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Hair endocannabinoids predict physiological fear conditioning and salivary endocannabinoids predict subjective stress reactivity in humans

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Abstract

On the basis of substantial preclinical evidence, the endogenous cannabinoid system has been proposed to be closely involved in stress reactivity and extinction of fear. Existing human research supports this proposal to some extent, but existing studies have used only a narrow range of tools and biomatrices to measure endocannabinoids during stress and fear experiments. In the present study we collected hair and saliva samples from 99 healthy participants who completed a fear conditioning and intrusive memory task. Subjective, physiological and biological stress reactivity to a trauma film, which later served as unconditional stimulus during fear conditioning, was also measured. We found that salivary endocannabinoid concentrations predicted subjective responses to stress, but not cortisol stress reactivity, and replicated previous findings demonstrating a sex dimorphism in hair and salivary endocannabinoid levels. Hair 2-arachidonoyl glycerol levels were significantly associated with better retention of safety learning during extinction and renewal phases of fear conditioning, while hair concentrations of oleoylethanolamide and palmitoylethanolamide were associated with overall physiological arousal, but not conditional learning, during fear conditioning. This study is the first to test the relationship between hair and salivary endocannabinoids and these important psychological processes. Our results suggest that these measures may serve as biomarkers of dysregulation in human fear memory and stress.

Keywords: endocannabinoids; fear conditioning; intrusive memories; posttraumatic stress disorder; stress reactivity; hair; saliva; mass spectrometry

The endocannabinoid system is a lipid signalling system that is gaining increasing attention as an important mediator of many biological processes and functions (Kano et al., 2009; Ligresti et al., 2016). The endocannabinoid system consists of G-protein-coupled receptors (CB1 and CB2) that are present throughout both the peripheral and central nervous systems (Howlett et al., 2002), as well as endogenous ligands that activate these receptors. Arachidonoyl ethanolamide (AEA, Devane et al. (1992)) and 2-arachidonoyl glycerol (2-AG, Suguira et al. (1995)) are the primary endocannabinoid ligands. Other N-acylethanolamines (NAEs), such as oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) are endocannabinoid-like molecules and are structural congeners to AEA and 2-AG, but they do not typically interface directly with CB1 or CB2 (Ligresti et al., 2016).

An increasingly compelling literature suggests that endocannabinoids are involved in stress and are important regulators of stress hormone release (Balsevich et al., 2017; Hill & Tasker, 2012; Morena et al., 2016; Ney et al., 2022). In animal models (Morena et al., 2016) as well as human experimental models of stress (Crombie et al., 2019; Dlugos et al., 2012; Mayo, Asratian, Linde, et al., 2020; Mayo, Asratian, Lindé, et al., 2020) there is clear evidence that stress alters endocannabinoid tone. Evidence from animal models suggest that reductions in central nervous system AEA is necessary for glucocorticoid release, whereas increases in 2-AG are necessary to terminate the glucocorticoid response through negative feedback (Evanson et al., 2010; Hill & Tasker, 2012; Patel et al., 2004). Preliminary evidence suggests that these effects may be influenced by sex hormones (Atkinson et al., 2010; Ney et al., 2018), and that the relationship between endocannabinoid signalling and glucocorticoid activity may be influential in stress- and trauma-related disorders such as anxiety and posttraumatic stress disorder (Blessing et al., 2015; Hill et al., 2018; Mayo et al., 2021; Ney et al., 2019).

Further, substantial evidence suggests that the endocannabinoid system is involved in fear conditioning (Hill et al., 2018; Marsicano et al., 2002; Mayo et al., 2021; Ney, Akhurst, et al., 2021). Fear conditioning, which involves the acquisition, extinction, and return of conditioned fear, is fundamental to understanding the aetiology and maintenance of traumatic and anxiety disorders (Bouton et al., 2020; Lonsdorf et al., 2017). However, while the role of endocannabinoids in fear conditioning has been demonstrated using invasive biological methods in animals, it has been harder to establish in human fear models (Ney et al., 2022). Current human evidence suggests that inhibition of AEA's degradation enzyme improves fear extinction recall (Mayo, Asratian, Lindé, et al., 2020), and higher blood AEA concentration is associated with improved fear extinction (Crombie et al., 2021; Mayo, Asratian, Linde, et al., 2020; Ney, Matthews, et al., 2021; Spohrs et al., 2021), though also with higher fear responses (Weisser et al., 2022). Genetic evidence is mixed, with reports suggesting that genotypes affecting inhibition of cannabinoid receptors and AEA degradation enzymes either improve, impair, or have no effect on fear extinction (Dincheva et al., 2015; Heitland et al., 2012; Mayo, Asratian, Linde, et al., 2020; Ney, Matthews, et al., 2021; Zabik et al., 2021).

Although the endocannabinoid system is well characterised in the central nervous system (Kendall & Yudowski, 2016; Ligresti et al., 2016) as well as in blood (Fanelli et al., 2012; Hillard, 2017), there is substantially less understanding of how salivary and hair endocannabinoids relate to broader human biology. Recent advances in mass spectrometry have seen the development of methods for quantifying endocannabinoids in hair (Gao et al., 2021; Gao et al., 2020; Krumbholz et al., 2013; Mwanza et al., 2016; Ney, Felmingham, et al., 2021; Voegel et al., 2021) and saliva (Matias et al., 2012; Ney et al., 2020), though how they can be used as an index of biological functioning and pathology is not understood. Some studies have shown that group differences in the levels of hair endocannabinoids are associated with cocaine use, trauma, anorexia, and borderline personality disorder (Croissant

et al., 2020; Koenig et al., 2018; Tam et al., 2021; Voegel et al., 2022; Wilker et al., 2016; Wingenfeld et al., 2018), and salivary endocannabinoids are acutely responsive to stress and mastication (Mennella et al., 2018; Ney, Stone, et al., 2021), affected by chronic orofacial pain (Haviv et al., 2022), and higher in males compared to females (Haviv et al., 2022; Ney, Stone, et al., 2021). There is no current explanation for why salivary endocannabinoids are higher in males, whereas hair endocannabinoids appear to be elevated in females (Gao et al., 2022). While hair collection provides a longitudinal, retrospective measure of endocannabinoid concentrations, saliva appears to be acutely responsive to different stimuli (Krumbholz et al., 2013; Stalder & Kirschbaum, 2012). However, the above literature represents the extent of our knowledge to date on the relationship between these molecules and human disease, which is in stark contrast to other well characterised systems (such as glucocorticoids) that have proved extremely useful non-invasive research tools in relation to stress and disease (Andreano & Cahill, 2006; Bryant et al., 2013; Kirschbaum & Hellhammer, 1989; Kirschbaum & Hellhammer, 1994; Stalder & Kirschbaum, 2012; Staufenbiel et al., 2013).

Development of new biomarkers – in this case salivary endocannabinoids – requires sophisticated and comprehensive knowledge concerning factors that impact their activity, such as age, sex, stress levels, and relationship to other related biological systems, among many others. Emerging evidence suggests that salivary endocannabinoids are highly correlated with one another as well as with N-acyl ethanolamines (OEA, PEA) (Ney et al., 2021); however, this data needs replication and a comparison to hair levels has not been performed. No study has tested the effect of age on basal levels of salivary endocannabinoids, although there is some evidence that endocannabinoid tone may change over the aging process in the brain (Piyanova et al., 2015).

In the current study, we collected hair and saliva samples from healthy human participants who watched a series of violent film clips and completed a fear conditioning task. Saliva samples were collected immediately before and 20-minutes following film viewing. We measured AEA, 2-AG, OEA, PEA and cortisol in these samples. Based on previous evidence (Ney et al., 2020; Voegel et al., 2021; Voegel et al., 2022), we hypothesised that endocannabinoids and NAEs will be positively correlated with each other, and that males would have higher endocannabinoid levels compared to females in saliva (Haviv et al., 2022; Ney, Stone, et al., 2021) but lower endocannabinoid levels in hair samples (Gao et al., 2022). No hypothesis for the effect of age on salivary endocannabinoids was made, as this was completely exploratory. We hypothesised that higher levels of endocannabinoids and NAEs would be associated with lower stress reactivity and improved fear extinction during the conditioning task. We hypothesised that higher SUDS reactivity following film viewing would be associated with higher salivary cortisol responses, but that there would be no relationship between salivary endocannabinoids and salivary cortisol. We also hypothesised that higher hair cortisol would be associated with higher fear acquisition but lower fear renewal, due to the known effects of cortisol on threat-related memory consolidation (Brueckner et al., 2019; Merz et al., 2018). Overall, this study forms an important incremental contribution to an ongoing investigation of the characteristics of the endocannabinoid system in human saliva and hair.

Methods

Participants

Participants were 99 healthy undergraduate students (mean age = 21.2, SD = 5.5, 77 females) recruited through flyers and course-related advertisements at the Queensland University of Technology. A screening questionnaire excluded prospective participants based

on current serious psychological, neurological, cardiac, or physiological disorders, current pregnancy, epilepsy, history of head trauma or loss of consciousness, cannabis use within the last month, and age older than 60 years. Further, prospective participants were excluded if they consumed violent media on a daily basis. Participants were reimbursed with either course credits or AUD$45 for their involvement in the study. The study was approved by the Queensland University of Technology Human Research Ethics Committee, and all participants provided fully informed consent prior to participation.

Measures

The state-trait anxiety inventory (STAI; Spielberger, 1983), intolerance of uncertainty scale (IUS-12; Carleton et al., 2007), depression anxiety and stress scales (DASS-21; Lovibond & Lovibond, 1995), and subjective units of distress scale (SUDS; Wolpe, 1969) were all used to measure current, recent, and general mood, anxiety, depression, and intolerance of uncertainty amongst our participants. These measures were collected so that they could be correlated with endocannabinoid concentrations, as in previous studies (Gao et al., 2022). The SUDS was used to measure subjective stress reactivity before and after viewing the trauma film. An intrusive memory diary was used to record the frequency of intrusive memories following the experiment and required participants to report the number of intrusive memories of experimental content that they experienced each day for the four days following the study.

Experimental Paradigm

The experimental paradigm involved participants watching a 10-min series of film clips chosen from commercial horror and action movies, as well as from news media. These clips contained non-sexual, grievous bodily harm from previously used media including

Irreversible (2002), *Hostel* (2005), a British road safety ad, a kitchen workplace and safety ad (Kunze et al., 2015; Landkroon et al., 2020; Landkroon et al., 2019; Miedl et al., 2020; Wegerer et al., 2013), as well as violent footage from *Drive* (2011), a news clip involving an escaped leopard attacking humans, and a news clip involving a violent robbery.

Sixteen-second clips that depicted the worst moments from the first four clips described above were subsequently used as unconditional stimuli during a fear conditioning paradigm. In the fear conditioning paradigm, participants completed habituation, acquisition, extinction, renewal, and re-acquisition phases. During acquisition, two geometric shapes (CS+) were immediately followed with 100% reinforcement by either an electric shock and an aversive film clip, or an aversive film clip alone, and a third geometric shape (CS-) was followed by 16-second neutral film clips of a man shovelling snow, a passing train, a café, or a man riding a horse. During extinction and contextual renewal, no CS was reinforced by any neutral or aversive stimuli. During re-acquisition, the CS+s were followed with 50% reinforcement by either an electric shock and an aversive film clip, or an aversive film clip alone. The CS- was followed by a neutral film clip 50% of the time during re-acquisition. There were 4 trials for each CS during habituation, 4 during acquisition, 5 during extinction, 4 during renewal, and 2 during reacquisition. CS duration was 6-seconds and the intertrial interval jittered between 10- and 14-seconds. Habituation, acquisition, renewal, and reacquisition were presented in Context A, which was either a bright colour or a background stock image scene of a street or an office room. Extinction was presented in Context B, which was either a different bright colour or the stock image scene (street or office) not used for Context A. Contexts were counterbalanced between participants, and the effect of contextual complexity will be explored thoroughly in another manuscript. Similarly, the full data from the fear conditioning and intrusive memory paradigm will be reported elsewhere as this data is substantial and cannot be reported within one manuscript. After fear conditioning, participants reported how

many intrusive memories they had of the experimental materials (i.e., anything from the experiment) during renewal and re-acquisition. Specifically, prior to completing the fear renewal and re-acquisition phase, participants were clearly instructed on the nature of intrusive memories and reporting perception and memory was clearly delineated by the experimenter. This manipulation emulates the memory triggering task used in Wegerer et al. (2013) where intrusive memories are intentionally triggered during the experiment by presenting the cue (i.e., the conditional stimuli CS+ and CS-) that at this stage are associated with the violent film clips. Participants were clearly instructed to report only intrusive memories and not moments when they were actually watching the film clips. Participants also completed the intrusive memory diary for 4 nights following the experiment, where they reported the frequency of intrusive memories of the film clips and fear conditioning materials.

Physiological Equipment

This study utilised a direct current approach to measuring SCR; entailing a constant low-level voltage that is passed between two adhesive-electrodes placed on the thenar and hypothenar regions of a participant's non-dominant palm (Boucsein et al., 2012). Raw form SCR recordings were captured using the Biopac MP150 and then scored in AcqKnowledge v3.9.1. In order to better preserve data integrity, SCR non-responders were classified as any participant with less than two responses above 0.02μS in any given phase (n=12). Respiration was also recorded to control for movement artifacts in SCR recordings via a Biopac TSD201 respiratory belt and a RSP100c respiration amplifier. SCRs were scored manually using the trough-to-peak method. SCRs were scored using EzyScore (manuscript under review), which is a semi-automated scoring software for event-related skin conductance responses. SCRs were classified as responses occurring from 1 until 7 seconds post-CS onset, which is the

entire interval response (Luck & Lipp, 2016; Pineles et al., 2009). Spontaneous skin conductance fluctuations during the baseline physiology recording were manually identified and counted by a trained research assistant. Spontaneous skin conductance fluctuations were defined as an identifiable skin conductance response (i.e., displaying a canonical skin conductance response peak shape) that was at least 0.02µS in amplitude (Lonsdorf et al., 2019). The electric shock device was a Digitimer with a concentric shock electrode, which delivered three 2-millisecond shocks 16-milliseconds apart, which is perceived as a discrete stimulus. Programming of the experimental task was achieved using DMDX 6.1.8.2 (Forster & Forster, 2003).

Hair extraction

Hair samples were first cut to 3cm from the scalp, weighed in milligrams, and washed using 2 mL isopropanol for 3 minutes. Next, 20 µL of an isotope labelled standard solution containing 20 ng/mL d4-AEA, 25 ng/mL d5-2AG, 20 ng/mL d4-OEA, and 50 ng/mL d4-cortisol was added. Hair samples were then incubated overnight in 1.6 mL LC-MS grade methanol. On the following day, the samples were evaporated to dryness using a vacuum evaporator and re-constituted in 100 µL acetonitrile and then 15 µL methanol, 5 µL of which was injected into a Sciex QTRAP 6500 and analysed using tandem mass spectrometry (LC-MS/MS). The column was a Waters Acquity BEH C18 column. Mobile phase A was 2mM ammonium acetate and mobile phase B was acetonitrile. Runtime of the method was 12 minutes, and full details of the method are described in Ney, Felmingham, et al. (2021).

Saliva extraction

1mL of saliva was collected per sample using the passive drool method. After saliva samples were collected, they were frozen at -20^{o}C until the day of analysis. Saliva samples

were first vortexed and then centrifuged, after which 500 µL of saliva was pipetted into a fresh microfuge tube for each sample. Next, 20 µL of an isotope labelled standard solution containing 20 ng/mL d4-AEA, 25 ng/mL d5-2AG, 20 ng/mL d4-OEA, and 50 ng/mL d4-cortisol were added. 1.4 mL freezer-cold 1:1 methanol:acetone solution was then added to each sample. Following thorough homogenisation using a vortex mixer, samples were placed in a -20ºC freezer for 60 minutes to facilitate protein precipitation. Samples were again centrifuged and the supernatant transferred to fresh tubes, evaporated to dryness, reconstituted in 150 µL methanol, evaporated again, and reconstituted to the final solution of 30 µL 50% methanol. 15 µL of the final solution was injected into the Sciex QTRAP 6500. The mass spectrometry conditions were otherwise the same as the hair analysis and can be found fully described in (Ney et al., 2020). Both of our methods are linear, though due to the increased sensitivity of the QTRAP 6500 compared to the Waters Xevo® TQ we previously used, our limits of quantification are lower in the current analyses (manuscript in preparation).

Procedure

Participants were initially screened online and eligible participants were invited to the Emotion, Learning, and Psychophysiology laboratory at Queensland University of Technology for a 1.5 hour testing session. All sessions were conducted between 11am and 5pm, to control for circadian rhythm of salivary analytes (Vining et al., 1983). After providing informed consent, participants were connected to the respiration and skin conductance equipment and completed the first saliva sample and SUDS rating. Next, participants watched the 10-min film while having their physiology recorded. Immediately after the film, participants rated their distress on the SUDS again and then completed the questionnaire battery. They provided a hair sample and then the next saliva sample (collected

20-minutes post film viewing), before doing a shock workup to calibrate the shock intensity for fear conditioning. Starting at 0mA, shock intensity was increased in increments of 0.1mA until reaching a level that participants determined was "unpleasant, but not painful". After the shock workup, 3 minutes of baseline physiology recording was performed. Then, participants completed the fear conditioning task. At the end of the fear conditioning task, participants reported how many intrusive memories of the experimental materials they had during renewal and re-acquisition and were instructed to complete the intrusive memory diary for the following four nights. Specific instructions were given to participants: they were informed about the exact nature of an intrusive memory and were instructed to only report intrusions that met these criteria (i.e., not deliberate, spontaneous, "in the mind's eye", and may occur while you are doing other things). Participants were required to report the frequency of their intrusive memories retrospectively at the end of each day. Text messages and email reminders to complete the intrusive memory diary were sent to participants at 4pm daily over the next 4 days.

[INSERT FIGURE 1 HERE]

Statistical Analysis

Absolute quantification of AEA, 2-AG, OEA, PEA and cortisol was performed using the isotope dilution method. We did not have a labelled standard for PEA. Due to its close structural similarity with OEA, we used d4-OEA as the labelled standard for PEA. Specifically, PEA and OEA have identical N-acyl structures, with the difference between the molecules being an additional 26 dalton mass on OEA due to a slightly longer N-acyl fatty acid chain and the presence of a N-acyl double bond. In our mass spectrometry method, the multiple reaction monitoring (MRM) daughter ion for quantification of both OEA and PEA is

62.1 m/z and elution of the two targets occurs within 12 seconds of one another. Therefore, the use of d4-OEA as a surrogate standard for PEA is justified chemically. 2-AG and its isomer 1-AG are chromatographically resolved using our method however these values were summed and reported as 2-AG, as is standardly practice (Hillard, 2017). Quantification of the analytes was achieved using Skyline-daily v21.1.9.348 (Pino et al., 2020).

Baseline endocannabinoids were tested between sex using one-way ANOVAs and across age using linear regression. Between one and three extreme outlying values (i.e., more than 3 standard deviations from the mean) were identified for each analysis and removed. Correlations between analyte concentrations were performed using Pearson's Correlation Coefficient (*r*). False Discovery Rate (FDR) corrections were applied at $Q = .05$ to account for multiple comparisons (Benjamini & Hochberg, 1995). Stress reactivity of SUDS and salivary measurements were tested using repeated measures ANOVAs, with a 2-level factor of Time. Average skin conductance level during the entire 10-minute duration of the film was calculated for each participant, as was standard deviation (i.e., variability of skin conductance during the film) and slope of overall increase/decrease of skin conductance level during the film. Correlations of endocannabinoid concentrations with stress reactivity were assessed separately using regression, with the change in stress reactivity between Time 1 and Time 2 as the outcome variable and the post-stress analyte concentration (e.g., salivary 2-AG) as the independent variable. Only post-stress endocannabinoid levels were used in these models for the following reasons: (1) no change was anticipated between time points in salivary endocannabinoids due to past data suggesting a fast-acting response (Ney et al., 2020; Ney, Stone, et al., 2021); and (2) due to their temporal proximity to the stress outcome measures (SUDS rating and cortisol response), relative to the endocannabinoid baseline measurement. However, the relationship between the pre-stress salivary endocannabinoid measures and subjective stress reactivity are reported for full transparency in the Supplementary Material.

Mixed ANOVAs with SCR as the outcome measure were used to test the relationship between CS type: 3(CS+s, CS-), Trial (2, 4, or 5 depending on phase) and hair endocannabinoid level (continuous variable). Separate analyses were run for each phase. Correlations between anxiety measures (IUS-12, STAI-T), mood assessments (STAI-S, DASS-21) and intrusive memories with hair endocannabinoid concentrations were also conducted using Pearson's *r*, with FDR corrections applied. Analyses were performed in SPSS for Windows (v27), and figures were produced in R using *ggplot2*.

Results

Demographics

There were 21 males and 72 females in the sample, and 6 participants did not provide sex information (N = 99). A total of 69 participants provided hair samples (57 female), and all participants provided saliva samples. The mean age of participants was 21.2 years (SD = 5.5), with a range of 17 – 49 years of age. The average STAIS, STAIT, IUS, depression, anxiety, and stress scores were 45.3 (SD = 10.7), 43.6 (SD = 10.9), 31.2 (SD = 9.2), 9.3 (SD = 8.4), 9.6 (SD = 8.1), and 15.0 (SD = 8.8), respectively.

Baseline endocannabinoids: Relationship with sex and age

Baseline saliva AEA was significantly higher in males: $F(1,90) = 5.70$, $p = .019$, $\eta_p^2 = .06$, as was OEA: $F(1,89) = 11.84$, $p < .001$, $\eta_p^2 = .12$. Baseline 2-AG was not significantly different between sexes: $F(1,89) = 0.84$, $p = .361$, $\eta_p^2 = .01$, and neither was cortisol: $F(1,91) = 0.29$, $p = .592$, $\eta_p^2 = .003$ or PEA: $F(1,88) = 3.90$, $p = .052$, $\eta_p^2 = .04$. Sex-related cortisol and endocannabinoid levels are reported in Table 1. 2-AG was higher in older participants: $F(1,94) = 5.94$, $p = .017$, $\eta_p^2 = .06$, as was OEA: $F(1,94) = 5.85$, $p = .017$, $\eta_p^2 = .06$ and PEA: $F(1,93) = 7.51$, $p = .007$, $\eta_p^2 = .07$. Conversely, cortisol level was significantly lower with

increased age: $F(1,96) = 5.66$, $p = .019$, $\eta_p^2 = .06$, but AEA was not significantly correlated with age: $F(1,93) = 1.89$, $p = .172$, $\eta_p^2 = .02$. The relationship between baseline saliva levels of significant analytes, as described above, is visualised in Figure 2.

Hair AEA was significantly higher in females compared to males: $F(1,67) = 9.64$, $p = .003$, $\eta_p^2 = .13$, as was 2-AG: $F(1,67) = 4.64$, $p = .035$, $\eta_p^2 = .06$, OEA: $F(1,67) = 8.02$, $p = .006$, $\eta_p^2 = .11$, and PEA: $F(1,67) = 7.18$, $p = .009$, $\eta_p^2 = .10$. Sex-stratified cortisol and endocannabinoids levels are reported in Table 1. Hair cortisol was higher in females, but the difference did not reach significance: $F(1,67) = 3.06$, $p = .085$, $\eta_p^2 = .04$. Hair AEA was not associated with participant age: $F(1,72) = 1.30$, $p = .258$, $\eta_p^2 = .02$, nor were 2-AG: $F(1,72) = 0.10$, $p = .757$, $\eta_p^2 = .001$, OEA: $F(1,72) = 0.06$, $p = .802$, $\eta_p^2 = .001$, PEA: $F(1,72) = 0.09$, $p = .770$, $\eta_p^2 = .001$, or cortisol: $F(1,72) = 0.39$, $p = .537$, $\eta_p^2 = .006$.

Table 1. Saliva and hair endocannabinoid and cortisol concentrations (in pg/mL), stratified by sex and overall

	Male	Female	Total
Saliva			
AEA*	2.2 (1.7)	1.3 (1.6)	1.5 (1.6)
2-AG	274.9 (255.4)	223.9 (212.8)	235.7 (222.9)
OEA***	295.4 (158.1)	171.3 (140.0)	198.6 (150.8)
PEA	212.6 (86.1)	165.6 (98.1)	176.5 (97.1)
Cortisol	1779.7 (952.2)	1603.5 (1406.2)	1643.3 (1314.8)
Hair			
AEA**	0.5 (0.2)	0.9 (0.6)	0.8 (0.5)
2-AG*	19.4 (7.2)	41.6 (48.7)	38.0 (43.9)
OEA**	390.7 (231.9)	1645.4 (1862.4)	1369.2 (1712.5)
PEA**	477.3 (132.8)	1632.8 (1648.6)	1382.4 (1529.5)
Cortisol	2.3 (2.6)	5.8 (8.3)	4.9 (7.5)

Note: AEA = arachidonoyl ethanolamide, 2-AG = 2-arachidonoyl glycerol, OEA = oleoylethanolmaide, PEA = palmitoylethanolamide. Mean values are reported with standard deviation in brackets. *$p < .05$, **$p < .01$, ***$p < .001$ for sex comparisons

[INSERT FIGURE 2 HERE]

Correlations between biological measurements

Bivariate correlations between saliva and hair measurements were performed and are visualised in Figure 3. Since many comparisons were performed, FDR corrections for multiple comparisons were applied. Hair AEA (r = .35, p = .003), hair OEA (r = .89, p < .001), and hair cortisol (r = .39, p < .001) were significantly positively correlated with hair PEA levels. Salivary AEA was positively and significantly correlated with salivary levels of 2-AG (r = .52, p < .001), OEA (r = .70, p < .001), and PEA (r = .55, p < .001). Similarly, salivary levels of 2-AG were significantly positively correlated with OEA (r = .48, p < .001) and PEA (r = .40, p < .001), and salivary OEA was significantly correlated with salivary PEA (r = .77, p < .001).

Hair OEA was positively correlated with hair AEA (r = .24, p = .042) and hair cortisol (r = .27, p = .020), hair AEA was correlated with salivary cortisol (r = -.27, p = .020) and hair PEA was negatively associated with salivary AEA (r = -.24, p = .048), but these differences did not survive corrections for multiple comparisons. There were no significant relationships between hair and salivary measures.

[INSERT FIGURE 3 HERE]

Stress reactivity

Cortisol levels did not reliably increase following film viewing (samples collected 25 minutes post-film viewing): $F(1,91) = 0.37$, p = .543, η_p^2 = .004. However, SUDS ratings significantly increased from pre- to post-film viewing: $F(1,97) = 129.32$, p < .001, η_p^2 = .59. Since salivary endocannabinoids only seem to show rapid reactivity to stress (Ney et al., 2020; Ney, Stone, et al., 2021), we did not expect to see any effect of film viewing on salivary endocannabinoid levels. As expected, AEA: $F(1,91) = 1.19$, p = .279, η_p^2 = .01 and

2-AG: $F(1,92) = 0.57$, $p = .454$, $\eta_p^2 = .01$ levels did not change between measurements; however, both OEA: $F(1,94) = 12.21$, $p < .001$, $\eta_p^2 = .11$ and PEA: $F(1,91) = 14.74$, $p < .001$, $\eta_p^2 = .14$ levels decreased significantly between measurements. This may have reflected a reduced arousal tone 20-minutes following film viewing when participants had been exposed to most of the experimental conditions and were no longer apprehensive about the experiment. Skin conductance level was slightly increasing during the film on average and was on average 5.00μS ($SD = 0.83$). Further, regression analysis showed that participants with higher skin conductance level had higher skin conductance variability during the film (indexed by standard deviation of skin conductance level throughout film viewing): $F(1,91) = 17.51$, $p < .001$, $\eta_p^2 = .16$ and had a more positive slope compared to participants with lower skin conductance level during the film: $F(1,91) = 15.39$, $p < .001$, $\eta_p^2 = .14$, suggesting that skin conductance was increasing rather than decreasing throughout the film for these participants.

Correlations with stress reactivity

Post-film salivary endocannabinoid concentrations were regressed against change in SUDS and cortisol (i.e., established measures of stress reactivity) before and after film viewing. Only post-stress endocannabinoid levels were analysed because these were temporally proximal to the cortisol and SUDS measurements (relative to the baseline measurement) and we do not think that it is biologically plausible that salivary endocannabinoids have a role in initiating the stress response itself. See Supplement for reporting of the pre-stress salivary endocannabinoids in relation to subjective stress reactivity. The change in SUDS ratings was significantly associated with post-film salivary 2-AG levels: $F(1,90) = 4.33$, $p = .040$, $\eta_p^2 = .05$ (not significant after correction for multiple comparisons using FDR), such that higher distress following the film clip was associated with lower levels

of salivary 2-AG. Similarly, post-film salivary AEA was negatively associated with changes in SUDS ratings from pre- to post-film viewing: $F(1,90) = 10.08$, $p = .002$, $\eta_p^2 = .10$, with higher AEA associated with lower SUDS change. Higher levels of hair cortisol were associated with higher differential SUDS responses: $F(1,72) = 6.27$, $p = .015$, $\eta_p^2 = .07$. There were no other associations between SUDS ratings and other biological measurements (all $p > .05$). Further, only post-film salivary 2-AG was associated with cortisol responses following film viewing: $F(1,86) = 4.81$, $p = .031$, $\eta_p^2 = .05$, such that higher salivary 2-AG was associated with higher cortisol responses. However, this did not survive correction for multiple comparisons using FDR.

Skin conductance was recorded while participants were viewing the film clips. Skin conductance was analysed in three different ways: (1) overall skin conductance level throughout the film clip, (2) skin conductance variability throughout the film clip, and (3) rate of increase or decrease (i.e., slope) of skin conductance throughout the film clip. More stressed participants should have higher skin conductance levels, more variability throughout the film, and smaller slope of skin conductance level, indicating lower rate of reduction/habituation of skin conductance throughout the film viewing. Hair AEA, 2-AG, and cortisol levels were not significantly associated with skin conductance level during the film (all *ps* > .2); however, higher hair PEA was associated with lower skin conductance variability: $F(1,72) = 4.06$, $p = .048$, $\eta_p^2 = .05$ and level: $F(1,72) = 3.52$, $p = .065$, $\eta_p^2 = .05$ during the film, though the latter was not significant. Similarly, hair OEA was associated with overall skin conductance level ($p = .070$) as well as variability ($p = .072$) during the film, though these effects are tentative as they did not reach significance. Of the salivary measures, higher post-film PEA was associated with lower skin conductance slope: $F(1,89) = 3.92$, $p = .051$, $\eta_p^2 = .04$ (not significant), whereas higher post-film cortisol was positively associated with higher skin conductance level: $F(1,94) = 5.43$, $p = .022$, $\eta_p^2 = .05$ as well as

reduced skin conductance slope: $F(1,94) = 11.41$, $p = .001$, $\eta_p^2 = .11$. No other significant effects were observed.

[INSERT FIGURE 4 HERE]

Association with fear conditioning

The full data from the fear conditioning and intrusive memory paradigm will be reported elsewhere, since these results were part of a larger project validating the use of this paradigm in humans. In the full data, an additional conditional stimulus was used in all phases of the experiment, though this manipulation was part of a research question unrelated to the current data. Otherwise, the conditioning procedure follows established methods in this emerging paradigm (e.g., Franke et al., 2021; Wegerer et al., 2013). Further, since salivary measures of endocannabinoids were not proximal to fear conditioning, no analyses were conducted between these variables.

Baseline spontaneous skin conductance fluctuations

There was an average of 16.8 ($SD = 11.5$) spontaneous skin conductance fluctuations during the baseline physiology recording (immediately prior to fear conditioning). Analytes measured in Time 2 saliva samples and hair samples were all not significantly correlated with the number of spontaneous fluctuations during baseline (all $ps > .1$).

Fear conditioning

AEA. Mixed model ANOVAs revealed that hair AEA levels were not significantly associated with CS type, trial, or the CS type × trial interaction during habituation, fear acquisition, extinction, renewal, or reacquisition (all $p > .1$).

2-AG. Higher hair 2-AG levels were significantly associated with lower SCRs to the CS- during both extinction: $F(2,134) = 4.19$, $p = .017$, $\eta_p^2 = .06$ (Note: result does not survive

FDR correction for multiple comparisons) and renewal: $F(2,134) = 4.56$, $p = .012$, $\eta_p^2 = .06$ (Figure 5), suggesting better retention of safety learning and/or better generalisation of safety learning in the extinction context in participants with high hair 2-AG. Higher hair 2-AG was significantly associated with lower SCRs during re-acquisition: $F(1,67) = 9.69$, $p = .003$, $\eta_p^2 = .13$, but not learning effects (all $p > .05$). There were no significant 2-AG effects during acquisition or habituation (all $p > .05$).

OEA. There were significant negative relationships between hair OEA and SCRs during habituation: $F(1,68) = 4.06$, $p = .048$, $\eta_p^2 = .06$ (result does not survive FDR correction for multiple comparisons), acquisition: $F(1,68) = 7.43$, $p = .008$, $\eta_p^2 = .10$, extinction: $F(1,68) = 11.44$, $p = .001$, $\eta_p^2 = .14$, renewal: $F(1,68) = 6.75$, $p = .011$, and reacquisition: $F(1,68) = 11.46$, $p = .001$, $\eta_p^2 = .14$.

PEA. Hair PEA was negatively associated with SCRs during habituation: $F(1,68) = 3.86$, $p = .054$, $\eta_p^2 = .05$ (not significant), acquisition: $F(1,68) = 7.24$, $p = .009$, $\eta_p^2 = .10$, extinction: $F(1,68) = 8.12$, $p = .006$, $\eta_p^2 = .11$, renewal: $F(1,68) = 5.83$, $p = .018$, $\eta_p^2 = .08$ (result does not survive FDR correction for multiple comparisons), and reacquisition: $F(1,68) = 10.25$, $p = .002$, $\eta_p^2 = .13$.

There were no conditioning effects for either OEA or PEA (all $p > .05$).

Cortisol. There was a non-significant negative relationship between hair cortisol and SCRs during extinction: $F(1,68) = 3.58$, $p = .063$, $\eta_p^2 = .05$ and a negative effect during renewal: $F(1,68) = 4.46$, $p = .038$, $\eta_p^2 = .06$ that did not survive FDR correction for multiple comparisons, but no effects during habituation, acquisition or reacquisition (all $p > .05$).

[INSERT FIGURE 5 HERE]

Associations with anxiety and depression, perceived mood, and intrusive memories

There were no significant associations between hair AEA, 2-AG, OEA, PEA, or cortisol with any of the questionnaire data, or the number of reported intrusive memories (all $p > .1$). Saliva endocannabinoid concentrations were not expected to be associated with these outcomes due to their fast-acting responsiveness, and when tested this assumption was upheld (all $p > .1$).

Discussion

The current study tested the relationship between salivary and hair levels of endocannabinoids and stress reactivity after watching a trauma film and during fear conditioning. Age and sex were both predictors of endocannabinoid concentrations, and endocannabinoid measurements were closely positively correlated within, but not between, biological matrices. Higher salivary 2-AG and AEA, as well as lower hair cortisol, were significantly associated with lower subjective distress following film viewing, and higher salivary 2-AG was associated with higher saliva cortisol responses to the film. While higher hair PEA and OEA were significantly associated with lower SCRs throughout the fear conditioning paradigm, higher hair 2-AG levels were associated with lower CS- but not CS+ responses during both extinction and renewal. These findings provide new insights into the relationships between endocannabinoids and stress, as well as how hair and salivary endocannabinoids are characterised within individuals.

The research field of endocannabinoids is rapidly evolving. The recognised roles of these critical lipid mediators have been identified in an increasing number of biological functions over the past decade, and the number of disease states that the endocannabinoid system has been proposed to be involved in has also increased (Di Marzo & Petrosino, 2007; Ligresti et al., 2016; Meccariello, 2020). We previously showed that endocannabinoids can be accurately and sensitively quantified in human saliva using mass spectrometry (Ney et al.,

2020) and there are now a number of published methods allowing endocannabinoid quantification in human hair (Gao et al., 2020; Krumbholz et al., 2013; Mwanza et al., 2016; Ney, Felmingham, et al., 2021; Voegel et al., 2021). Knowledge of what endocannabinoids within these matrices indicate is slowly growing, with recent research suggesting that salivary endocannabinoids are sexually dimorphic and stress responsive (Haviv et al., 2022; Ney, Stone, et al., 2021), and hair endocannabinoids are associated with various mental disease states (Croissant et al., 2020; Koenig et al., 2018; Tam et al., 2021; Voegel et al., 2022; Walther et al., 2023; Wilker et al., 2016).

The data presented in the current study add substantially to our existing knowledge concerning the characteristics and role of endocannabinoids in saliva and hair samples. Firstly, we replicated previous research that found ethanolamide-based salivary endocannabinoids – but not 2-AG – are higher in males compared to females (Haviv et al., 2022; Ney, Stone, et al., 2021). Conversely, hair endocannabinoid concentrations were recently reported to be higher in females compared to males in a sample of over 700 participants (Gao et al., 2022), which was replicated in the present data. Although the opposing hair and saliva endocannabinoid sex-based analyses in the current study are both replications of existing research, there is still not a satisfactory explanation for why this effect is observed. Future research that identifies the cause for this difference may produce a breakthrough in our understanding of endocannabinoid metabolism and synthesis between the sexes. We found the first evidence that salivary endocannabinoids may increase with age, which replicates some previous data from plasma samples where AEA, 2-AG and OEA tended to be higher in older participants (Fanelli et al., 2012). These findings show that hair and saliva samples show distinct profiles that can discriminate between age and sex. However, it is critical that future studies elicit the tonic stability of salivary

endocannabinoids, both through circadian rhythm studies and through test-retest studies over short periods of time where no stimulation is anticipated.

As has been previously reported, our study showed that both hair and salivary endocannabinoids were highly and positively correlated within, but not between, sample matrices (Fanelli et al., 2012; Gao et al., 2022; Ney, Stone, et al., 2021). This suggests that hair endocannabinoid levels are not related to salivary endocannabinoid levels (at least when only one salivary measure is assessed), which raises the question as to the driving factor of individual variability in salivary endocannabinoids. It is possible that enzymic differences in the biosynthetic pathway or difference in arachidonic acid tone explain individual differences between endocannabinoid measurements; however, neither of these possibilities have been tested to date. We previously found that salivary endocannabinoids were not correlated with plasma endocannabinoids before and after a psychosocial stress task (Ney, Stone, et al., 2021); however, this result needs replication as those samples were measured on a less sensitive instrument compared to the present study and many of those values were below the limit of detection. Further, no study to date has tested the relationship between hair and blood levels of endocannabinoids, which may show closer correlations compared to saliva.

The endocannabinoid system has been shown in a vast, international series of clinical as well as preclinical studies to be a key driver of stress reactivity as well as emotional and fear memory consolidation (Balsevich et al., 2017; Hill et al., 2018; Hill & Tasker, 2012; Laricchiuta et al., 2013; Morena et al., 2016). The present data suggest that salivary endocannabinoids (2-AG and AEA) are significantly and negatively correlated with subjective ratings of distress after viewing violent media, and that higher salivary 2-AG is correlated with higher cortisol responses to this media. However, the 2-AG results did not survive corrections for multiple comparisons and need replication. Higher levels of hair PEA were associated with lower skin conductance level during the film, whereas higher saliva

cortisol was associated with higher skin conductance level during the film. This again suggests that salivary endocannabinoids – in particular PEA and potentially also 2-AG – are valid markers of stress reactivity (Ney, Stone, et al., 2021). However, our violent film was not sufficiently stressful to invoke a genuine biological stress response in the majority of our participants, and we did not collect a saliva sample immediately after film viewing. Given that salivary endocannabinoids appear to be rapidly responsive to stress (Ney et al., 2020; Ney, Stone, et al., 2021), this means that we were unable to properly gauge whether endocannabinoids in the current study responded to the film itself. This should be the topic of future studies.

The current study also tested the relationship between hair endocannabinoid levels and performance on a fear conditioning task. Interestingly, we found that higher hair levels of 2-AG were associated with increased retention of safety learning and/or generalisation of safety learning during extinction and renewal, whereas higher hair levels of OEA and PEA were associated with lower overall physiological reactivity throughout the experiment, which also appeared to be the case during film viewing. Higher hair cortisol was also associated with lower physiological reactivity, but not learning, during extinction and renewal. These findings are somewhat consistent with proposals developed in the animal literature; namely, that extinction learning and retention require specific activation of the cannabinoid receptor 1 (Hill et al., 2018; Marsicano et al., 2002; Ney, Matthews, et al., 2021). OEA and PEA do not interact directly with cannabinoid receptors, whereas 2-AG does (Ligresti et al., 2016; Suguira et al., 1995). Therefore, we would not expect a direct relationship between OEA, PEA, and learning during fear conditioning. However, OEA and PEA are involved in stress, anxiety and depression, and the current study supports the notion that they are a marker for physiological reactivity during a mildly anxiety-inducing task (Crupi et al., 2013; De Gregorio et al., 2019). Overall though, there was not compelling and consistent evidence that

2-AG was associated with fear conditioning throughout the task, so the above hypotheses are only partially supported. There were no effects of hair cortisol or hair AEA during fear conditioning in the present data, and none of the biological markers was associated with baseline skin conductance spontaneous fluctuations prior to the experiment. Further, the salivary endocannabinoid measurements in the current study were not proximal to the fear conditioning segment of the experiment and we did not conduct analyses between these variables. This was a main limiting factor of the current experimental design. Future research should collect saliva samples during and immediately following fear conditioning to test the relationship between salivary endocannabinoids and fear conditioning.

Although some (but not all, see Gao et al., 2022) previous studies have found relationships between endocannabinoids and responses to mood and depression questionnaires (Walther et al., 2023), in the present study we did not find any significant relationships between these measures. We also did not observe any relationship between hair endocannabinoid concentrations and the number of intrusive memories reported by participants. However, since the paradigm we used was not a standard intrusive memory experiment, these findings may not generalise to more conventional methods. Replication is needed in a larger sample and using a standard trauma film paradigm. Importantly, d4-OEA was used as a surrogate labelled standard for PEA, since we did not have a labelled PEA standard at the time of analysis. Although chemically this choice was justifiable, it is possible that these results are not entirely accurate since this approach has not been previously validated. The study is also limited by the fact that time-of-day data was not collected. Although recruitment was limited to a time period that controls for cortisol circadian rhythm, endocannabinoids in blood show fluctuation during this time period (Hanlon et al., 2020; Hanlon et al., 2015, 2016). Since endocannabinoids in blood samples do not correlate with endocannabinoids in saliva (Ney et al., 2021), it is unlikely that the circadian rhythm will be

the same; however, due to lack of data on this and the absence of time-of-day data in the current study this is a limiting factor for the present analyses. Similarly, endocannabinoid concentrations in human biofluids are affected by body-mass index (BMI, see Hillard, 2017) and this data was not collected in the present study. Future studies will need to collect data and control for secondary variables such as BMI. Other secondary variables that should be examined include clinical status, as it is unknown whether salivary endocannabinoid tone and stress reactivity are differentially affected by presence of a psychiatric diagnosis.

In summary, we found some evidence suggesting that hair 2-AG levels may be associated with retention of safety learning during fear conditioning. Our findings also add substantially to our knowledge of the characteristics of saliva endocannabinoids, with age, sex, physiological, and stress effects identified. Future studies should test the relationship between salivary endocannabinoids and fear conditioning by including proximal saliva samples and test the relationship between hair endocannabinoids and intrusive memories in a standard trauma film task.

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Figure Captions

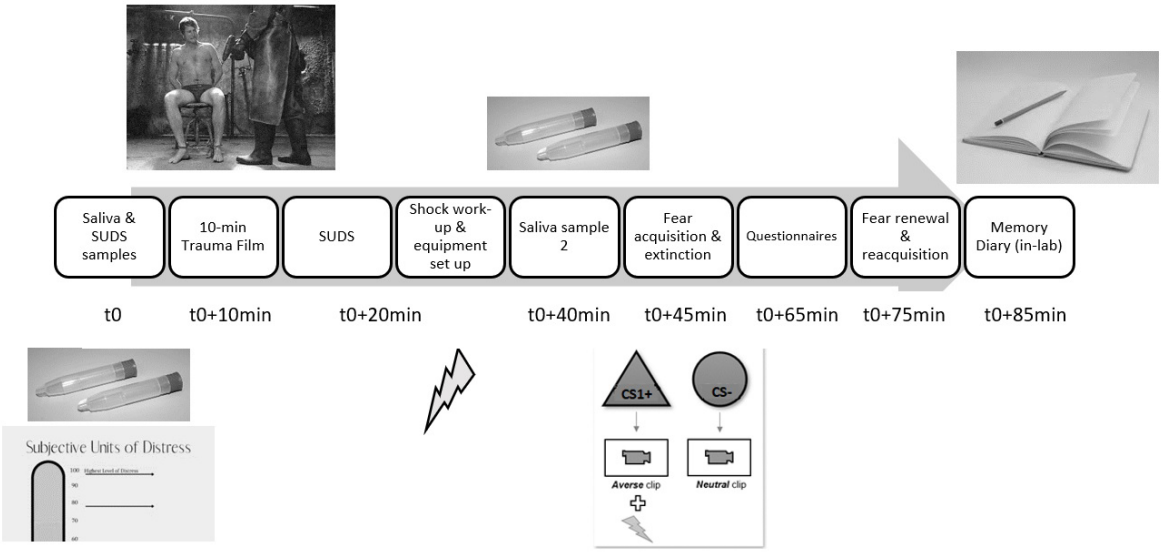

Figure 1. Schematic representation of the experiment. Participants provided saliva samples before and 20-minutes following watching a 10-minute trauma film. Subjective Units of Distress Scale responses were recorded before and immediately after the trauma film. Fear acquisition, renewal and reacquisition were conducted in Context A and extinction in Context B. Memory diary was completed in lab following renewal and reacquisition, as well as at the end of each day for 4 days following the experiment.

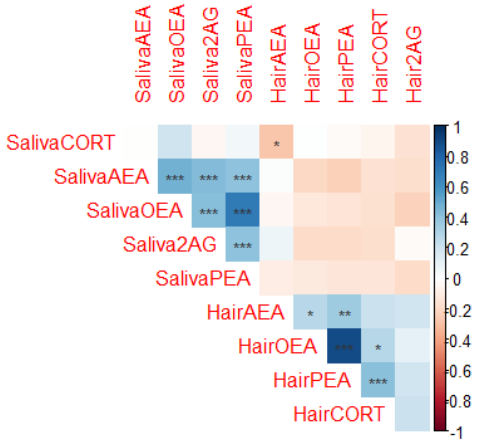

Figure 2. Baseline salivary AEA, 2-AG, OEA, PEA, and cortisol were correlated with each other but not hair levels of these analytes. ***p < .001, **p < .01, *p < .05.

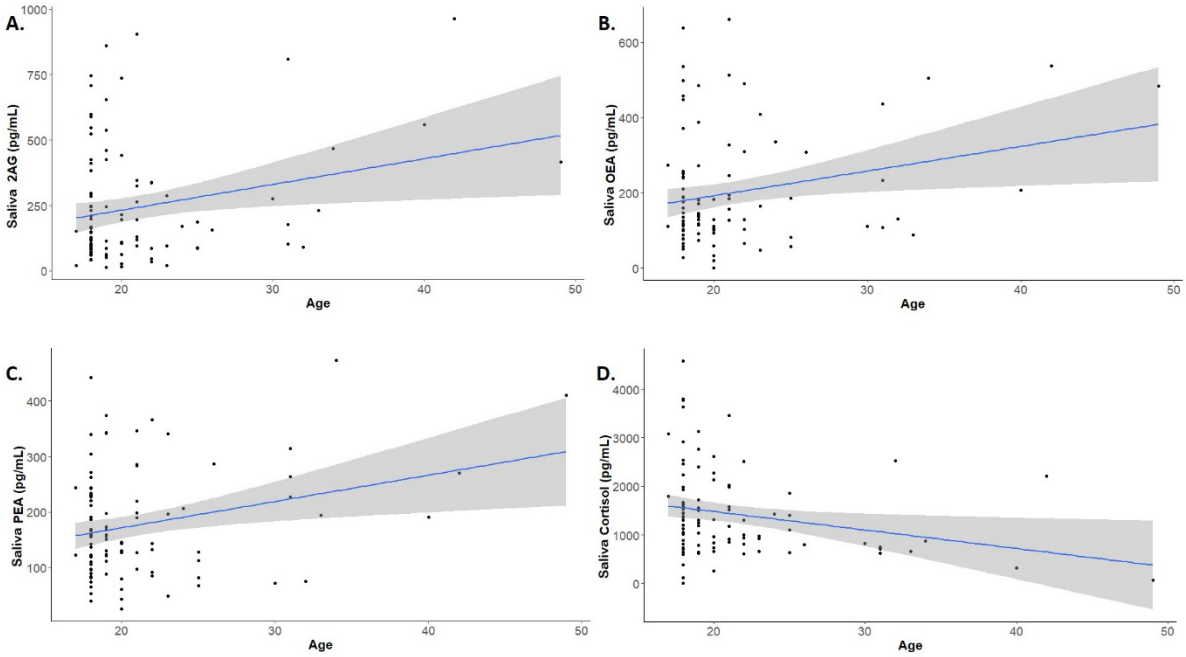

Figure 3. Baseline salivary 2-AG (Panel A), OEA (Panel B), and PEA (Panel C) were significantly higher with older age, whereas baseline salivary cortisol was significantly lower with older age (Panel D). Band is 95% confidence interval.

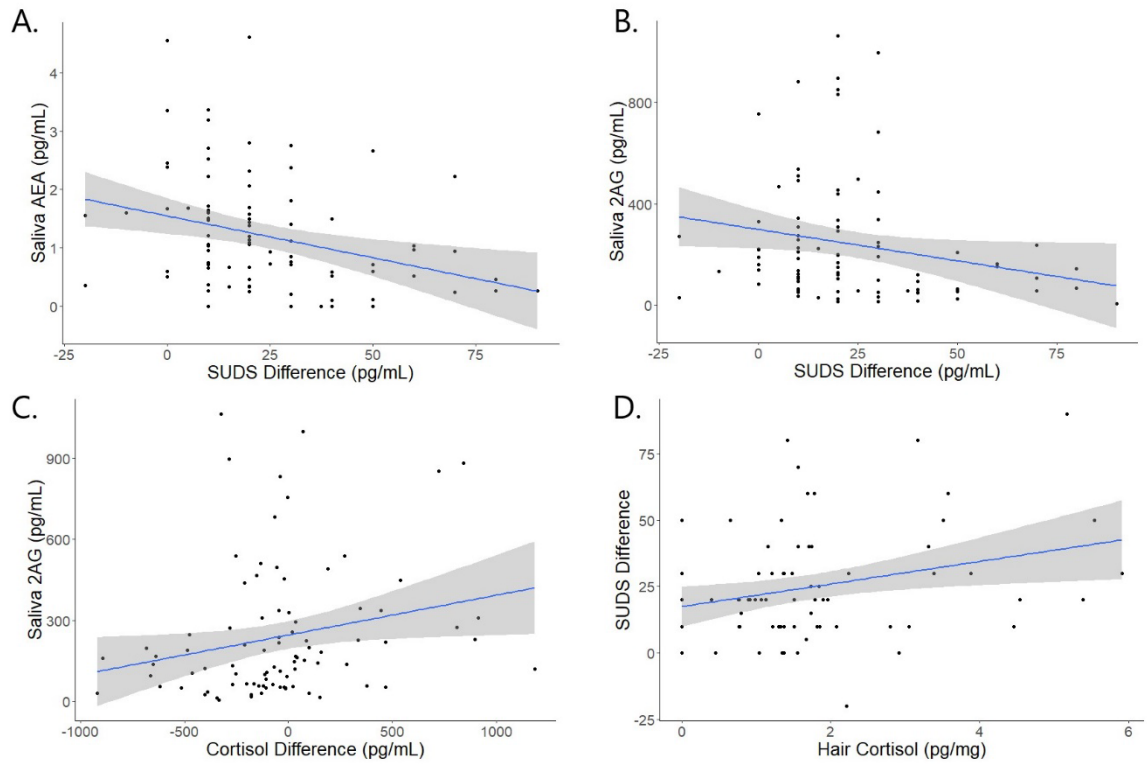

Figure 4. Post-stress salivary AEA (Panel A) and post-stress salivary 2-AG (Panel B) were significantly negatively associated with subjective distress in response to trauma film viewing. Conversely, higher difference between baseline and post-stress salivary cortisol levels were associated with higher post-stress salivary 2-AG levels (Panel C). Higher hair cortisol level was associated with higher distress to the film (Panel B). Post-stress 2-AG and AEA were used in the analysis rather than

the baseline to post-stress difference because no time-difference was anticipated or observed. Band is 95% confidence interval.

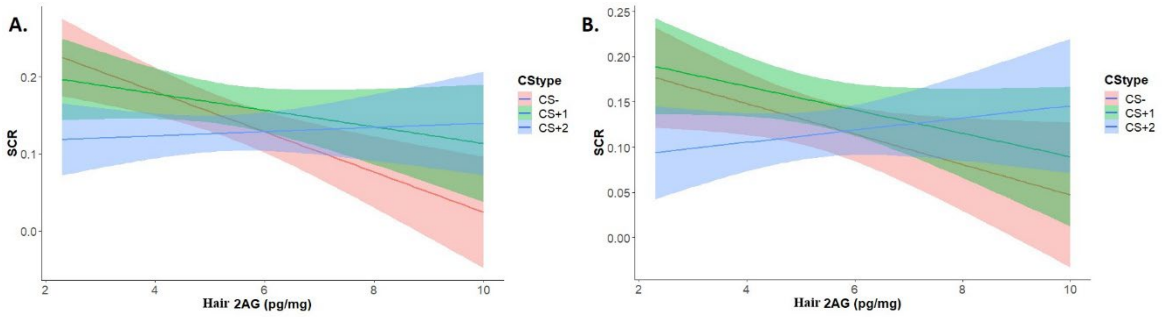

Figure 5. Higher hair levels of 2-AG are associated with significantly better retention of safety learning during extinction (Panel A) and significantly lower return of fear during renewal towards the safety stimulus but not the threat stimulus (Panel B). SCR = Skin conductance response. Band is 95% confidence interval.