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Date:
2021-10-01

Citation:
Ney, L. J., Matthews, A., Hsu, C. M. K., Zuj, D. V., Nicholson, E., Steward, T., Nichols, D., Graham, B., Harrison, B., Bruno, R. & Felmingham, K. (2021). Cannabinoid polymorphisms interact with plasma endocannabinoid levels to predict fear extinction learning. Depression and Anxiety, 38 (10), pp.1087-1099. https://doi.org/10.1002/da.23170.

Persistent Link:
https://hdl.handle.net/11343/298657

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Cannabinoid polymorphisms interact with plasma endocannabinoid levels to predict fear extinction learning

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Acknowledgements

This work was supported by an NHMRC Program grant to KLF (APP1073041).

The authors have no conflicts of interest to report.

Research data are not shared.

Abstract

Background: The endocannabinoid system is gaining increasing attention as a favourable target for improving posttraumatic stress disorder (PTSD) treatments. Exposure therapy is the gold-standard treatment for PTSD, and fear extinction learning is a key concept underlying successful exposure.

Methods: This study examined the role of genetic endocannabinoid polymorphisms in a fear extinction paradigm with PTSD compared to healthy participants (*N*=220). Participants provided saliva for genotyping, completed a fear conditioning and extinction task, with blood samples taken before and after the task (*n*=57). Skin conductance was the outcome and was analysed using mixed models.

Results: Results for cannabinoid receptor type 1 polymorphisms suggested that minor alleles of rs2180619 and rs1049353 were associated with poorer extinction learning in PTSD participants. The minor allele of the fatty acid amide hydrolase (FAAH) polymorphism rs324420 was associated with worse extinction in PTSD participants. Subanalysis of healthy participants (*n*=57) showed the FAAH rs324420 genotype effect was dependent on plasma arachidonoyl ethanolamide (AEA) level, but not oleoylethanolamide or 2-arachidonoyl glycerol. Specifically, higher but not lower AEA levels in conjunction with the minor allele of FAAH rs324420 were associated with better extinction learning.

Conclusions: These findings provide translational evidence that cannabinoid receptor 1 and AEA are involved in extinction learning in humans. FAAH rs324420's effect on fear extinction is moderated by AEA plasma level in healthy controls. These findings imply that FAAH inhibitors may be effective for targeting anxiety in PTSD, but this effect needs to be explored further in clinical populations.

Keywords: Endocannabinoids, fear extinction, fear conditioning, posttraumatic stress disorder

Background

Post-traumatic stress disorder (PTSD) is a chronic mental health condition that develops in some individuals following exposure to trauma. Symptoms of PTSD include re-experiencing of the traumatic event, hyperarousal, negative cognitions and mood, and avoidance behaviours (American Psychiatric Association, 2013). Underlying the inability to recover from trauma is impairment of the learning process called fear extinction, which reflects an individual's ability to learn that a previously threatening stimuli or situation is no longer threatening (Lonsdorf et al., 2017; Zuj et al., 2016). Fear extinction is one of the key processes that contributes to the development and refinement of exposure therapies for PTSD, which are best-practice treatments for the disorder (Graham et al., 2014; Milad & Quirk, 2012; Rothbaum & Davis, 2003). Therefore, understanding the biological and cognitive factors that compromise fear extinction is a key goal for PTSD research as it has the potential to develop new ways to facilitate exposure therapy (Zuj & Norrholm, 2019).

The endogenous cannabinoid (endocannabinoid) system is a critical mediator of stress responding increasingly recognised for its role in PTSD aetiology and symptomology (Hill et al., 2018; Ney, Matthews, et al., 2018, 2019). The endocannabinoid system is comprised of cannabinoid receptors that are responsive to both exogenous and endogenous cannabinoid molecules such as arachidonoyl ethanolamine (AEA) and 2-arachidonoyl glycerol (2-AG). Fatty acid amide hydrolase (FAAH) is the primary degrading enzyme for AEA and its inhibition can elevate AEA levels. Many of the endocannabinoid effects on the central nervous system are believed to be mediated by the cannabinoid type 1 receptor (CB1R), though all

endocannabinoids that have been characterised display affinity for multiple molecular pathways, such as transient receptor potential cation channels and serotonin pathways (Ligresti et al., 2016; Oultram et al., 2021).

The relevance of endocannabinoids to PTSD is partly explained by their critical role in fear extinction learning (Hill et al., 2018; Morena et al., 2016). Specifically, impairment of endocannabinoid signalling in animal models of extinction learning results in poorer fear extinction recall and heightened PTSD-like symptomology (Hill et al., 2018; Marsicano et al., 2002). These effects are mediated by CB1R, such that blockade of CB1R results in impaired extinction and restoration of this receptor rescues extinction (Kamprath et al., 2006; Marsicano et al., 2002). This effect appears to extend to human studies where peripheral levels of endocannabinoids (AEA, 2-AG, and the N-acyl-ethanolamide oleoylethanolamide) tend to be lower in individuals with higher PTSD symptom severity (Hill et al., 2013; Spagnolo et al., 2016; Wilker et al., 2016). Similarly, augmentation of endocannabinoid signalling in humans has resulted in improved fear extinction in some studies. Specifically, Hammoud et al. (2019) and Rabinak et al. (2013) found that delta-9-tetrahydrocannabinol, a CB1R agonist, improves physiologically measured fear extinction. Similarly, Mayo et al. (2019) found that FAAH inhibitor administration to healthy participants elevated AEA levels and resulted in improved fear extinction learning and recall. Endocannabinoid levels have also been shown in multiple studies to fluctuate in response to acute psychosocial stress in healthy (Dlugos et al., 2012; Mayo et al., 2018; Ney, Stone, et al., 2021), but 2-AG does not in PTSD participants (Crombie et al., 2019). This further supports the notion that PTSD symptomology may be associated on a mechanistic level with impaired endocannabinoid signalling.

Further evidence for endocannabinoid signalling in PTSD comes from studies of variants of cannabinoid receptor and FAAH genes. Healthy (non-PTSD) human carriers of the A allele variant of the FAAH-reducing single nucleotide polymorphism (SNP) rs324420 (also known as c.385C>A or Pro129Thr, here called FAAH rs324420) have enhanced fear extinction learning and decreased anxiety levels compared to CC homozygotes (Dincheva et al., 2015; Gee et al., 2016; Gunduz-Cinar et al., 2013; Hariri et al., 2009; Spagnolo et al., 2016). This effect is particularly prominent in AA homozygotes and is likely driven by Pro129Thr conversion in this SNP resulting in a more easily degradable FAAH enzyme, yielding increased AEA levels in the carriers (Mayo et al., 2018; Sipe et al., 2002). Investigation of the rs1049353 SNP – located on the CNR1 gene – has suggested that A allele carriers display increased emotional response and memory of emotional images following stress compared to GG homozygotes (Wirz et al., 2018). It was reported that were no effects of rs1049353 during fear conditioning or extinction learning (Heitland et al., 2012). However, A carriers of rs2180619, also located on the CNR1 gene, were associated with reduced extinction learning (Heitland et al., 2012). This SNP is part of the promotor region of the CNR1 gene (Zhang et al., 2004) and GG homozygotes have been associated with reduced cannabinoid receptor 1 expression (Lazary et al., 2009). Hence, mutations of FAAH rs324420 has been associated with better fear extinction, whereas CNR1 rs2180619 has been associated with poorer fear extinction and rs1049353 mutations have been implicated in higher emotional reactivity. This body of work suggests that modulating the endocannabinoid system through cannabinoid receptor agonists or FAAH inhibition could potentially improve fear extinction learning impairments in PTSD (Hill et al., 2018; Ney, Matthews, et al., 2019).

No study to date has tested the effects of cannabinoid receptor related genetic variants on fear extinction in PTSD participants. Further, no study has reported the association between peripheral endocannabinoid levels and fear extinction learning. In the current study, we collected plasma for endocannabinoid measurements and CNR1 SNPs rs1049353 and rs2180619, as well as FAAH rs324420 from PTSD, trauma-exposed and non-trauma exposed participants who completed a fear extinction paradigm, where physiological responses to threatening as well as safety cues were measured. We hypothesised that participants with mutations at FAAH (i.e., A allele carriers) would have better extinction learning, whereas CNR1 rs2180619 and rs1049353 mutations (G allele and A allele, respectively) would have poorer extinction learning. In the sub-analysis of healthy participants, we hypothesised these effects would be moderated by AEA and 2-AG levels, with lower endocannabinoid levels associated with worse extinction outcomes.

Method

Participants

This study was conducted at both the University of Tasmania and the University of Melbourne. 261 participants aged 18-61 were recruited to the study from universities, private psychology clinics, and the community. All participants provided salivary gene samples. Participants were also separated into three groups based on responses to the questionnaires outlined below: PTSD (*n*=52), trauma-exposed controls (TE, *n*=110), and healthy controls without a trauma history (NTE, *n*=99). A trauma-exposed group was discriminated from healthy controls due to a known effect of trauma on fear learning independent of PTSD (Dunsmoor et al., 2017; Harnett et al., 2018; Stark et al., 2015). Participants were excluded if they had neurological, cardiac, or physiological conditions (current or historical), were

diagnosed with a psychiatric condition (current or historical, except for PTSD), were pregnant or lactating, had a history of head trauma, had heavy alcohol use, or were using illicit substances or psychoactive medications. Alcohol use was screened using the Alcohol Use Disorder Identification Test (Saunders et al., 1993) and psychiatric conditions were screened using: (a) the Depression, Anxiety, and Stress Scales (Lovibond & Lovibond, 1995) and (b) self-report of current or historical psychiatric illnesses. A total of 79 participants had blood samples collected (final sample n=57 after exclusions described later in methods). The addition of blood samples was an amendment in the final year of the study and a substantial proportion of the sample was not collected due to COVID restrictions beginning in 2020. The study was approved by the University of Melbourne and Tasmanian Medical Human Research Ethics Committees. All procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Measures

PTSD Checklist – Civilian Version (PCL-C). The PCL-C is an ordinal measure of PTSD symptom severity, based on the DSM-5 (American Psychiatric Association, 2013). The PCL-C consists of 20 five-point, self-report items, which index intrusive memory, hyperarousal, and avoidance behaviour over the previous month. The PCL-C can be used for preliminary diagnostic profiling. PCL-C scores over 40 were classified as PTSD (Weathers et al., 1993). PTSD participants were also included if they had a formal PTSD diagnosis. Participants reporting a formal diagnosis of PTSD were mostly recruited from advertisements at private psychology clinics. Diagnoses were reported by participants as having been administered by their licensed clinicians.

Traumatic Experiences Questionnaire (TEQ). The TEQ is an 11-item, dichotomous answer, questionnaire that counts and assesses the number and type of criterion A traumatic events experienced over the lifetime (Vrana & Lauterbach, 1994). Participants who reported a traumatic experience but did not reach the PTSD cut-off score were classified as TE participants, whereas participants without a traumatic experience were NTE participants.

Procedure

Experimental sessions were conducted at the University of Tasmania and University of Melbourne. All testing was completed between 11am and 6pm. Participants refrained from caffeine, food, and nicotine for a minimum of one hour prior to the study. When arriving at the laboratory, participants were briefed about the study, provided written informed consent, provided a baseline blood sample (for participants who agreed to provide blood) and a saliva sample for DNA analysis (DNA Genotek Inc., Canada). They also completed the PCL-C and the TEQ. Next, participants had skin conductance electrodes attached to the first and third palmar phalanges of the left hand. The stimulus isolator was attached to the right hand between the thumb and index finger. Recording was made using a 16/35 Powerlab (ADInstruments, Australia), including a galvanic skin response amplifier and stimulus isolator. Skin conductance response (SCR) was recorded as microsiemens (μS) from the galvanic skin response trace on LabChart v7.3.7 (ADInstruments, Australia). The fear extinction paradigm software was scripted on Inquisit by the researchers on a separate computer that was paired to the LabChart computer. Metal-plated (dry) skin conductance electrodes and a stimulus bar (ADInstruments, Australia) were used for the study. Participants were required to choose a shock level that was subjectively "highly uncomfortable, but not painful" (Grillon, 2002; Orr et al., 2000; Schmitz &

Grillon, 2012). This was achieved by undergoing a series of 500ms shocks starting at 2 milliamps (mA) and increasing or decreasing in 0.5mA increments to a maximum of 10mA and a minimum of 1mA. All participants included in the study located an appropriate shock level within this range. Participants received the instruction that their right hand should remain motionless throughout the experiment.

Participants then underwent a differential fear conditioning and extinction task comprising habituation, fear conditioning, and fear extinction learning phases (Supplementary Figure 1) (Briscione et al., 2014; Zuj & Norrholm, 2019). Participants watched two coloured circles appear on a computer screen whilst receiving a 500ms mild electric shock that was paired with one of the stimuli in the fear conditioning phase (the conditioned stimulus (CS+)) and occurred immediately following CS+ offset (Milad et al., 2005; Milad et al., 2010). The other coloured circle (CS-) was never paired with the shock. The CS+ therefore becomes the danger signal and the CS- the safety signal. The CS+ and CS- were presented in a pseudo-randomized order, and never presented more than two times in a row. CS duration was 12 seconds with an intertrial interval jittering between 12 and 21 seconds (average 16 seconds). During habituation, four red and four blue circles were presented with no subsequent shocks. At the beginning of all following phases, participants were told that they may or may not receive shocks. During the fear conditioning phase, five red and five blue circles were presented, with one circle (the CS+) being immediately followed by a shock at 100% reinforcement. The extinction learning phase was separated into early and late phases, which were separated by an instruction screen where participants were told to keep their hand still during the study. During each phase of extinction learning, each CS type was presented five times and no shocks were delivered. Participants who provided blood samples had a

blood sample drawn immediately following late extinction learning. All participants were thanked for their time, debriefed and reimbursed with either cash or course credits.

Plasma endocannabinoid analysis

AEA, 2-AG, and oleoylethanolamide (OEA) were quantified in plasma samples according to previously described methods (Ney, Felmingham, et al., 2020). Briefly, samples were collected into Lithium Heparin tubes by a trained phlebotomist and immediately centrifuged and frozen at -80^{o}C until analysis. Prior to analysis, 1mL of 1:1 ethylacetate:cyclohexane was added to 400µL of plasma and stable isotopic standards were added. The mixture was vortexed, centrifuged and the resulting organic layer was transferred to a fresh HPLC vial, dried under nitrogen and reconstituted in 100µL acetonitrile. 2µL of the final 30µL solution was injected into a Waters Acquity® H-class UPLC system (Waters Corporation, Milford, MA) coupled to a Waters Xevo® TQ triple quadrupole mass spectrometer (Waters Corporation). Analyses were conducted in positive electrospray ionisation mode using multiple reaction monitoring.

Genetic analysis

Saliva was collected using the passive drool method into OG-500 Oragene salivary DNA kits (DNA Genotek Inc., Canada). DNA in saliva samples was extracted, purified, and analysed using standard methods (DNA Genotek Inc., 2012). Genotypes of the CNR1 rs2180619 and rs1049353 polymorphisms as well as FAAH rs324420 polymorphism were identified using quantitative polymerase chain reaction (qPCR). qPCR was conducted by Australian Genome Research Facility (AGRF, Melbourne, Australia) using the Illumina method and interpreted using the Forward data, which complies with the dbSNP classification call style. Genotyping was

repeated to ensure accuracy, with the proportion of concordance > 99%. 21 participants did not achieve appropriate concordance or did not have genetic material collected and were unable to be assayed. These participants were not included in the analyses. There were no specified cases of overlapping genotypes. Genotypes that included the less common alleles (i.e. A in rs324420, G in rs2180619 and A in rs1049353) were combined as is standard practice (Lonsdorf & Kalisch, 2011). Analyses of minor homozygotes was not possible in the current sample as there were only 5 and 8 AA homozygotes for rs324420 and rs1049353, respectively, in the final 220 participants.

Skin conductance responses

Skin conductance was recorded using a 22 mVrms, 75 Hz constant-voltage coupler (AD Instruments), sampled at 512 Hz and stored at 64 Hz. SCRs were estimated using PsPM toolbox v4.0 in MATLAB version 9.7 (Bach & Friston, 2013; Bach et al., 2013), with peak amplitude scored during the first interval response (0.9s to 5s following CS onset) following subtraction of the baseline level 2 s preceding CS onset. A bidirectional Butterworth filter with a low pass of 1.5Hz and a 0.5Hz high pass was used to filter noise from the raw SCR trace data. In total, 36 participants were excluded due to non-responding (50% or more SCR less than 0.01μS) or due to technical difficulties with the equipment, such as electrical failure equipment during the experiment in several participants. These participants were not included in the analyses.

Statistical Analysis

After excluding non-responders and participants without genetic data, *N*=220 participants remained (NTE *n*=84, TE *n*=93, PTSD *n*=43). Post-hoc sensitivity analysis using G*Power 3.1.9.4 suggested that an effect size of *f*=0.09 was required to reach

statistical significance with α=0.05 and β=0.80. Demographics and genotypes were compared with univariate ANOVAs or chi-square tests, where appropriate. Data analysis of SCRs was synonymous to our recent approaches (Ney, Vicario, et al., 2021), so as to conform to standardization of fear extinction analysis (Ney, Laing, et al., 2020; Ney, Wade, et al., 2018). Specifically, linear mixed effects were used to analyse SCR data during habituation, fear conditioning, early extinction learning, and late extinction learning phases separately. *Stimulus type* (CS+, CS-), *group* (PTSD, TE, NTE), *genotype* (dichotomous and analysed separately for CNR1 rs1049353, CNR1 rs2180619, and FAAH rs324420), *trial* (1-5, but 2-5 for conditioning since no conditioning effect could have occurred on trial 1), and their interactions were added as fixed effects. *Subject* was entered as a random slope and intercept. First-order autoregressive was chosen as the covariate structure and models were estimated using Restricted Maximum Likelihood. Given the evidence supporting that sex plays an important role in fear extinction and PTSD (Glover et al., 2012; Glover et al., 2015; Gogos et al., 2019; Lebron-Milad et al., 2012; Li & Graham, 2017; Merz et al., 2018; Ney, Gogos, et al., 2019), *sex* was added as a covariate to these analyses. For the sub-analysis, the factor of *group* was included as a covariate and *analyte level* (AEA, 2-AG, OEA) was added as a continuous predictor to the existing models. As there was no change in endocannabinoid level between blood measurements (p=.798 multivariate analysis), the second blood measurement was used due to its immediacy to extinction learning. Due to the high number of comparisons during the sub-analysis, false discovery rate corrections for multiple comparisons were used (Benjamini & Hochberg, 1995). Analyses were conducted in SPSS v27 (Armonk, NY, USA).

Results

Demographics and Clinical Data

Following exclusion of participants due to lack of genotyping data or poor-quality SCR data, *N*=220 participants remained. These exclusions are described in the methods sections. Mean scores, standard deviations and test statistics are summarized in Table 1 for demographic and clinical data across NTE, TE and PTSD groups. Overall, there were no significant differences between the groups in terms of gender. However, age and PCL scores were significantly different between groups (both $p<.001$). Post-hoc analyses revealed that PTSD participants had higher PTSD symptoms compared to both control groups ($p<.001$). They were older than NTE ($p<.001$) but not TE participants ($p=.179$). TE participants were significantly older than NTE participants ($p=.001$) and had higher PCL ($p<.001$) scores. Age was therefore added as a covariate. Shock intensity did not differ between groups ($p=.438$). Mann-Whitney U tests revealed that AEA levels were marginally higher in A allele carriers compared to CC homozygotes of FAAH ($p=.080$), whilst OEA levels were significantly higher in A carriers ($p=.003$, mean values in Supplementary). Tests of allele frequency against the Hardy-Weinberg equilibrium revealed that expected cell counts were met for FAAH rs324420 ($\chi^2=2.34$, $p=.126$) and CNR1 rs2180619 ($\chi^2=1.16$, $p=.281$), though there were more A allele carriers of rs1049353 ($\chi^2=6.21$, $p=.013$).

Table 1. Demographic and genetic data between the experimental groups

	NTE (*n*=84)	TE (*n*=93)	PTSD (*n*=43)	Total (*N*=220)
Age	22.13 (6.27)	27.24 (10.30)	30.36 (12.40)	25.90 (9.96)
Gender	47 (56%) F	55 (59%) F	30 (70%) F	132 (60%) F
	37 (44%) M	38 (41%) M	13 (30%) M	88 (40%) M

PCL	12.25 (10.08)	19.92 (11.30)	48.15 (12.75)	22.57 (17.26)
Shock Intensity	2.39 (0.92)	2.16 (0.89)	1.96 (0.62)	2.22 (.87)
FAAH rs324420	25 (30%) A	25 (27%) A	18 (42%) A	68 (31%) A
	59 (70%) CC	68 (73%) CC	25 (58%) CC	152 (69%) CC
CNR1 rs1049353	23 (27%) A	28 (30%) A	20 (47%) A	71 (32%) A
	61 (73%) GG	65 (70%) GG	23 (53%) GG	149 (68%) GG
CNR1 rs2180619	40 (48%) G	51 (55%) G	25 (58%) G	116 (53%) G
	44 (52%) AA	42 (45%) AA	18 (42%) AA	104 (47%) AA

Note: PCL = Posttraumatic Disorder CheckList 5, NTE = Non-trauma exposed participants, TE = trauma exposed participants, PTSD = participants with posttraumatic stress disorder. Continuous variables are means (standard deviation).

Effect of group status on SCR data

There were no effects of stimulus type or group during habituation (Supplementary Figure 2). During conditioning, there was a significant stimulus effect, $F(1,708)=65.61$, $p<.001$, with significantly higher SCR amplitudes to CS+ compared to CS- stimuli. During early extinction learning, there was a stimulus effect that approached significance, $F(2,481)=3.55$, $p=.060$, with higher CS+ compared to CS- responses. This effect was superseded by a significant interaction effect between stimulus and group, $F(2,481)=3.71$, $p=.025$, such that higher CS+ responding compared to CS- was observed in the PTSD and TE groups but not the NTE group. There was also a significant trial effect ($p<.001$), such that SCRs to both stimuli reduced over early and late extinction learning. There were no further interaction effects and there were no further significant effects during the late extinction learning phase. This suggests that extinction learning was most efficacious in the NTE group during early extinction but was equivalently achieved across groups during late extinction.

Effect of genotype on SCR data

Mixed models with trial, stimulus type, and group as fixed factors were run separately for each phase and each genotype. For CNR1 rs1049353, there were no significant effects during habituation, conditioning or early extinction learning. During late extinction learning, there was a significant stimulus*trial*group*genotype interaction, $F(8,1318)=2.32$, $p=.018$, such that there was higher responding to the CS+ in PTSD A allele carriers compared to other groups (Figure 1C-E). There was also a significant stimulus*trial*genotype interaction, $F(4,954)=3.21$, $p=.012$, but this was superseded by the four-way interaction. For CNR1 rs2180619, there were no significant effects observed during any phase, implying no effect on the rate of fear acquisition or extinction.

For FAAH rs324420, there was a significant stimulus*trial*genotype interaction during conditioning, $F(4,1356)=2.97$, $p=.019$, with higher CS+ responding on the first trial in A carriers (Figure 1A). There was a significant stimulus*group*genotype interaction during late extinction learning, $F(2,676)=6.04$, $p=.003$, such that higher responding towards the CS- was observed in the PTSD group who were A allele carriers (Figure 1B).

Insert Figure 1 here

Effect of plasma endocannabinoids on SCR data

Due to low numbers of PTSD subjects who were genotyped, had adequate SCR data, and had blood samples taken ($n=9$), data were only analysed from healthy participants (TE and NTE, $n=57$). There were no significant differences between TE and NTE groups in AEA ($p=.734$), 2-AG ($p=.497$) or OEA ($p=.931$). Results from mixed models SCR analyses are fully described in Table 2, with significant effects highlighted following correction for multiple comparisons (Benjamini & Hochberg,

1995). For these analyses, AEA level is included as a continuous predictor variable in a mixed model.

There were strong interaction effects between FAAH rs324420 and AEA level. However, no effects were observed in interaction with 2-AG, which was expected given that FAAH degrades AEA and not 2-AG. Therefore, 2-AG results are not reported. There was no and minimal evidence for the effect of AEA or 2-AG in interaction with either CNR1 rs2180619 or rs1049353, respectively. Results for AEA in interaction with these polymorphisms are reported in the Supplementary Material.

For FAAH rs324420 A allele carriers, higher AEA was associated with lower SCR responses across all phases (Figure 2; note that the Figure is illustrated using simple slopes analyses to simplify visualisation of a continuous predictor (Hayes, 2013)). No significant effects were observed during habituation. During conditioning, this effect was primarily driven by reduced responding to the CS- in participants with higher AEA level and was observed primarily in A allele carriers. Similarly, during early extinction learning higher AEA was associated with lower SCRs, but only in A allele carriers and primarily in response to the CS+. This effect was replicated in late extinction learning, but not on the first trial of the phase (Figure 3).

Insert Figures 2 and 3 here

There were no significant effects or interaction effects with FAAH rs324420 when the factor of AEA plasma level was substituted for OEA plasma level (see Supplementary Material).

Table 2. Interaction between post-extinction plasma AEA level, stimulus type, trial, and FAAH rs324420 genotype across experimental phases

	Conditioning	**Early Extinction Learning**	**Late Extinction Learning**

Stim*AEA	$F(1,134)=1.69$, $p=.195$	$F(1,115)=2.52$, $p=.115$	$F(1,162)=0.05$, $p=.828$
Trial*AEA	$F(4,282)=2.95$, $p=.021$	$F(4,267)=2.37$, $p=.053$	$F(4,254)=10.49$, $\mathbf{p<.001}$
Genotype*AEA	$F(1,42)=5.78$, $p=.021$	$F(1,42)=11.54$, $\mathbf{p=.001}$	$F(1,42)=2.18$, $p=.147$
Stim*Genotype*AEA	$F(1,134)=0.94$, $p=.335$	$F(1,115)=7.52$, $\mathbf{p=.007}$	$F(1,162)=0.53$, $p=.466$
Trial*Genotype*AEA	$F(4,282)=2.63$, $p=.035$	$F(4,267)=3.07$, $p=.017$	$F(4,254)=12.99$, $\mathbf{p<.001}$
Stim*Trial*AEA	$F(4,275)=7.44$, $\mathbf{p<.001}$	$F(4,259)=2.20$, $p=.069$	$F(4,257)=1.15$, $p=.335$
Stim*Trial*Genotype*AEA	$F(4,275)=7.92$, $\mathbf{p<.001}$	$F(4,259)=2.53$, $p=.041$	$F(4,257)=2.08$, $p=.085$

Discussion

In the current study, we tested the effect of endocannabinoid polymorphisms and plasma endocannabinoid levels on fear conditioning and extinction learning in a group of healthy and PTSD participants. Participants with A alleles on CNR1 SNP rs1049353 had poorer extinction learning and this effect was mostly due to higher CS+ responding in PTSD A allele carriers. Contrary to what we predicted in the PTSD group, A allele carriers of FAAH rs324420 displayed generalisation of fear during extinction learning, indicated by higher CS- responding. Interestingly, our plasma sub-analyses in healthy participants revealed that FAAH rs324420 (A carrier versus CC homozygote) was associated with better conditioning and extinction learning but only if AEA levels were high, when AEA was entered as a continuous

variable to the mixed model. These findings replicate and extend existing literature on the effect of endocannabinoids in PTSD (Dincheva et al., 2015; Gunduz-Cinar et al., 2013; Hill et al., 2018; Mayo et al., 2018; Mayo et al., 2019; Ney, Akhurst, et al., 2021; Rabinak et al., 2013) and are the first human evidence of an association between plasma endocannabinoids and fear extinction.

Our genetic results were partially consistent with the current literature indicating that impaired endocannabinoid signalling is associated with poorer extinction learning (Hill et al., 2018; Marsicano et al., 2002; Morena et al., 2016). Specifically, CB1R is associated with these effects (Ganon-Elazar & Akirav, 2009; Hartley et al., 2016; Kamprath et al., 2006), and this is believed to occur largely through the endocannabinoids AEA and 2-AG (Hill et al., 2018). Consequently, existing research has supported the notion that polymorphisms resulting in higher CB1R density and AEA levels are associated with better outcomes in fear extinction tasks in animals (Bowers & Ressler, 2015; Kamprath et al., 2006; Marsicano et al., 2002; Morena et al., 2018; Ney, Akhurst, et al., 2021) and in humans (Dincheva et al., 2015; Heitland et al., 2012; Mayo et al., 2018; Mayo et al., 2019). We found that extinction learning was impaired in PTSD participants who were A allele carriers of the CNR1 coding polymorphism rs1049353. Available literature also suggests that A carriers of rs1049353 are more likely to develop PTSD (Lu et al., 2008; Mota et al., 2015) and here we found that A carriers with PTSD had poorer extinction learning compared to GG homozygotes and controls. However, no effects were observed for CNR1 promotor SNP rs2180619. These findings therefore contrast to the only other study that has assessed these polymorphisms in relation to fear extinction. Specifically, this study reported that G carriers of rs2180619 were associated with better extinction learning, and that no effects were observed for rs1049353 (Heitland

et al., 2012). Similarly, it has been reported that the G allele of rs2180619 is associated with higher anxiety, higher rate of substance abuse disorders, lower CB1R expression, poorer attentional control, and poorer working memory (Lazary et al., 2009; Ruiz-Contreras et al., 2014; Zhang et al., 2004). Further research is needed to understand why these studies do not converge, but we can speculate that data from the CNR1 SNPs overall suggest involvement of CB1R in human fear extinction learning. How the CNR1 polymorphisms affect CB1R receptors is yet to be determined, though there is some indication that variation of these SNPs are associated with differential CB1R expression (Lazary et al., 2009; Zhang et al., 2004).

Despite previous studies strongly supporting a positive role for the A allele of FAAH in fear extinction learning and extinction recall (Dincheva et al., 2015; Mayo et al., 2018), stress responding (Gunduz-Cinar et al., 2013; Spagnolo et al., 2016), and PTSD (Spagnolo et al., 2016), we found minimal effects of the polymorphism in our sample. During late extinction learning, we observed higher responses to the CS- in A carriers of the PTSD group. This effect was opposite to what we anticipated and as the first study to test the effect of rs324420 in PTSD participants during fear extinction, this implies a potential disease by gene interaction during extinction learning that has not previously been identified. Generalisation of the fear response to the safety cue is well documented in PTSD samples and is understood to have a neurobiological basis (Grillon & Morgan, 1999; Jovanovic et al., 2012; Jovanovic et al., 2010; Levy-Gigi et al., 2015; Morey et al., 2015; Orr et al., 2000; Peri et al., 2000; Zuj et al., 2017). Further, it generally occurs when safety stimuli are of similar appearance to threat stimuli and when the threat loses its predictability (Grillon et al., 2009; Lissek & van Meurs, 2015), as was the case in our study. Critically, previous studies that have reported a rs324420 effect on fear extinction learning only recruited healthy

participants (Dincheva et al., 2015), suggesting that the A allele substitution on FAAH rs324420 may be a risk factor for fear generalisation in PTSD participants exclusively. Conversely, recent research has suggested that the fear resilient phenotype might only be present in AA homozygotes (Mayo et al., 2018). Our sample did not contain sufficient AA homozygotes to replicate this analysis and this may explain why our findings were not consistent with previous literature.

Interestingly, robust genotype by plasma AEA interactions were observed during conditioning and extinction learning in healthy participants. These data revealed that A carriers of FAAH rs324420 had better fear extinction learning only if they had higher post-extinction AEA levels. Conversely, lower AEA levels were associated with worse extinction learning performance. This moderation effect was absent for CC homozygotes. This data is consistent with Mayo et al. (2018) who concluded that potentiation of AEA by substitution of both rs324420 C alleles with A alleles contributed to improved stress resilience and fear extinction. Our findings compliment and extend the findings from this study by directly correlating peripheral AEA levels with the rs324420 effect on fear extinction learning. Further, since AEA and OEA levels have been reported to be decreased with heightened PTSD symptom severity (Hill et al., 2013; Spagnolo et al., 2016; Wilker et al., 2016), it is possible that the effect of rs324420 on fear extinction learning in PTSD participants in the current study was associated with endocannabinoid levels in A carriers that are inconsistent with those of healthy participants. However, whether this finding is able to explain the data from our PTSD sample – a psychiatric population – will need to be investigated in future studies. Specifically, it is important to note that the FAAH rs324420 polymorphism appears to interact with life adversity, such that A carriers who experience childhood stress have higher long-term anxiety and depression (Lazary et

al., 2016). In addition, increased corticotrophin-releasing hormone signalling combined with the A allele of rs324420 is associated with higher anxiety (Demers et al., 2015; Harris et al., 2019), and it has be hypothesised that FAAH rs324420 effects might be modulated by trauma exposure and/or resilience (deRoon-Cassini et al., 2020). While this interaction could explain our PTSD results – which were inconsistent with the existing literature – it likely also has implications for whether our sub-analysis can generalise to clinical populations.

Since the A allele substitution results in lower FAAH enzyme, our subanalysis suggests that previously identified FAAH rs324420 effects are indeed moderated by AEA in healthy humans. Further to this, the FAAH rs324420 effects in the current study were not moderated by closely related OEA or 2-AG. Since OEA shares many of the molecular pathways and close chemical similarity to AEA, asides from affinity for CB1R (Ligresti et al., 2016), it is likely that activation of the endocannabinoid system through CB1R, rather than competing pathways, was a catalyst for the FAAH rs324420 effects. This is an important and novel translation of animal findings for the critical role of CB1R in fear extinction in humans (Kamprath et al., 2006; Marsicano et al., 2002) and is complimented by significant effects of CNR1 rs1049353 on fear extinction in the current study. However, it should similarly be noted that there are FAAH substrates besides AEA and OEA that we did not measure that may have been involved in the fear extinction outcomes. Regardless, this is the first direct evidence of this effect in humans and reflects a significant advance in the translational knowledge of endocannabinoid effects on fear extinction in humans.

There were several limitations in the current study. Firstly, our sample size was modest, with only 43 PTSD participants and 57 participants who were in the sub-analysis. Fear extinction paradigms require large sample sizes due to large individual

differences and variability of responding resulting in small effect sizes (Bach & Melinscak, 2020; Ney, Wade, et al., 2018). Relatedly, we were unable to separate rs324420 AC from AA genotypes due to low numbers of the latter genotype and this may have affected our ability to replicate recent research describing a more prominent effect in the AA group (Mayo et al., 2018). More importantly, our sub-analysis included only healthy participants and may not generalise to our PTSD sample. Blood sampling was added as an amendment to the project in its final year during which only a small number of PTSD participants were recruited, and data collection was terminated prematurely due to COVID restrictions. As a consequence, it is possible that these data are incompatible with psychiatric populations. Future research should recruit a higher number of PTSD participants providing blood samples to test this possibility. Our data is also correlational and we cannot confirm whether the plasma endocannabinoid levels were a cause or result of the paradigm performance. More clinical trials utilising cannabinoids are needed to establish causality. Finally, we employed candidate loci research, which is now usually surpassed by genome-wide association studies. However, when genetic mutations have an observable biochemical effect and are used to explain complex behavioural phenotypes, as in the current study, single candidate loci research can inform precision medicine with higher specificity than GWAS (Mayo et al., 2018; Moore, 2017; Pulley et al., 2012). In the current study, we found that A carriers of rs324420 had higher OEA and AEA levels and we were thus able to examine these effects on fear extinction with high precision.

In conclusion, we found the first evidence for negative effects of CNR1 genetic variations on fear extinction learning specific to PTSD. We also found evidence for a moderating role of plasma AEA level on the effect of FAAH

polymorphism rs324420 on fear extinction learning in healthy humans. This finding was not replicated when substituting OEA or 2-AG level, suggesting that the rs324420 effect is associated with the endocannabinoid system through AEA availability, potentially as well as other analytes that were unmeasured in our study. These findings provide new evidence supporting the potential for augmentation of fear extinction-based treatments for PTSD via direct involvement of the endocannabinoid system. This might be achieved by direct agonism of cannabinoid receptors or by enzyme inhibitors, such as FAAH inhibition, which would upregulate endocannabinoid levels. However, our data suggest that larger PTSD samples are critically needed to evaluate the transfer of endocannabinoid effects between healthy and clinical populations.

Author Contributions

LJN, CMH, DVZ, DN, TS, BG, BH, and EN acquired, analysed and interpreted the work.

LJN, AM, DVZ, RB, and KF contributed to the conception and design of the work.

LJN drafted the work and all authors provided critical revisions.

Final approval and agreement to be accountable for all aspects of the work was committed by all authors.

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Figures

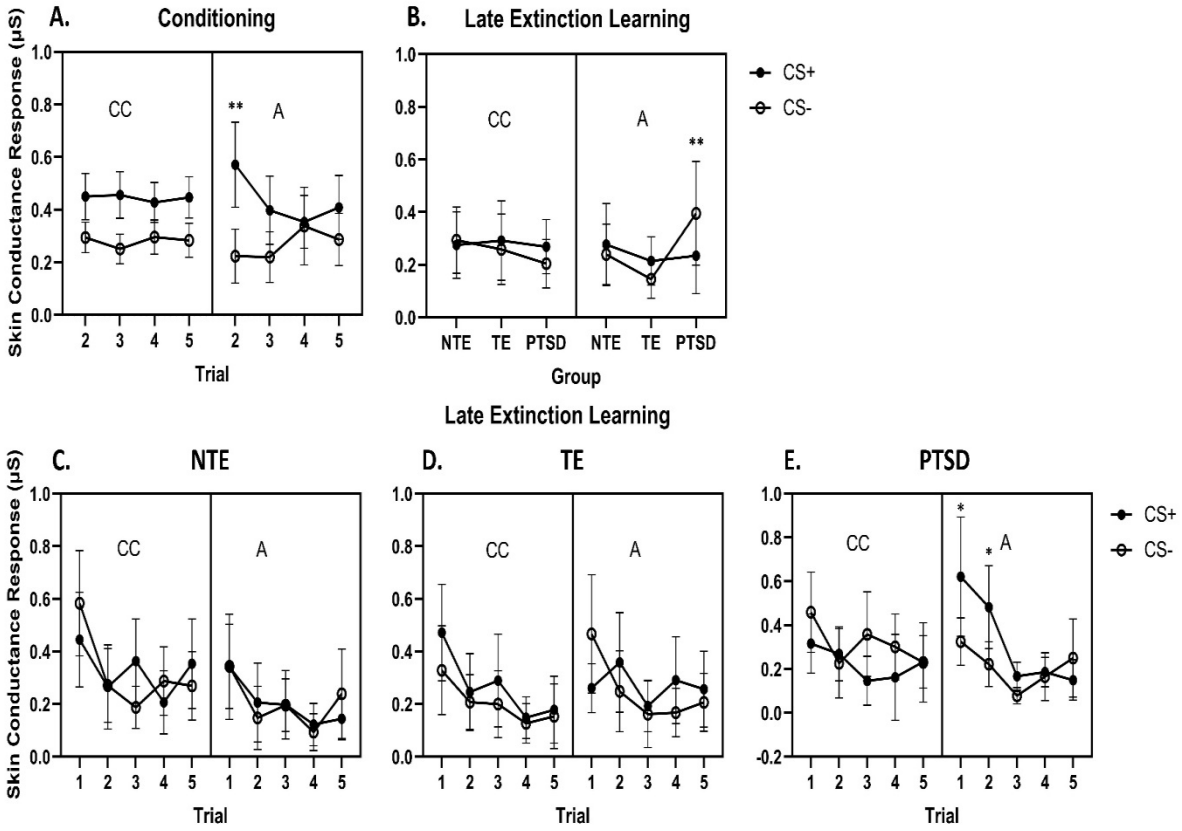

Figure 1. Interactions between genotype, group, stimulus, and trial during late extinction learning and conditioning. Panels A is FAAH rs324420 during conditioning (trials 2-5) and Panel B is FAAH rs324420 during late extinction (mean

of trials 1-5 for CS+ and CS-). Panels C-E are for the CNR1 SNP rs1049353 during late extinction (trials 1-5 for CS+ and CS-). A = A allele carrier, CC = CC homozygote, NTE = non-trauma exposed, TE = trauma exposed, PTSD = PTSD participant. Increased reactivity to the CS+ during conditioning in A carriers compared to CC homozygotes for FAAH rs324420 (Panel A). Increased reactivity to the CS- was observed in rs324420 A allele PTSD participants during late extinction (Panel B). A allele rs1049353 PTSD participants had increased reactivity to the CS+ on trials one and two during late extinction (Panel E). Error bars are 95% confidence intervals. *p<.05, **p<.01. N=220: n=84 NTE, n=93 TE, n=43 PTSD.

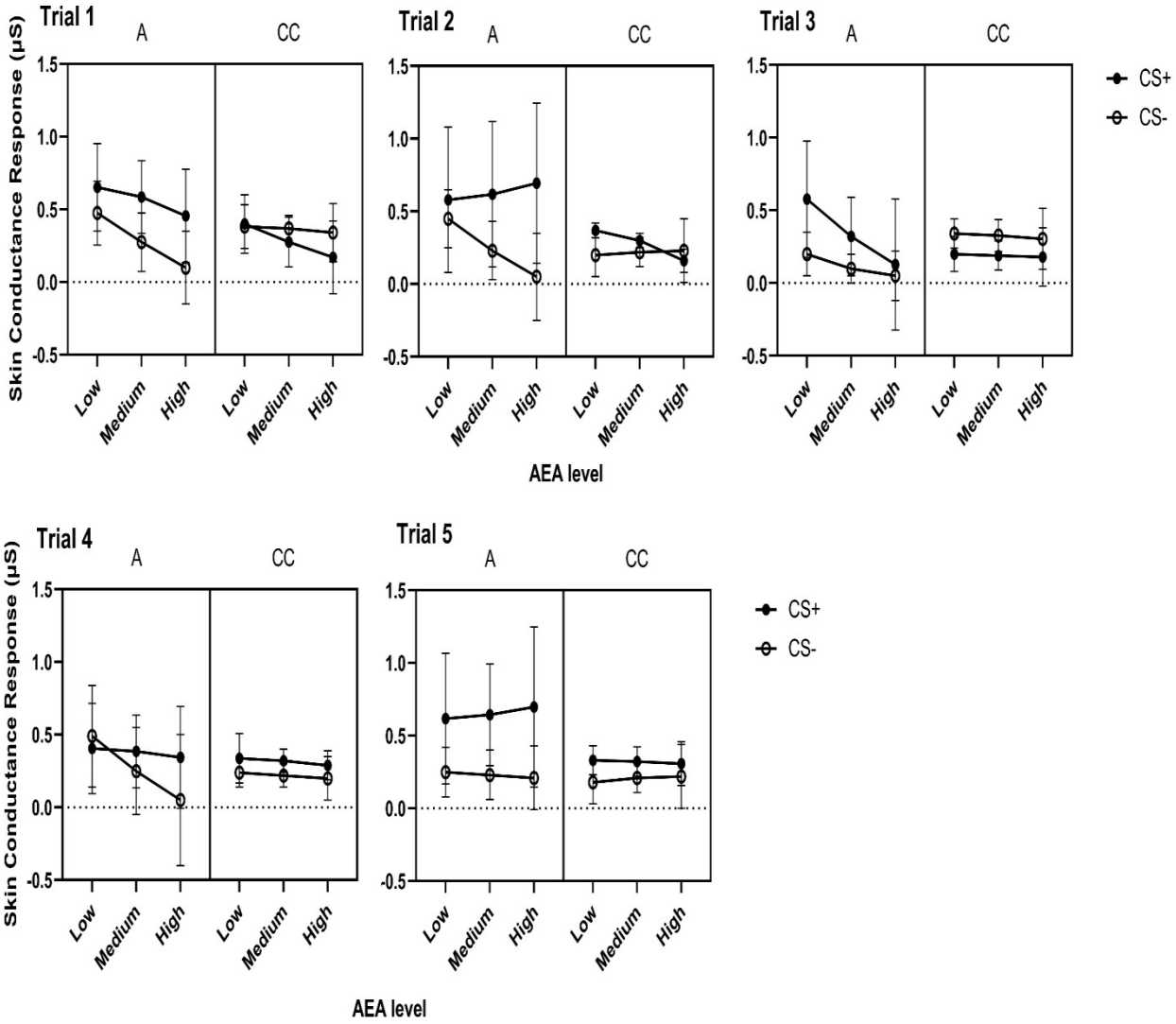

Figure 2. Higher CS+ compared to CS- responding was observed over most conditioning experimental trials (Trials 1-5) in the A allele but not CC homozygote group for FAAH rs324420. Errors bars are 95% confidence intervals. Simple slopes analyses (using 1 standard deviation above and below the mean for high and low levels, respectively) are used to illustrate the findings from the mixed model, where AEA level was a continuous predictor. N=57.

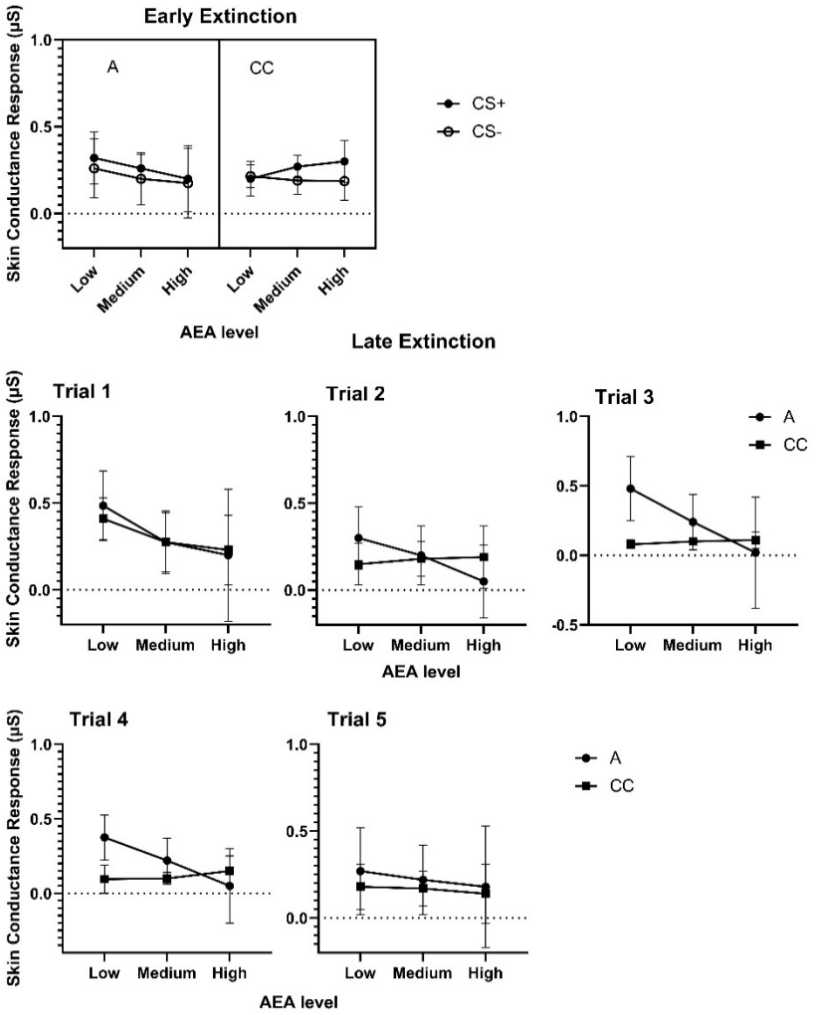

Figure 3. AEA level interacts with FAAH rs324420 genotype to predict SCRs (p=.007) during early extinction learning (top panel, containing CS+ and CS-). During most trials of late extinction learning (bottom two panels, containing SCRs from combined CS+ and CS- responses), rs324420 A carriers responded higher if AEA levels were low but lower if AEA levels were high (p<.001). Error bars are 95% confidence intervals. Simple slopes analyses (using 1 standard deviation above and below the mean for high and low levels respectively) are used to illustrate the findings from the mixed model, where AEA level was a continuous predictor. N=57