (η2) reported during the extinction (day 2) obtained by performing a repeated measures ANOVA between val/val and val/met BDNF carriers ($F(1,46)= 5.25$, $p = .027$, $\eta2 = 0.10$). We took $\eta2 = 0.10$ to calculate the Effect size $f = 0.333$. We then input this value in G*Power 3.1.9.2 tool (Faul et al., 2007) to compute sample size and assumed an alpha error of 0.05, beta risk of less than 0.2 and 2 groups, obtaining 28 participants required for each group.

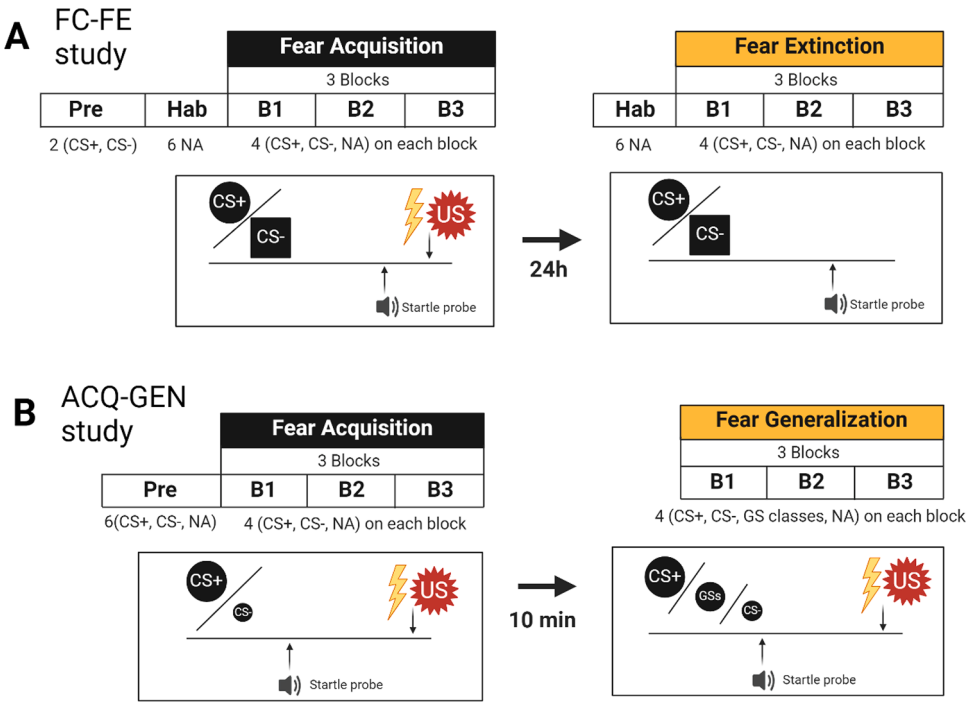

Fig. 1. Diagram of both studies included. Panel A shows FC-FE study, Panel B shows ACQ-GEN study. B1,B2,B3: block, CS+ : reinforced CS, CS-: non-reinforced CS, NA: noise alone, GS: generalization stimuli, US: unconditioned stimulus, pre: pre-acquisition trials, hab: habituation phase.

2.2. *Conditioned stimuli (CS)*

2.2.1. *FC-FE study*

Two geometric figures were used as CSs, a square and a circle. One stimulus (CS+) was followed by the US, while a second stimulus (CS-) was not. The figure used as CS+ , and the one used as CS- were counterbalanced between participants. Stimuli were presented for 8000 ms on a computer monitor.

2.2.2. *ACQ-GEN study*

Ten rings of gradually increasing size served as conditioned stimuli (CS) and generalization stimuli (GS). The smallest ring had a 5.08 cm diameter, and subsequent rings increased by 15 %. During fear acquisition, the smallest and largest rings were used as CSs. For half of the participants, the smallest ring was the CS+ (paired with the US before its offset), and the largest was the CS- (not paired); for the other half, this pairing was reversed. Stimuli were presented for 8000 ms on a computer monitor.

2.3. *Unconditioned stimuli (US) and startle probes*

In both studies, the US was an electric shock of 100 ms delivered to the non-dominant forearm and its intensity was selected by the participant. The US was generated by a stimulator (Grass Instruments S48, USA), isolated (SIU5), and transmitted via a constant current unit (CCU1) to a bipolar bar electrode (EP10–621, Technomed Europe, Netherlands). The startle probe was a 50 ms burst of white noise at 102 dB, with near-instantaneous rise time, delivered binaurally through high-fidelity headphones. Stimulus presentation and timing were controlled with the software Affect 4.0 (KU Leuven, Belgium) (Spruyt et al., 2009) or Presentation (Neurobehavioral Systems, Inc., USA).

3. Procedures

3.1. *FC-FE study*

On day 1 the participants underwent the following procedures: they gave a 2-ml saliva sample, the electrodes were attached, the intensity of the US was calibrated (to an intensity that was “highly uncomfortable but not painful”), and the headphones for the startle probes were placed. Participants were instructed that they could receive an electric shock during the experiment which they would be able to predict if they paid close attention to the presentation of the figures. They were also explicitly asked to ignore the startle probes and to assess the risk of shock as online expectancy risk ratings during every CS presentation using a 0–9 scale (from “no risk of shock” to “maximum risk of shock”). The scale for risk assessment was available for scoring during the 8 s of stimuli presentation. Still, the participants were asked to rate them as fast as possible. During the *pre-acquisition* phase, two non-reinforced presentations of each CS occurred; followed by the *habituation* phase with 6 presentations of the startle probe alone. During the *fear acquisition* phase, the CS+ had an 83 % reinforcement rate (US presented 500 ms before CS+ offset). The CS- was never paired with the US. A fixation cross appeared during the intertrial intervals (ITIs). Trial order was pseudorandomized and counterbalanced across CS, with no more than two consecutive presentations of the same CS. Startle probes were presented in 66 % of the trials 1000 ms before CS offset, and in 68 % of ITIs. ITIs and inter-probe intervals ranged from 10 to 14 s, and 18–22 s respectively. The acquisition phase consisted of 12 presentations of each stimulus CS+ , CS-, and noise alone (NA) (defined as ITI periods with startle probe presentations). After the task, participants rated the discomfort associated with the startle probe and the US (0 = no discomfort, 9 = maximum discomfort), and answered a question regarding contingency awareness (“The electric stimulus appeared along with a) the circle, b) the square, c) there was no association, d) I don’t know”).

On day 2, participants followed the same procedures as for day 1 except for the US calibration and they were instructed that they would predict better the appearance of the electric shock if they recalled what they had experienced in the previous session. This was followed by the *habituation* phase with 6 presentations of the startle probe alone and the *fear extinction* phase, with 12 presentations of each stimulus CS+ , CS-, and NA. No US was administered during day 2.

3.2. ACQ-GEN study

Upon arrival, participants were given written instructions about the experiment and signed the informed consent. They were not instructed about the CS-US contingency but were told that they might learn to predict the shock if they paid attention to the presented stimuli. Next, buccal mucosa was collected with a cotton swab, the electrodes were placed, and the intensity of the US was adjusted. During US calibration the trials needed for intensity selection were noted. After placing the headphones, 9 startle probes were presented to reduce initial startle reactivity (*habituation*). Startle probes were presented 4 or 5 s after the beginning of odd trials; inter-probe intervals ranged from 18 to 25 s. During even trials, online ratings of the perceived risk of shock were obtained (1 = no risk, 2 = moderate risk, 3 = high risk), 1 or 2 s after trial onset. The *pre-acquisition* consisted of 6 presentations of each stimulus CS+ , CS-, and (ITI), the CS+ was unreinforced. *Fear acquisition* consisted of 12 CS+ (9 of them co-terminating with a US), 12 CS-, and 12 ITI trials. Trial order was pseudorandomized and counterbalanced across CS, with no more than two consecutive presentations of the same CS. *Fear generalization* consisted of 12 CS+ (6 of them co-terminating with a US), 12 CS-, 12 ITI, and 6 trials from each of the eight GS sizes. Following the methods in Lissek et al. (2008), to balance the presentations of GS, CS+ , CS- and ITI, responses from two consecutive GSs were averaged (therefore, the responses to the first and second GSs were averaged as the response to the now constituted Class 1 (C1), the third and fourth GSs as the Class 2 (C2), and subsequently), resulting in four classes of responses to GSs (Class 1, Class 2, Class 3, and Class 4), leading to 12 trials of each stimulus class. Trial order was pseudorandomized and counterbalanced across CS, with no more than two consecutive presentations of the same CS, or of GSs belonging to the same class. There was a 10-minute break between the acquisition and the generalization. After the experiment, participants rated the discomfort produced by the US and the startle probe on a 1 (no discomfort) to 9 (maximum discomfort) scale; and answered a multiple-choice question regarding contingency awareness ("The electric stimulus usually appeared: a) in the presence of the smallest ring; b) in the presence of the biggest ring; c) randomly; d) I don't know.

3.2.1. Physiological Recordings and Response Definition

Physiological data were recorded using a BIOPAC MP 150 (BIOPAC Systems Inc., USA) and AcqKnowledge 4.1.0 software. SCR was recorded by attaching two Ag-AgCl electrodes filled with hydrogel to the middle and index fingers of the non-dominant hand. The signal was sampled at 125 Hz and SCR magnitudes were computed as the difference between the maximum SCR and the value at response onset, detected 1–6 s after stimulus onset. Only trials with deflections starting between 1 and 4 s after stimulus onset were considered valid. When two clearly separated peaks in the SCR were visible, only the maximum SCR response of the first peak was considered. Trials with no response, or magnitudes < 0.01 μS, were considered no-response trials and scored as 0. Trials with excessive baseline or artifacts were discarded. Startle blink responses were measured by recording the electromyographic activity (EMG) of the orbicularis oculi using two 0.5 cm Ag-AgCl surface electrodes. The raw EMG signal was sampled at 2 kHz and filtered (analog 50-Hz notch filter; and digital infinite impulse response band-pass filter, cut-off frequencies of 28 and 500 Hz), rectified, and smoothed (20-ms moving window average). Fear-potentiated startle responses were considered valid if the elevation in the EMG observed after the startle probe started between 20 and 100 ms, with its peak occurring between 20 and 150 ms (Blumenthal et al., 2005). Startle amplitudes were computed by subtracting the peak response value in microvolts (μV) from the mean response in the 50 ms preceding the startle probe presentation. Trials where no response was detected were scored as 0. Trials with excessive baseline or artifacts were discarded. SCR data were normalized by applying a square root transformation and startle data were normalized using T-scores (calculated using all probes for each day).

For the FC-FE study, after visual inspection (performed blind to genotype), participants showing non-valid SCR responses in 70 % of US trials during fear acquisition were classified as physiological non-responders (n = 1) and all SCR trials for these participants were scored as missing values and discarded. Similarly, individuals showing non-valid responses in 70 % of habituation startle probes were also classified as physiological non-responders and excluded from fear-potentiated startle analyses (n = 2 FC, n = 0 FE). Non-responders were identified using raw SCR and raw fear-potentiated startle data (Lonsdorf et al., 2019). Moreover, 13 participants declined to participate in the day-2 session and their data was not included. The final sample was made up of 97 participants.

In the ACQ-GEN study, participants were excluded from specific analyses if all the trials for one type of stimulus in a given block were rejected [n = 1 (FPS) and n = 5 (SCR) during acquisition; n = 2 (FPS) and n = 6 (SCR) during generalization]. Participants were excluded from risk rating analyses if they reported the same value throughout a particular experimental phase or due to technical problems (n = 1 during acquisition and n = 1 during generalization). Finally, participants were excluded from SCR analyses if they did not show any response throughout the session (n = 9 during acquisition and n = 5 during generalization). The final sample was made up of 101 participants.

3.3. Genotyping

Samples were collected into polypropylene tubes using the passive drool method, or cotton swabs, and frozen at −80°C until analysis. DNA was extracted using the ATP TM Genomic DNA Mini Kit Tissue (ATP Biotech Inc, Taiwan) or Real Extraction DNA Kit (Durviz S.L.U, Spain). DNA was quantified and diluted with DNAse free water to 5 ng/ul. The rs2267735 *PAC1R* was determined using the TaqMan Genotyping Master Mix (Applied Biosystems, USA) and the TaqMan SNP Genotyping Assay, human #4351379 assay C__1340532_10 (Thermo Fisher Scientific, Spain). The final volume for qPCR reaction was 10 ml, containing 10 ng of genomic DNA. Cycling parameters included 10 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C and 1 min at 60°C for annealing/ extension. PCR plates were read in an ABI 7500 fast system instrument with 7500 Software v2.3 (Applied Biosystems, USA).

3.4. Data analysis

Statistical analyses were performed with IBM SPSS 25.0. Mean values or ranks for the variables age, STAI-T, US calibration trials, US intensity, US and startle discomfort, and percentage of contingency-aware individuals were compared between genotypes using two-tailed T-tests or Mann-Whitney U tests, and frequencies using Chi-squared tests. Data were analyzed separately for each experimental phase (FC-FE study: pre-acquisition, fear acquisition, fear extinction; ACQ-GEN study: pre-acquisition, fear acquisition, and fear generalization) and for each measure (FPS, SCR, and risk ratings), using repeated-measures analyses of variance (ANOVAs; GLM procedure). Greenhouse-Geiser corrections were used when appropriate. Significant interactions were followed by pairwise comparisons with the Least Significant Difference procedure. The level of significance was set to $p < .05$ (two-tailed). We report η_p^2 as an estimate of effect size.

3.4.1. FC-FE study

Fear acquisition and fear extinction were split into three blocks (B1, B2, B3). Each block was calculated as the mean of four consecutive startle responses/SCR responses/risk ratings within a stimulus category (CS+, CS-). For startle data, CS+ potentiation (CS+ vs. ITI) and CS discrimination (CS+ vs. CS-) were calculated. CS discrimination was calculated for SCR and risk ratings. Stimulus (CS+, CS-, ITI for FPS; CS+, CS- for SCR and risk ratings) and block (B1, B2, B3) were included as within-subjects factors, and genotype (CC, G carrier) as between-

subjects factor.

3.4.2. ACQ-GEN study

The same procedure was used for the acquisition phase of this study, FPS and risk ratings data was split into three blocks (B1, B2, B3), of four startles each, but due to technical problems during the recording of one trial, SCR was divided into two blocks (B1, B2) to avoid the loss of data. The same within and between-subjects factors as in the FC-FE study were defined. In the fear generalization phase, stimulus (CS-, Class 1, Class 2, Class 3, Class 4, and CS+) was introduced as a within-subjects factor, and genotype as a between-subjects factor.

Since no significant effect of group in whole sample analyses was seen with the CC/G carrier division, separate analyses for each genotype were performed supplementary and results can be found graphically represented.

See Supplementary Table 2 for a summary of the acronyms used and their meanings.

All schematics were created with BioRender.com

4. Results

4.1. Genotype frequencies and participant's characteristics

4.1.1. FC-FE study

23 out of 97 participants (23.72 %) were homozygous for the G allele in the rs2267735 *ADCYAP1R1*, 49 (50.51 %) had GC genotype, and 25 (25.77 %) were homozygous for the C allele. (Allele frequencies: G allele = 48.97 %, C allele = 51.03 %). Hardy-Weinberg equilibrium was verified for the present population, $\chi 2 = 0.01$, $p = .99$. Given that the risk phenotype is associated with the C allele, and similar to previous studies (Jovanovic et al., 2020; Ressler et al., 2011), the GG and GC participants were combined into a G-carrier genotype group (n = 72). The two genotype groups did not differ in terms of age, STAI-T, US intensity, US trials required for calibration, US discomfort, startle discomfort, and the proportion of contingency-aware participants. Participant's characteristics by genotype group are presented in Table 1.

4.1.2. ACQ-GEN study

27 out of 101 participants (26.7 %) were homozygous for the G allele, 49 (48.5 %) had the GC genotype, and 25 (24.8 %) were homozygous for the C allele. (Allele frequencies: G allele = 50.99 %, C allele = 49.01 %). Hardy-Weinberg equilibrium was verified for this population, $\chi 2 = 0.09$, $p = .96$. Participants with the G allele were combined in a G-carrier group (n = 76). The two genotype groups did not differ in terms of age, STAI-T, US intensity, US trials required for calibration, US discomfort, startle discomfort, and the proportion of contingency-aware participants. Participant's characteristics by genotype group are presented in Table 2.

Table 1
Participant's characteristics in the FC-FE study. Data are presented as mean ± SD or number of subjects and percentage, n (%). STAI-T: trait section from the Spanish Version of the State-Trait Anxiety Inventory (range 0–60), US: unconditioned stimulus. Discomfort ratings ranged from 0 (no discomfort) to 9 (maximum discomfort). T-tests, Mann-Whitney U, or Chi-squared tests were used.

	CC (n = 25)	G carriers (n = 72)	*p*
Age, in years	22.5 ± 4.6	23.2 ± 4.2	0.221
STAI-T, 0–60	22.3 ± 9.3	21.6 ± 9.4	0.865
US calibration trials, n	4.2 ± 1.3	4.0 ± 1.2	0.629
US intensity, in mA	4.0 ± 1.3	3.6 ± 1.1	0.275
US discomfort, 0–9	7.1 ± 0.7	7.2 ± 0.7	0.481
Startle probe discomfort, 0–9	7.5 ± 1.6	6.9 ± 1.6	0.054
Contingency-aware individuals, n (%)	21 (93.9)	67 (95.8)	0.647

Table 2
Participant's characteristics in the ACQ-GEN study. Data are presented as mean ± SD or number of subjects and percentage, n (%). STAI-T: trait section from the Spanish Version of the State-Trait Anxiety Inventory (range 0–60), US: unconditioned stimulus. Discomfort ratings ranged from 1 (no discomfort) to 9 (maximum discomfort). T-tests, Mann-Whitney U, or Chi-squared tests were used.

	CC (n = 25)	G carriers (n = 76)	*p*
Age, in years	22.3 ± 2.5	22.3 ± 2.6	0.878
STAI-T, 0–60	23.5 ± 13.4	20.3 ± 11.7	0.564
US calibration trials, n	4.24 ± 1.4	4.49 ± 1.7	0.871
US intensity, in mA	3.3 ± 0.7	3.4 ± 0.9	0.676
US discomfort, 1–9	8.12 ± 1.0	7.92 ± 1.0	0.481
Startle probe discomfort, 1–9	7.1 ± 1.7	7.1 ± 1.8	0.940
Contingency-aware individuals, n (%)	23 (92)	70 (90)	0.732

4.2. FC-FE study: Pre-acquisition

Pre-acquisition analyses were performed to ensure there were no pre-existing differences between groups. No significant main or interaction effects for stimulus or genotype were found for fear-potentiated startle, skin conductance response, or risk ratings ($p > .05$).

4.3. FC-FE study: Fear acquisition

No significant main or interacting effects with genotype were found ($p > .05$).

4.3.1. Fear-potentiated startle

There was evidence of successful fear acquisition (i.e., higher response to the CS+ than to the CS−) as shown by a significant main effect of stimulus ($F(2,186) = 61.40$, $p < .001$, $\eta^2_p = 0.398$) with significant CS+ potentiation (CS+ vs. NA, $p < .001$), and CS discrimination (CS+ vs. CS-, $p < .001$). A Stimulus*Block interaction ($F(4,372) = 3.89$, $p = .004$, $\eta^2_p = 0.040$) showed faster habituation responses to the CS- and NA than to the CS+ . No genotype main effect ($F(1,93) = 1.29$, $p = .26$, $\eta^2_p = 0.014$, observed power = 0.203), nor Stimulus*Genotype ($F(2,186) = 0.99$, $p = .38$, $\eta^2_p = 0.011$, observed power = 0.220), Block*Genotype ($F(2,186) = 1.29$, $p = .28$, $\eta^2_p = 0.014$, observed power = 0.277) or Stimulus*Block*Genotype ($F(4,372) = 0.80$, $p = .53$, $\eta^2_p = 0.009$, observed power = 0.256) interactions were seen (Fig. 2, Supplementary Table 3).

4.3.2. Skin conductance response

There was evidence of successful fear acquisition by a main effect stimulus ($F(1,95) = 37.96$, $p < .001$, $\eta^2_p = 0.286$) with significant CS discrimination (CS+ vs. CS-; $p < .001$). No genotype main effect ($F(1,95) = 0.03$, $p = .86$, $\eta^2_p = 0.000$, observed power = 0.053), nor Stimulus*Genotype ($F(1,95) = 0.64$, $p = .43$, $\eta^2_p = 0.007$, observed power = 0.125), Block*Genotype ($F(2,190) = 0.22$, $p = .78$, $\eta^2_p = 0.002$, observed power = 0.082, Greenhouse-Geisser $\varepsilon = 0.882$) or Stimulus*Block*Genotype ($F(2,190) = 2.26$, $p = .11$, $\eta^2_p = 0.023$, observed power = 0.435, Greenhouse-Geisser $\varepsilon = 0.918$) interactions were seen (Fig. 3, Supplementary Table 3).

4.3.3. Risk ratings

A main effect of stimulus ($F(1,95) = 564.80$, $p < .001$, $\eta^2_p = 0.856$) with significant CS discrimination (CS+ vs. CS-; $p < .001$) evidenced successful acquisition. A Stimulus*Block interaction was also seen ($F(2,190) = 62.92$, $p < .001$, $\eta^2_p = 0.398$). No genotype main effect ($F(1,95) = 0.06$, $p = .81$, $\eta^2_p = 0.001$, observed power = 0.057), nor Stimulus*Genotype ($F(1,95) = 0.20$, $p = .66$, $\eta^2_p = 0.002$, observed power = 0.072), Block*Genotype ($F(2,190) = 0.11$, $p = .89$, $\eta^2_p = 0.001$, observed power = 0.066, Greenhouse-Geisser $\varepsilon = 0.934$) or

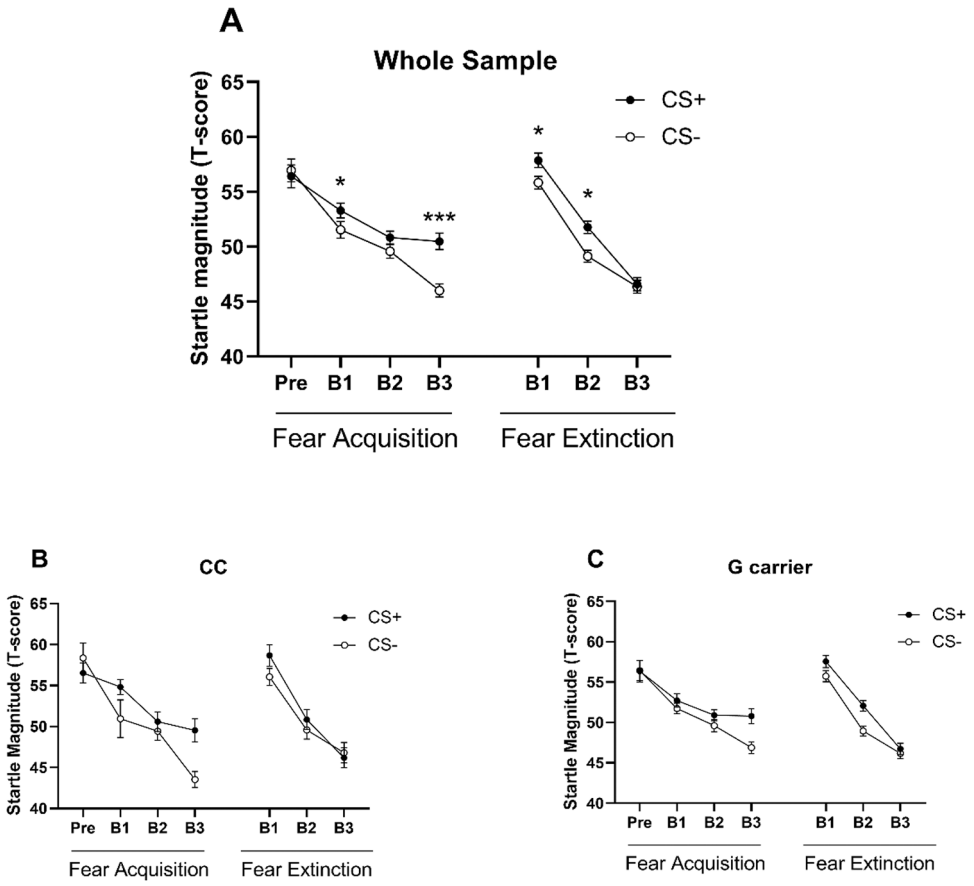

Fig. 2. FC-FE study. Fear potentiated startle: fear acquisition and extinction. Panel A shows whole sample data, panel B shows CC women, panel C shows G carrier women. B1,B2,B3: block, CS+ : reinforced CS, CS-: non-reinforced CS, pre: pre-acquisition trials. * Indicates CS discrimination (i.e., higher responses to CS+>CS-). * = p < 0.05, * * = p < 0.01, * ** = p < 0.001.

Stimulus*Block*Genotype ($F(2,190)= 0.30$, $p = .74$, $\eta_p^2= 0.003$, observed power= 0.097) interactions were seen (Supplementary Fig. 1, Supplementary Table 3).

4.4. FC-FE study: Fear extinction

No significant main or interacting effects with genotype were found ($p > .05$).

4.4.1. Fear-potentiated startle

During fear extinction, a main effect for stimulus ($F(2,190)= 73.71$, $p < .001$, $\eta_p^2= 0.437$) was seen, with significant CS+ potentiation ($p < .001$) and CS discrimination ($p = .018$). A Stimulus*Block interaction ($F(4,380)= 13.16$, $p < .001$, $\eta_p^2= 0.122$) showed a significant decrease in CS potentiation (B1, $p < .001$; B2, $p < .001$; B3, $p = .014$), and CS discrimination across blocks (B1, $p = .041$; B2, $p = .011$; B3, $p = .96$). No genotype main effect ($F(1,95)= 0.00$, $p = .96$, $\eta_p^2= 0.000$, observed power= 0.050), nor Stimulus*Genotype ($F(2,190)= 0.32$, $p = .73$, $\eta_p^2= 0.003$, observed power= 0.101), Block*Genotype ($F(2,190)= 0.08$, $p = .93$, $\eta_p^2= 0.001$, observed power= 0.062) or Stimulus*Block*Genotype ($F(4,380)= 0.49$, $p = .74$, $\eta_p^2= 0.005$, observed power= 0.167) interactions were seen (Fig. 2, Supplementary Table 3).

4.4.2. Skin conductance response

There was a main effect of stimulus ($F(1,94)= 22.84$, $p < .001$, $\eta_p^2= 0.196$) with significant CS discrimination ($p < .001$). A Stimulus*Block interaction ($F(2,188)= 4.87$, $p = .009$, $\eta_p^2= 0.049$) showed a significant decrease in CS discrimination across blocks (B1, $p < .001$; B2, $p = .022$; B3, $p = .047$). No genotype main effect ($F(1,94)= 0.01$, $p = .92$, $\eta_p^2= 0.000$, observed power= 0.051), nor Stimulus*Genotype ($F(1,94)= 0.96$, $p = .33$, $\eta_p^2= 0.010$, observed power= 0.163), Block*Genotype ($F(2,188)= 0.33$, $p = .68$, $\eta_p^2= 0.003$, observed power= 0.097, Greenhouse-Geisser $\varepsilon= 0.808$) or Stimulus*Block*Genotype ($F(2,188)= 0.19$, $p = .83$, $\eta_p^2= 0.002$, observed power= 0.079) interactions were seen (Fig. 3, Supplementary Table 3).

4.4.3. Risk Ratings

A main effect of stimulus ($F(1,95)= 126.83$, $p < .001$, $\eta_p^2= 0.572$) with a significant Stimulus*Block interaction was found ($F(2,190)= 165.50$, $p < .001$, $\eta_p^2= 0.635$, Greenhouse-Geisser $\varepsilon= 0.798$), showing decreasing CS discrimination across blocks (B1, $p < .001$; B2, $p < .001$; B3, $p = .064$). No genotype main effect ($F(1,95)= 0.01$, $p = .93$, $\eta_p^2= 0.000$, observed power= 0.051), nor Stimulus*Genotype ($F(1,95)= 0.03$, $p = .87$, $\eta_p^2= 0.000$, observed power= 0.053), Block*Genotype ($F(2,190)= 0.65$, $p = .52$, $\eta_p^2= 0.007$, observed power= 0.158) or Stimulus*Block*Genotype ($F(2,190)= 1.12$, $p = .32$, $\eta_p^2= 0.012$, observed power= 0.220, Greenhouse-Geisser $\varepsilon= 0.798$) interactions were seen (Supplementary Fig. 1, Supplementary Table 3).

4.5. ACQ-GEN study: Pre-Acquisition

No significant main or interacting effects with genotype were found ($p > .05$).

4.6. ACQ-GEN study: Fear acquisition

No significant main or interacting effects with genotype were found ($p > .05$).

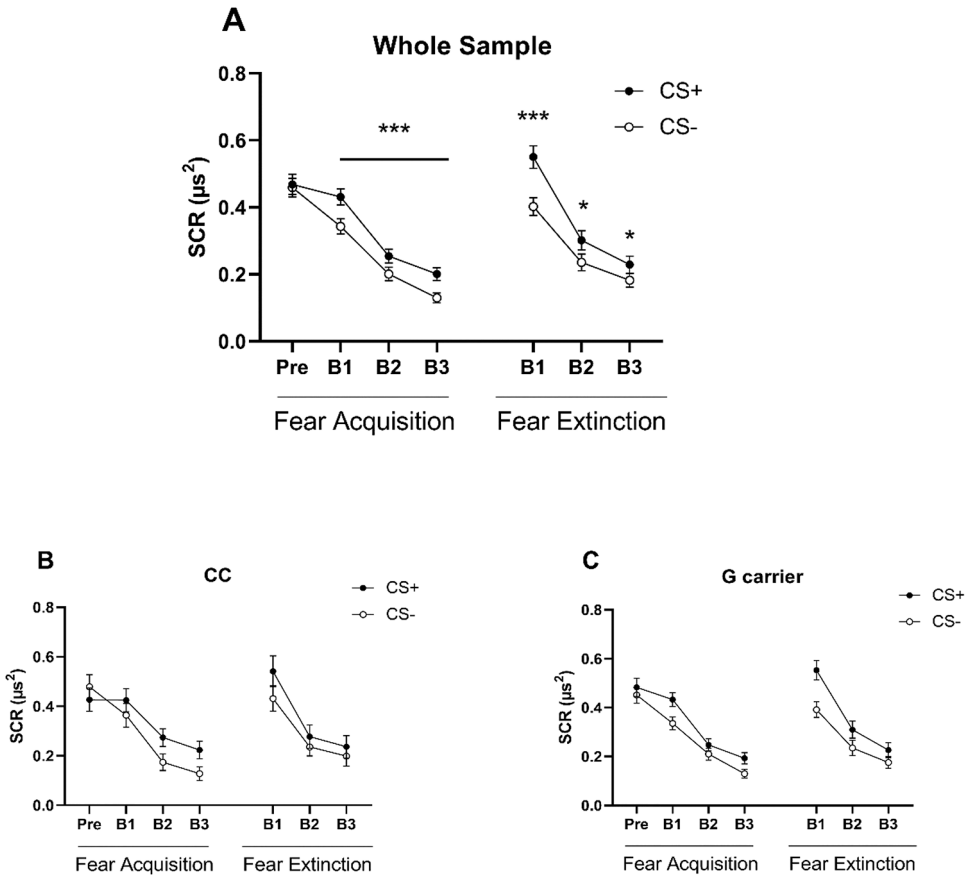

Fig. 3. FC-FE study. Skin conductance response: fear acquisition and extinction. Panel A shows whole sample data, panel B shows CC women, panel C shows G carrier women. B1,B2,B3: block, CS+ : reinforced CS, CS-: non-reinforced CS, pre: pre-acquisition trials. * Indicates CS discrimination (i.e., higher responses to CS+>CS-). * = p < 0.05, * * = p < 0.01, * ** = p < 0.001.

4.6.1. Fear-potentiated startle

There was evidence of successful fear acquisition by a significant main effect of stimulus ($F(2,194) = 16.25$, $p < .001$, $\eta_p^2 = 0.144$) with significant CS+ potentiation ($p < .001$) and CS discrimination ($p = .007$). A Stimulus*Block interaction ($F(4,388) = 2.91$, $p = .021$, $\eta_p^2 = 0.029$) showed significant CS+ discrimination at the second block (B1, $p = .37$; B2, $p = .007$; B3, $p = .13$). No genotype main effect ($F(1,97) = 3.20$, $p = .077$, $\eta_p^2 = 0.032$, observed power= 0.425), nor Stimulus*Genotype ($F(2,194) = 1.90$, $p = .15$, $\eta_p^2 = 0.019$, observed power= 0.391), Block*Genotype ($F(2,194) = 0.39$, $p = .68$, $\eta_p^2 = 0.004$, observed power= 0.113) or Stimulus*Block*Genotype ($F(4,388) = 1.54$, $p = .19$, $\eta_p^2 = 0.016$, observed power= 0.476) interactions were seen (Fig. 4, Supplementary Table 4).

4.6.2. Skin conductance response

There was evidence of fear acquisition by a main effect stimulus ($F(1,87) = 8.71$, $p = .004$, $\eta_p^2 = 0.091$) without interactions with block or genotype. No genotype main effect ($F(1,87) = 0.63$, $p = .43$, $\eta_p^2 = 0.007$, observed power= 0.123), nor Stimulus*Genotype ($F(1,87) = 2.59$, $p = .11$, $\eta_p^2 = 0.029$, observed power= 0.356), Block*Genotype ($F(1,87) = 0.79$, $p = .38$, $\eta_p^2 = 0.009$, observed power= 0.142) or Stimulus*Block*Genotype ($F(1,87) = 1.50$, $p = .23$, $\eta_p^2 = 0.017$, observed power= 0.227) interactions were seen (Supplementary Table 4).

4.6.3. Risk Ratings

Analyses showed a main effect of stimulus ($F(1,99) = 118.70$, $p < .001$, $\eta_p^2 = 0.545$), and a Stimulus*Block interaction ($F(2,198) = 10.50$, $p < .001$, $\eta_p^2 = 0.096$, Greenhouse-Geisser $\varepsilon = 0.937$) showed that responses to CS+ were higher than to CS- since early fear acquisition ($p < .001$). No genotype main effect ($F(1,99) = 1.10$, $p = .30$, $\eta_p^2 = 0.011$, observed power= 0.180), nor Stimulus*Genotype ($F(1,99) = 0.12$, $p = .74$, $\eta_p^2 = 0.001$, observed power= 0.063), Block*Genotype ($F(2,198) = 1.98$, $p = .14$, $\eta_p^2 = 0.020$, observed power= 0.407) or Stimulus*Block*Genotype ($F(2,198) = 0.45$, $p = .64$, $\eta_p^2 = 0.005$, observed power= 0.120, Greenhouse-Geisser $\varepsilon = 0.937$) interactions were seen (Supplementary Table 4).

4.7. ACQ-GEN study: Fear generalization

We tested the effect of genotype on CS type (CS+, CS-, and the 4 class types C1, C2, C3, C4) (See Fig. 1 for a detailed overview of the paradigm).

No significant main or interacting effects with genotype were found ($p > .05$).

4.7.1. Fear potentiated startle

Analyses showed a main effect of stimulus ($F(5,435) = 8.86$, $p < .001$, $\eta_p^2 = 0.092$, Greenhouse-Geisser $\varepsilon = 0.848$). Startle responses decreased as the stimulus differed from the CS+ and this pattern was similar among genotypes as shown by a non-significant interaction of Stimulus*Genotype ($F(5,435) = 1.71$, $p = .14$, $\eta_p^2 = 0.019$, observed power= 0.540, Greenhouse-Geisser $\varepsilon = 0.848$), and a non-significant effect of genotype ($F(1,87) = 3.17$, $p = .079$, $\eta_p^2 = 0.035$, observed power= 0.421). All generalization stimuli showed significant differences with the CS+ (CS- vs. CS+ $p < .001$; C1 vs. CS+ $p < .001$; C2 vs. CS+ $p < .001$; C3 vs. CS+ $p = .017$; C4 vs. CS+ $p = .032$) (Supplementary Table 4).

4.7.2. Skin conductance response

Similar results were observed for this measure, with a main effect of

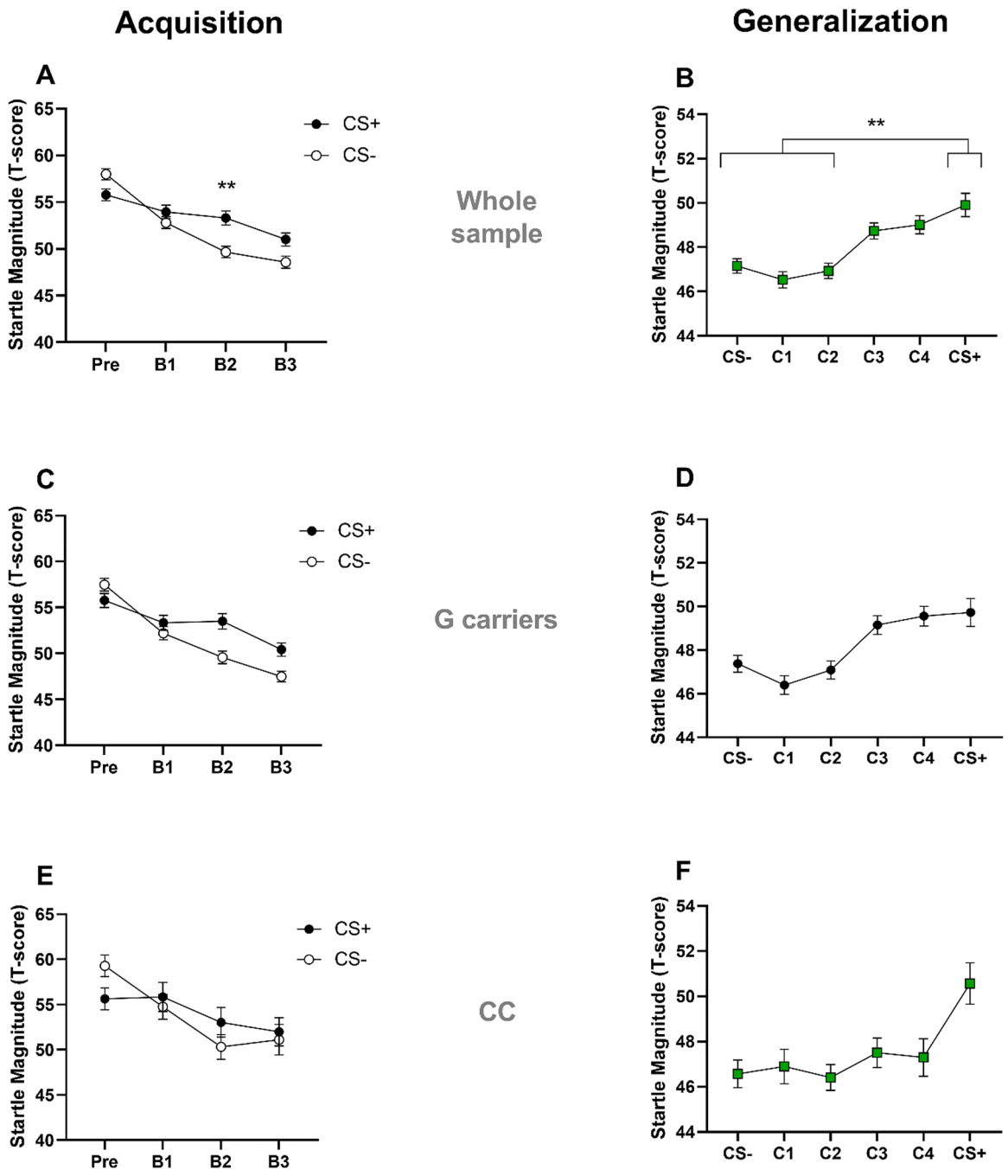

Fig. 4. ACQ-GEN study. Fear potentiated startle: fear acquisition and generalization. Panel A shows acquisition in the whole sample, panel B shows generalization in the whole sample, panel C shows acquisition in G carrier women, panel D shows generalization in G carrier women, panel E shows acquisition in CC women, panel F shows generalization in CC women. B1,B2,B3: block, CS+ : reinforced CS, CS-: non-reinforced CS, pre: pre-acquisition trials. In the acquisition graphs * Indicates CS discrimination (i.e., higher responses to CS+>CS-). In the generalization graphs * indicates differences between stimulus class. * = p < 0.05, * * = p < 0.01, * ** = p < 0.001. Asterisks above bars in the generalization graphs indicate differences between the CS+ and the rest of the stimuli.

stimulus ($F(5,390)= 5.37$, $p = .001$, $\eta^2_p= 0.064$, Greenhouse-Geisser $\varepsilon= 0.682$) Only CS-, C1 and C4 differed from the CS+ (CS- vs. CS+ $p = .020$; C1 vs. CS+ $p = .045$; C2 vs. CS+ $p = .18$; C3 vs. CS+ $p = .11$; C4 vs. CS+ $p = .045$). Responses to the CS- and C1 were smaller than those to the CS+ , and responses to the C4 were higher. No genotype main effect ($F(1,78)= 0.15$, $p = .70$, $\eta^2_p= 0.002$, observed power= 0.067), nor Stimulus*Genotype ($F(5,390)= 0.10$, $p = .97$, $\eta^2_p= 0.001$, observed power= 0.070, Greenhouse-Geisser $\varepsilon= 0.682$) interactions were seen (Supplementary Table 4).

4.7.3. Risk ratings

Subjective risk ratings followed the same pattern of results with a main effect of stimulus ($F(5,445)= 104.75$, $p < .001$, $\eta^2_p= 0.541$, Greenhouse-Geisser $\varepsilon= 0.445$) All generalization stimuli showed significant differences with the CS+ (CS- vs. CS+ $p < .001$; C1 vs. CS+ $p < .001$; C2 vs. CS+ $p < .001$; C3 vs. CS+ $p < .001$; C4 vs. CS+ $p < .001$). No genotype main effect ($F(1,89)= 0.51$, $p = .48$, $\eta^2_p= 0.006$, observed power= 0.109), nor Stimulus*Genotype ($F(5,445)= 0.20$, $p = .84$, $\eta^2_p= 0.002$, observed power= 0.082, Greenhouse-Geisser $\varepsilon= 0.445$) interactions were seen (Supplementary Table 4).

5. Discussion

A vast majority of the world population will be exposed to at least one traumatic event through their lifetime (70 %), without necessarily developing a psychiatric disorder resulting from it (Kessler et al., 2017). Several studies have tried to map the factors posing individuals at risk, but the picture remains heterogeneous. A variety of environmental, individual and trauma-specific factors have been proposed including previous mental disorders, injuries resulting from trauma, repetitive trauma or intense experiences like interpersonal violence (Garcia-Esteve et al., 2021; Kessler et al., 2014; Möller et al., 2018; Scott et al., 2018). Not only are some factors contributing to the development of PTSD, others like higher education levels, access to emotional and social support, or even younger age can help buffer the effects of trauma (Ditlevsen & Elklit, 2010; Folayan et al., 2024). In translational studies, researchers have shown that genetics also contribute to the sustained behavioral alterations after trauma, involving not only PACAP-PAC1R but other neuropeptide and neurotransmitter systems (Cohen et al., 2006; Wang et al., 2021; Zannas et al., 2016).

Thus, the aim of this study was to investigate whether the alterations in fear processing seen in highly traumatized women with the rs2267735 CC risk genotype were also present in women with no trauma/low trauma load. We found in two different studies using two different cohorts that the CC risk genotype was not associated with difficulties in CS discrimination or CS generalization. These findings provide insight into the question whether the CC risk status is linked to a pre-existent fear processing impairment, and therefore, to a pathway for PTSD risk.

Studies have shown that patients with PTSD have impairments in FC and FE (Norrholm et al., 2011; Rougemont-Bücking et al., 2011). A meta-analysis reported that the "C" allele of rs2267735 conferred significant risk for PTSD in women (Lind et al., 2017). We hypothesized we would find alterations in fear processing in a sample of women carrying the risk genotype but without severe trauma, as this could suggest it may be related to a pre-existing vulnerability phenotype. However, our findings refuted our initial hypothesis. These results are nevertheless important as they suggest that the given risk is not related to fear processes alone, but rather involved in the potentiation of fear secondary to traumatic stressors. A process likely associated with the stress-priming effects of PACAP-PAC1R over hypothalamic and limbic structures, as we previously demonstrated in an animal model (Velasco et al., 2022).

PTSD is associated with an overgeneralization of fear responses to safe stimuli resembling conditioned aversive cues (Kaczkurkin et al., 2017). We expected to see this phenomenon in CC women based on the close relationship between this genotype and PTSD. However, our results showed no statistically significant evidence for this.

The visual inspection of the graphs showed an arguable pattern, where CC women seemed to have better discrimination than G carriers, displaying more selective startle to the CS+ . A phenomenon that may be related to the lower CS discrimination in CC women during the fear conditioning phase. However, alternative explanations may also include the fact that CC women could require more extensive fear training, as in the generalization phase of ACQ-GEN there was still partial reinforcement of the CS+ . Also, successful discrimination during FC was observed in the FC-FE study which used two very different shapes as CS+ and CS-, which may be easier to discriminate compared to the same ring stimuli with different sizes. Further, low levels of discrimination during learning and high discrimination during tests may be related to an overall alteration in adaptive responses to fear cues.

The paradigms used here include both fear-potentiated startle and skin conductance response measures. The integration of both complementary measures provides a comprehensive approach to the neurophysiological basis of the processes studied. Also, it allows us to compare findings with a greater variety of studies focusing on a single measure. Although both are general fear measures, it's fundamental to consider the specific aspects of fear processing reflected by them. fear-potentiated startle is thought to reflect aversive learning (Grillon, 2008; Öhman and Mineka, 2001). In turn, skin conductance response is usually associated with contingency awareness and general arousal (Lovibond and Shanks, 2002).Whole-sample analyses in the FC-FE study pointed towards a successful fear acquisition, fear expression, and fear extinction; therefore, supporting the validity of our task as a proper protocol for studying fear processing in humans.

It is worth highlighting that the lack of statistical significance promoted by the genotype might be at least partially attributed to the differences of our participants and their demographics compared to other studies. Most studies that detect rs2267735 as a risk genotype come from the Grady Trauma Project, which is mainly composed of low-income, highly traumatized, Afro-American women, in a completely different socio-cultural environment (Ressler et al., 2011; Stevens et al., 2014; Velasco et al., 2022). Another study with positive effects analyzed data from the children of these traumatized women (Jovanovic et al., 2012). Conversely, the manuscripts analyzing participants with the demographics features closer to ours, describes negative effects for this risk genotype in two cohorts (Chang et al., 2012; Pohlack et al., 2015). Previous research has shown that, on a molecular level, stress exposure increases Adcyap1r1 mRNA expression in brain regions involved in fear processing (Mercer et al., 2016). In our study, which includes participants with low or no trauma history, we hypothesize that the absence of stress may prevent sufficient activation of the neurobiological pathways needed to reveal genotype differences. Unlike studies in PTSD patients, where trauma and stress exposure may enhance activity in these pathways, triggering the effects associated with the risk genotype.

Among the limitations of these findings, our paradigms used discrete and non-ecological stimuli, which may not reproduce fully real-life-threatening stimuli. The acquisition paradigms we used had slight variations in reinforcement rate which impeded pooled analyses. A factor that may influence the translatability of these results is the longevity of the fear memory, as in PTSD and other real-life situations memories tend to be older and more consolidated between acquisition and generalization or extinction. To note, the effects we are trying to detect are small, as shown by other studies using SNP genotypes to define groups. These small genetic variations rarely show drastic behavioral/ psychophysiological effects, instead they often show more of a small modulatory role. We must acknowledge that a bigger sample size may have allowed for a more detailed characterization of the phenotype. It is possible that the sample of CC participants (around 1/3) of the G carrier sample in our cohorts was insufficient for detecting very low statistical differences. This difference is due to the frequency of each allele, with the G allele having a 48.5 % probability of appearing (Wang et al., 2021). Both alleles show similar probabilities, but grouping together GG and GC genotypes under G-carriers leads to this size difference between groups. In addition, we could not include the hormonal status of our participants in the analyses, despite reports of CC genotype interaction with estradiol dynamics and a longitudinal study in children showing differences between genotypes only in girls who had experienced puberty (Jovanovic et al., 2020; Mercer et al., 2016). Based on the estradiol-mediated dynamics regarding this SNP we consider that future studies should take into consideration this variable and compare if these findings are dependent on hormonal status.

This study addressed the question whether the CC genotype alters fear processing independently of chronic or traumatic stress. Our results showed that fear processing and fear generalization are unaltered in women with the rs2267735 risk genotype and a low trauma/ trauma naïve background, which suggest that these alterations are dependent on the cumulative addition of environmental and genetic factors. The limitations of this study warrant careful consideration of the findings, as well as their generalization to other populations.

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Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT in order to correct grammar and improve readability in specific sentences. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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No images, figures of artwork were created with Generative AI or AI-assisted technologies

Declaration of Competing Interest

The authors declare no conflict of interest

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The FC-FE study was planned and conceptualized by R.A., R.T. and E. R.V. The The ACQ-GEN study was planned and conceptualized by M.A.F. and D.T.R. E.R.V. made the behavioral experiment and genotyping for the FC-FE study, D.T.R made the behavioral experiment for the ACQ-GEN study, B.A did the genotyping for the ACQ-GEN study. Data was analyzed by J.F.N and E.R.V. The paper was written by R.A., J.F.N. and E.R.V. and commented on and discussed by all authors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopsycho.2024.108981.

Data availability

Data will be made available on request.

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