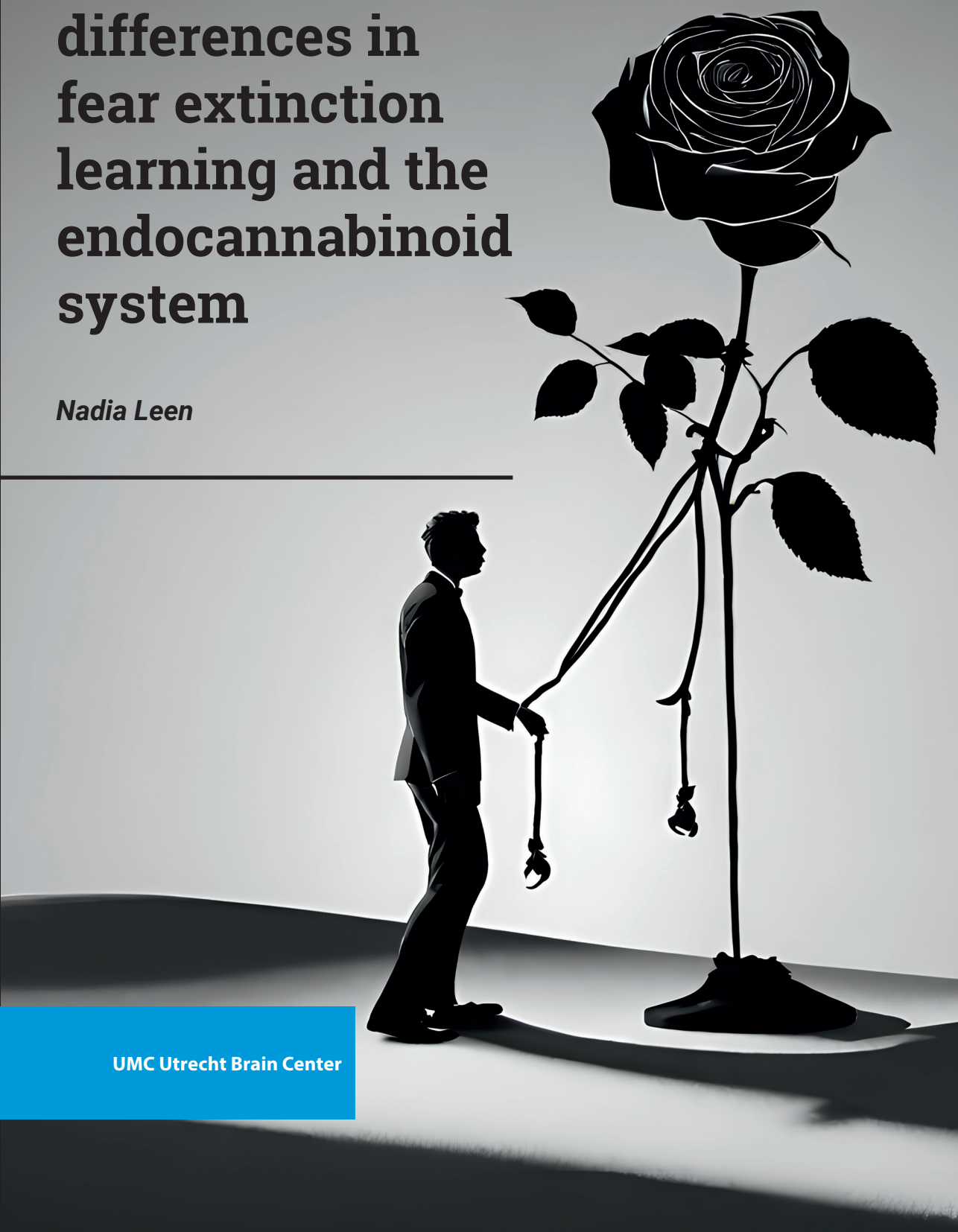
Individual differences in fear extinction learning and the endocannabinoid system

Nadia Leen



UMC Utrecht Brain Center

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Individuele verschillen in angstuitdoving en het endocannabinoïdensysteem

Nadia Leen

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'Indeed as I learned, there were on the little prince's planet – as on all planets – good plants and bad plants. Therefore, there were good seeds from good plants, and bad seeds from bad plants. But seeds are invisible. They sleep deep in the heart of the earth's darkness, until one is seized with the desire to awaken. Then it will stretch itself and begin timidly to push a little sprig inoffensively upward toward the sun. If it is only a sprout of radish or the spring of a rose-bush, one would let it grow. But when it is a bad plant, one must destroy it as soon as one recognizes it.'

Antoine de Saint-Exupéry, The Little Prince

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CHAPTER 1

Introduction

Anxiety, trauma- and stressor related disorders (collective referred to as anxiety related disorders hereafter) are prevalent, not only in the Netherlands but also in the rest of the world. The prevalence of anxiety related disorders across the world is estimated between 2.5-7%, making it one of the most prevalent mental health disorders[1]. It is estimated that around 33.7% of adults experience any anxiety related disorder at some point in their lives[2]. Within certain professions, for example in the military, individuals are more at risk to develop these disorders because they are at higher risk of exposure to stressful and sometimes even traumatic situations. For example, six months after military deployment 8.6% of veterans displayed post-traumatic stress disorder (PTSD) symptoms[3]. Getting more insight into the development, maintenance and treatment of anxiety, particular for people who have a higher chance of being exposed to stressful and traumatic events, can help to prevent individuals from the development of these disorders and may help to find new ways to improve treatment response. This is particularly necessary, as current psychological and pharmacological treatments are insufficient for approximately 40% of patients[4,5].

The aforementioned observations raise a couple of interesting questions with regard to the development, maintenance and treatment of anxiety related disorders. First, what mechanism lies beneath in the development of anxiety in some, but not other individuals? Second, can we predict who will respond to certain kind of treatments? Third, if patients do not respond to certain treatments do alternative therapies exist that can be used to treat these patients? Although there are several different systems underlying the development, maintenance and treatment of anxiety related disorders, one specific neuromodulatory system has gained more interest over the last 20 years[6]. This neuromodulatory system is thought to be critical in the extinction of unwanted fear memories, namely the endocannabinoid system[7]. In addition, today the endocannabinoid system is seen as a promising new target in the treatment of anxiety related disorders[7,8].

Fear conditioning and extinction learning

One of the golden standards in the field of anxiety related disorders is the fear conditioning and extinction paradigm. Conditioning was first described by Ivan Pavlov in 1897 and is nowadays known as the classical conditioning paradigm[9]. In Pavlov's famous experiment with dogs, the sound of a bell was accompanied with the presentation of food. The dogs learned to associate the sound of the bell with the presentation of food. This learned association led to the production of saliva in dogs in anticipation of the presentation of food, when hearing the sound of the bell.

Based on Pavlov's experiment, John B. Watson conducted the Little Albert experiment in 1920[10]. This experiment demonstrated that emotional responses could be classically conditioned in humans. In his experiment a young child was conditioned to fear a white rat, by accompanied the presentation of the white rat with a loud sound. The fear to the white rat also generalized to similar objects like a cute little rabbit. The paradigm

evolved since it was first described by Ivan Pavlov and is now well established in both animals and humans as a model for anxiety[11]. In the fear conditioning paradigm that is currently the golden standard in human fear conditioning research a neutral cue (CS), e.g. a picture, is paired with an unconditioned, aversive stimulus (US), e.g. a shock or a loud noise[12]. This pairing will form an associative memory between the CS and US. Fear extinction can then be induced by presenting the CS without the US, leading to extinction of the fear response. Additionally, a safety stimulus (CS-; the stimulus not coupled with the US) is presented. However, although the CS- serves as a safety stimulus, some individuals may still show high fear responses during presentation of the CS-. The high fear responses to the CS- may be interpreted as fear generalization[13]. See also Figure 1 for a schematic overview of the fear conditioning procedure.

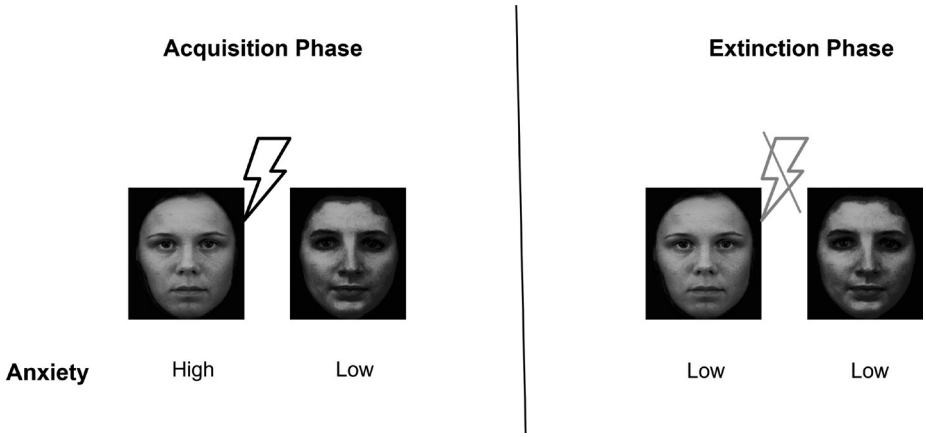

Figure 1. Schematic overview of a fear conditioning procedure.

The first-choice treatment for anxiety related disorders is based on these fear conditioning and extinction paradigms. In exposure therapy, which is often part of cognitive behavioral therapy, the patient is repetitively exposed to the feared stimuli in order to reduce the fear response[14]. However, two meta-analyses pointed out that there is a difference between healthy individuals and individuals with anxiety related disorders in their response to these fear conditioning and extinction paradigms[13,15]. Anxiety patients show a higher fear response to the CS+ during both fear acquisition and fear extinction[13]. Additionally, anxiety patients demonstrate a higher response to the CS- during acquisition[15]. This suggests the existence of two potential mechanisms for developing anxiety related disorders. First, a failure to extinguish fear because a fear once learned fails to extinguish when it is no longer predictive of an aversive outcome[11,16,17]. Second, fear generalization to stimuli that were present during conditioning, but have never been paired with the US[18].

Most of the time the fear conditioning and extinction paradigm is used to compare distinct groups (for example anxiety patients versus healthy controls). However, recently

there has been more focus on individual characterization of patterns in fear extinction learning[19–21]. Several studies have demonstrated that within different patient populations, individuals can be characterized by differences in fear (extinction) learning classes[19–21]. Which raises the question whether individuals belonging to these different classes, especially those within the maladaptive classes of poor extinction and generalization, also respond differently to current treatments for anxiety related disorders. More insight into underlying factors of poor extinction and the neurotransmitter systems involved, may help to get more insight into individual differences underlying the development of anxiety related disorders. Moreover, because the endocannabinoid system is involved in extinction learning, this system is a potential target for new pharmacotherapeutic approaches to the treatment of anxiety.

The endocannabinoid system

The endocannabinoid system is a neuromodulatory system that plays an important role in in the central nervous system and the extinction of aversive memories[7,22,23]. Additionally, it plays a role in the regulation of stress and anxiety like behavior and acts as a feedback loop to inhibit responses of the HPA-axis to stressors[24,25]. The as of yet known elements of the endocannabinoid system comprises the cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), the endogenous cannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and the catabolic enzymes for the degradation of AEA and 2-AG, fatty acid amide hydrolase (FAAH) for AEA and monoacylglycerol lipase (MAGL) for 2-AG[26]. AEA and 2-AG, are generated 'on demand', e.g. in reaction to stressful situations, and act in a retrograde manner to regulate neurotransmitter release, primarily through inhibition of GABAergic and glutamatergic neurotransmission[27–30].

In general, the endocannabinoid system is responsible for maintaining homeostasis in living, biological systems and regulating physiological processes through our endogenous cannabinoids (AEA and 2-AG)[30]. However, the endocannabinoid system can also be enhanced by exogenous compounds mimicking or strengthening the effects of AEA and 2-AG. The most well-known compounds are derived from the cannabis plant, namely Delta-9-tetrahydrocannabinol (THC) and Cannabidiol (CBD). While THC acts as a direct agonist to the CB1 receptor, one of the actions of CBD is inhibiting the FAAH enzyme which normally breaks down the endogenous cannabinoid AEA[31]. Besides the inhibition of the FAAH enzyme it is thought that CBD facilitates 5-HT1A and 5-HT2A receptor-mediated serotonergic neurotransmission, the putative cannabinoid receptor GPR55 and activate the potential vanilloid type[31–35]. In contrast to THC, CBD does not induce the numerous side-effects that can be seen with the use of THC. CBD does not induce psychomotor impairment or psychotomimetic effects[36,37]. Furthermore, CBD does not induce a change in heart rate and seems to attenuate the anxiogenic effect of THC[36–38]. These beneficial characteristics have contributed to the popularity of CBD. CBD, mainly in an oil solution, is commonly used for several different symptoms such as anxiety, sleep problems and depression, in both humans and animals[39–41]. The use of

CBD for both humans and animals for these variety of symptoms is not always evidence based. However, its popularity has led to the development of different compounds to study the inhibition of FAAH in both animal and human[42–45]. Besides these developments the question remains whether the CBD compound from the cannabis plant and synthetic developed FAAH inhibitors really improve anxiety and stress symptoms in humans.

As mentioned previously, the endocannabinoid system is activated by the release of endogenous cannabinoids (e.g. AEA and 2-AG). Studies demonstrated that blood levels of AEA and 2-AG are associated with anxiety, depression, stress and trauma symptoms in individuals with PTSD and depressive disorder[46–48]. Several studies have demonstrated differences in the endogenous endocannabinoid levels between healthy individuals and people with trauma and stressor related disorders[47,48]. Additionally, these differences in endocannabinoid levels (mostly a reduction in AEA or 2-AG in a PTSD group compared to controls) were accompanied by an upregulation of the CB1 receptor[49]. These differences in how the endocannabinoid system operates might indicate a vulnerability in individuals with PTSD or a change in the endocannabinoid system as reaction to trauma. However, much is still unclear since null-effects and opposite effects are also reported and sample sizes are relatively small[29,50]. Since preclinical studies suggest that extinction processes depend on endocannabinoid signaling, higher pretreatment endocannabinoid levels might be associated with better treatment outcome in PTSD.

When investigating whether endocannabinoids play a role in treatment response the question remains whether individual differences in the endocannabinoid system have been developed through trauma or are based on ones' genetic makeup. Besides growing interest into enhancing the endogenous endocannabinoid system by exogenous compounds, the enzyme for the degradation of AEA, FAAH, also gained in interest[51]. In general, the inhibition of FAAH mitigates anxiogenic effects of stress because it prevents reductions in AEA that normally accompany stress and anxiety[52]. The FAAH rs324420 polymorphism is an interesting candidate gene to study because of its potential protective effects on stress and anxiety during high environmental aversiveness as demonstrated in animal studies[45,52–54]. Individuals with the A-allele may be protected because this allele is associated with reduced FAAH activity, and corresponding elevated levels of AEA[55,56]. Several studies have demonstrated that this A-allele was associated with decreased self-reported anxiety, enhanced fear extinction learning and extinction recall, decreased threat related amygdala reactivity, increased fronto-amygdala connectivity, and that it protects against stress-induced decreases in AEA and negative emotional consequences of stress[57–62]. However, it is important to notice that research also suggests that FAAH inhibition is not anxiolytic per se, but that it also protects against anxiogenic effect of stress during high environmental aversiveness[45,52–54]. The association between genetic variations of the FAAH polymorphism and the development of anxiety and trauma related symptom was, however, never investigated in real life events but only in experimental settings.

Study aims and outline

This thesis about *Individual differences in fear extinction learning and the endocannabinoid system* has two closely related aims. First, to investigate individual differences in fear learning and the analysis of these differences in relation to treatment outcome for anxiety related psychopathology. Second, to investigate the role of the endocannabinoid system in anxiety related psychopathology.

In the section: *Fear conditioning and extinction learning* we investigated a newly developed fear conditioning and extinction task to separate different fear learning classes. In **Chapter 2** we tested this task in 300 healthy subjects to see whether we could distinguish between different fear learning classes. In **Chapter 3** we translated this research from a healthy to a clinical population of patients with various anxiety related disorders. In this study we investigated again whether we could distinguish between different fear learning classes and additionally if these classes were associated with treatment outcome and other clinical characteristics. We hypothesize that individuals within the maladaptive classes of poor extinction and generalization show less symptom reduction after treatment in comparison to the others classes.

In the section: *The endocannabinoid system* we investigate the role of the endocannabinoid system in anxiety related symptoms. **Chapter 4** consists of a systematic review and meta-analysis in which we investigated anxiolytic effects of compounds that can enhance the endocannabinoid system both in animals and humans. In the other two chapters we focused on functioning of the endocannabinoid system without enhancement from the outside, but by our own endogenous cannabinoids and our genetic makeup. In **Chapter 5** we investigated the endogenous endocannabinoids AEA and 2-AG and the effect of baseline levels of the endogenous cannabinoids on treatment outcome and association with clinical symptoms in veterans with PTSD. We hypothesize that higher baseline endocannabinoid levels are associated with a higher symptom reduction after treatment. In **Chapter 6** we investigated the endocannabinoid system at the level of genetics. We investigated whether the FAAH rs324420 polymorphism is related to the development of anxiety related symptoms after military deployment. We hypothesize that individuals with the A-allele, which is accompanied by increased AEA levels, would demonstrate less development of anxiety related symptoms after military deployment than individuals with the CC genotype.

I will conclude this thesis with an integration of all the findings in the last section: *Fear extinction learning and the endocannabinoid system.* In **Chapter 7** the general discussion I will focus on the integration of these two research topics. I will contemplate on how the endocannabinoid system can play a role in the understanding and treatment of anxiety related symptoms. Finally, the clinical implications and directions for future research are considered.

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CHAPTER 2

Trajectories of fear learning in healthy participants are able to distinguish groups that differ in individual characteristics, chronicity of fear and intrusions

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ABSTRACT

Background and objectives: Studies on the development and treatment of anxiety disorders mostly focus on the comparison of predefined groups. An alternative approach is to use data-driven latent class growth analyses (LCGA) to determine differentiation between groups based on particular mechanistic factors. This study validated the use of LCGA on responses in a compact fear conditioning task and whether specific characteristics are associated with maladaptive fear learning trajectories.

Methods: Healthy subjects (*N*=300) completed a fear conditioning task that included uninstructed and instructed acquisition and extinction phases. Subjective fearfulness and US expectancy were used as outcome measures. Latent classes in the responses to the CS+ (coupled with a scream) and the CS- (control stimulus) were determined based on trajectories across the experimental phases. State and trait anxiety were measured during testing, and return of fear and intrusions were measured one and six weeks later.

Results: Fear learning trajectories of poor extinction in responding to the CS+ and generalization of fear to the CS- were associated with higher state and trait anxiety. Individuals belonging to these trajectories reported more intrusions, fear and had higher US expectancy ratings after 1 week.

Limitations: Only 56% of participants completed the six weeks follow-up measures.

Conclusion: Fear learning trajectories are associated with individual characteristics, return of fear and intrusions. Next, this task will be implemented in clinical practice to assess its predictive power for the extent to which patients benefit from exposure treatments.

1. INTRODUCTION

Anxiety disorders are characterized by recurring fears or concerns[1]. Lifetime prevalence is estimated around 11.6%[2] and associated with enormous costs[3]. Many anxiety disorders have their roots in youth and will manifest in late adolescence/early adulthood[4]. The state of the art treatment for anxiety disorders is exposure therapy, a part of cognitive behavioral therapy (CBT)[5]. Although current psychological and pharmacological treatments are effective, state of the art treatments are insufficient for a subset (approximately 40%) of patients[6,7].

Theoretical understanding of mechanisms underlying the development, maintenance and treatment of anxiety disorders is largely based on classical fear conditioning in which a neutral cue (CS), e.g. a picture, is paired with an unconditioned, aversive stimulus (US), e.g. a shock[8]. This pairing will form an associative memory between the CS and US in the organism undergoing the procedure. Fear extinction can be induced by presenting the CS without the US, leading to extinction of the fear response. Exposure therapy, in which the patient is repetitively exposed to the feared stimuli, is thought to rely on this mechanism to reduce fear responses.

Clinical relevance of conditioning and extinction processes is derived from differences in how healthy people and anxiety patients respond to this kind of fear conditioning paradigm. A meta-analysis showed that anxiety patients have a higher fear response to CS+ (the stimulus coupled with the US) during fear acquisition and fear extinction[9]. However, a more recent update of this meta-analysis also demonstrated a higher response to the CS- (the stimulus not coupled with the US) during acquisition[10]. This suggests two potential mechanisms for developing exaggerated fears. The first is when a fear once learned fails to extinguish when it is no longer predictive of an aversive outcome. Accordingly, the development and maintenance of anxiety disorders has therefore been conceived as a failure to extinguish[11–13]. The second is when a fearful response to stimuli that have never been paired with an aversive consequence, such as the CS-, is developed. Elevated fear responses to the CS- index fear generalization, as they reflect a tendency to generalize fear responses to stimuli that are present during conditioning, but are never paired with the US. This conceptualization of generalization follows the description in Pavlov's 1927 lecture VII[14] of the initial generalization of conditioned responses to other stimuli that are present when conditioning starts (including the environment)[15]. In case this generalized response to the CS- is maintained throughout the experiment, this can be conceptualized as impaired safety learning. Generalization of fear responses has been suggested as an additional mechanism that poses a risk factor for the onset of anxiety disorders[16]. In sum, failure to extinguish and generalization of fear may play a role in the etiology of anxiety disorders. Moreover, considering the role that fear extinction plays in the beneficial effect of exposure therapy, failure to extinguish may be associated with a poorer treatment outcome.

Research into how anxiety patients may differ from healthy controls with respect to fear conditioning parameters has mainly focused on the comparison between these two groups. Another approach is to look at heterogeneity in data across individuals within a particular group of patients[17]. Recently, data-driven classification of subjects has been applied to fear conditioning data using latent class growth analyses (LCGA). A study showed that in PTSD patients patterns of conditioned startle responses can be divided into three classes: rapid extinguishers, slow extinguishers and non-extinguishers[18].

A similar classification was found in a previous fear conditioning study from our group that included anxiety patients who were assessed prior to therapy and healthy matched controls[19]. LCGA on the subjective ratings of fearfulness revealed three different classes for responding to the CS+: normal conditioners (50%), low fearful conditioners (32%) and poor extinguishers (18%). The latter group showed failure to extinguish fear, even after having received specific instructions that the shock would no longer come. Patients were also three times more likely than control subjects to be characterized as poor extinguisher[19]. A group that reported exaggerated fear in responding to the CS- throughout the experiment that was labeled as fear generalizers also included an overrepresentation of patients (39% of the patients versus 19% of controls). The subjective outcome measure of shock expectancy had a similar pattern of results, but the physiological fear-potentiated startle did not reliably differentiate between different classes of responders.

These results suggest that data driven analysis can reveal maladaptive fear learning trajectories that are clinically relevant because patients are more likely to display trajectories of poor extinction and generalization. The current study is aimed at replicating the trajectories found by Duits et al. (2021)[19] with a adapted version of the same fear conditioning task that is optimized with respect to ease of administration. A practical implication of the previous results is that subjective measurements appear to be more sensitive than physiological fear responses in picking up different classes[19]. The focus on subjective measures allows for shorter stimulus duration and intervals, and makes the task much shorter. To make the experiment even easier to apply, e.g. in a clinical setting, the electric shock (US)[19] is replaced by a loud female scream[20].

This study was conducted in a healthy population, with the primary aim to replicate the latent classes previously observed with a shorter fear conditioning task that uses only subjective measures[19]. The subsidiary aim was to investigate whether specific characteristics (e.g. state and trait anxiety are associated with the (mal)adaptive fear learning classes[21]. In order to examine the predictive validity with regard to clinically relevant parameters, follow-up measures of return of fear and intrusions were taken at one and six weeks after the experiment.

2. METHOD

2.1. Participants

Healthy participants (*N*=306) recruited amongst students at Utrecht University campus participated in the study. Exclusion criteria included self-reported neurological, cardiovascular and/or psychiatric disorders, use of psychoactive medication, and hearing problems. Analyses were conducted on 300 participants (female *N*=218, male *N*=83; age *M*=21.98, *SD*=3.16) because six participants did not finish the experiment. The study was approved by the Ethics Review Board of the Faculty of Social Sciences of Utrecht University.

2.2. Procedure

All participants were informed about the study and gave written informed consent. Participants then completed a couple of questionnaires and a fear conditioning task on a computer. One and six weeks after visiting the lab participants completed an online follow-up questionnaire. The entire procedure lasted 30 min. Participants received €3,- or course credits for participation.

2.3. Questionnaires

Trait and state anxiety were assessed with the State-Trait Anxiety Inventory (STAI-DY, the Trait part)[22] and a 6-item version of the state anxiety (STAI-6)[23].

2.4. Fear conditioning task

The fear conditioning task was adapted from Duits et al. (2021)[19]. The conditioned stimuli (CS's) were two female faces with a neutral facial expression displayed in either a blue or green color against a black background (http://pics.stir.ac.uk). There were two different versions of the task with a fixed order that was counterbalanced to prevent differences in CS-US learning. During the acquisition phases, the first and last CS+ were coupled with the US and no more than two of the same stimuli were presented consecutively. A trial consisted of one of the CS pictures shown for 4 s and the ITI screen for 2 s. A female scream (95 dB(A), duration 1s) was used as unconditioned stimulus (US) (adapted from Lau et al., 2008)[24] and presented through headphones 1,5/2,5 s after stimulus onset. The male faces used by Duits et al. (2021)[19] were replaced by female faces because of the female scream that was available for the experiment, and stronger acquisition of fear is expected when CS-US belongingness is stronger[25]. The task consisted of 5 phases (consisting of 2 blocks (except the pre-acquisition phase); ±1.5 min per block): pre-acquisition, acquisition (uninstructed and instructed) and extinction (uninstructed and instructed), see Fig. 1. During pre-conditioning and extinction phases, the CS's were presented in the absence of the US. In both acquisition phases, one of the two faces (CS+) was followed by the scream (6 out of 8 trials). Prior to each phase, a text screen was displayed. In the uninstructed phases, it contained a reminder to pay attention to the faces on the screen, but no explicit information about the CS-US relation. In the instruct-

ed phases the text screen included instructions about the CS-US contingency. Halfway acquisition (after two acquisition uninstructed blocks) they were presented with the CS + face and the text "The scream sound is only played during the presentation of the image presented above". Halfway extinction (after two extinction uninstructed blocks) the instruction was "You will no longer hear the scream in the next phase". After each block participants rated questions on a computerized VAS scales (range 0–100): How anxious/nervous the participants were in the displayed condition (fearfulness), whether they thought the occurrence of a scream was very unlikely/likely (US expectancy), how aversive they rated the scream (US) and how certain they were of their answers. The task was programmed using OpenSesame version 26[26].

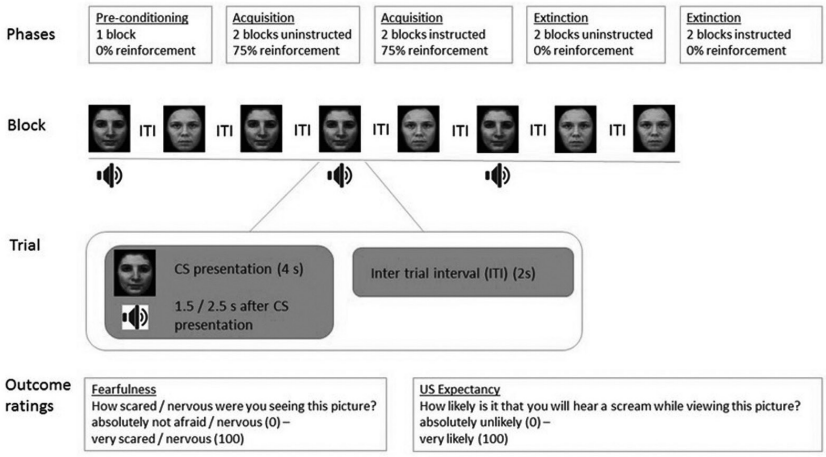

Fig. 1. Overview of the fear conditioning task. Each block consists of four CS- stimuli and four CS + stimuli.

2.5. Follow-up assessment

One and six weeks after the experiment participants completed an online questionnaire. They were asked to rate 'fearfulness' and 'US expectancy' as in the experiment when showing the two pictures from the experiment, see Fig. 1; outcome ratings. Intrusions were measured using 4 questions from the Impact of Events Scale[27,28]:'I thought about it when I didn't mean to', 'Pictures about it popped into my mind', 'I tried not to think about it' and 'Any reminder brought back feelings about it.'

2.6. Data analysis

Latent class growth analyses (LCGA) was conducted in MPlus (version 6.12)[29]. LCGA is a data-driven approach to investigate latent homogenous classes within a larger heterogeneous sample. This analysis characterizes individual differences in parameters reflecting participants' change in outcomes over time. Individuals are classified into latent classes based upon similar patterns over time[30,31]. LCGA was conducted with

one data point per block from the fear conditioning task for pre-acquisition and two for acquisition (uninstructed and instructed) and extinction (uninstructed and instructed), see Fig. 1. Analyses were conducted per outcome measure (fearfulness and US expectancy) and stimulus type (CS+ and CS-) because this allows separate analysis of the acquisition and extinction of the fear response to the CS+ and the responses to the CS-. These show strong variability across individuals, which we interpret as differences in the extent to which the fear evoked by the CS + generalizes to the CS-. The sample size of 300 participants was based on the recommendation by Zhang and Wang (2009)[32] who advise a sample size between N = 210 and N = 270 when using growth curve models to obtain a power of .8. The reliability of the loglikelihood estimation random sets starting value was set at 800 and the number of final optimizations at 200[33]. Model fit was compared between models with 1–6 trajectories, and model selection was based on three criteria: 1) Apparent drops in Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC)[34]; 2) A large entropy score and 3) The smallest number of classes that would still be theoretically meaningful[35]. These criteria were based on results from Galatzer-Levy et al. (2017)[18] and most closely followed Duits et al. (2021)[19]. Participants were assigned to the class for which they had obtained the highest probability according to the best fitting model.

Chi-square tests and one-way ANOVAs were applied to determine differences in characteristics between individuals assigned to the different classes (gender, age, STAI-state, STAI-trait, US aversiveness, certainty). To allow analysis of all the data acquired at the follow-up (larger attrition), one-way ANOVA's were conducted separately for the one and six week follow-up. Group differences in intrusions (section 3.5.1) were tested for all models for both outcome measures (fearfulness and US expectancy), and on both CS+ and CS- responses. Analyses of group differences in subjective fearfulness (section 3.5.2) and US expectancy (section 3.5.3) at follow-up were restricted to the classes that were based on the responses to that measure (fearfulness/US expectancy) in that experimental condition (CS+/CS-). Finally, repeated measure ANOVA's on possible time x class interaction effects are reported in the supplementary data. P-values were considered statistically significant at <0.05 and post-hoc Bonferroni correction was used to test the differences between groups. Analyses were carried out with IBM SPSS Statistics (version 23).

3. RESULTS

3.1. Classes based on the subjective fearfulness ratings to the CS+

LCGA demonstrated three distinct classes on CS + fearfulness rating, see Fig. 2 and Table 1. The selection of the 3-class model was based on a combination of substantial drops in BIC and AIC scores from the 2nd to the 3rd class model, peak in entropy scores, and congruency with previous studies. The largest class (56%, *N*=170) was labeled 'normal conditioners' and was characterized by an increase in fearfulness scores

during acquisition that decrease again during extinction phases. The second largest class labeled 'low fearful conditioners' (32%, *N*=95) reported low fearfulness scores during all phases. The third class labeled 'poor extinguishers' (12%, *N*=35) was primarily characterized by an sustained fearfulness to the CS+ during extinction phases, even after explicit instructions that the CS + would no longer be followed by the US. Statistical tests across classes per phase are reported in the supplementary data.

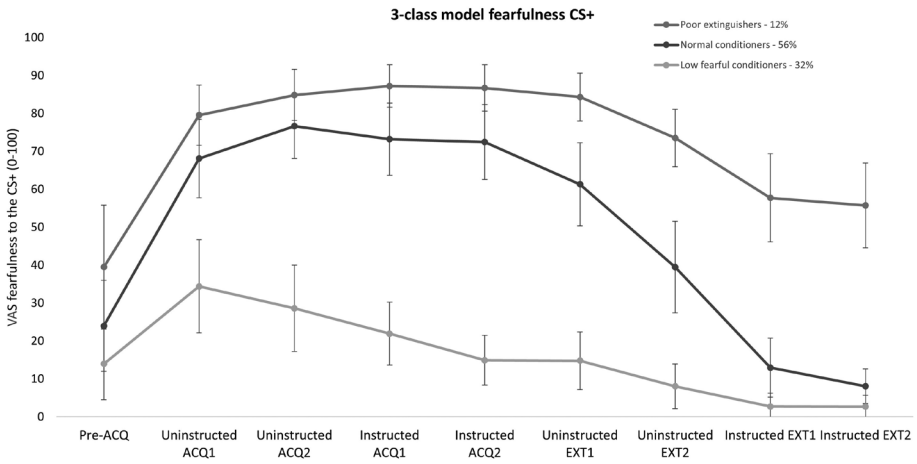

Fig. 2. Estimated means and standard deviation of the final model on fearfulness ratings to the CS+ in the 3-class model. ACQ1 = acquisition block 1; ACQ2 = acquisition block 2; EXT1 = extinction block 1; EXT2 = extinction block 2.

Table 1. Fit indices for one-to six class Latent Growth Models based on fearfulness ratings on the CS+.

No. of classes	*AIC*	*BIC*	*Entropy*	*Sample size per class based on most likely class membership*
1	25488	25555	NA	300
2	-1209	-1173	.959	90/203
3	**-502**	**-464**	**.975**	**95/170/35**
4	-215	-179	.918	92/114/69/25
5	-156	-119	.936	111/42/93/33/21
6	-108	-70	.922	74/35/35/94/21/41

AIC = Akaike Information Criterion; BIC = Bayesian Information Criterion; NA = not applicable. AIC and BIC values for 2 classes and up are expressed as reduction with respect to the model with one class less. Best fitting model values are displayed in bold.

3.1.1. Group characteristics fearfulness CS + classes

Classes differed significantly in gender, state and trait anxiety and US aversiveness, see Table 2. Women are more often categorized as normal conditioners than men, and men

are more often categorized as low fearful conditioners. Post-hoc testing revealed that low fearful conditioners reported lower state anxiety than both normal conditioners and poor extinguishers (both, p=<.001). For trait anxiety low fearful conditioners reported a lower score than normal conditioners (p=.009) and poor extinguishers (p=<.001). Poor extinguishers rated the US as more aversive than normal conditioners (p=.005) and low fearful conditioners (p=<.001). Normal conditioners reported a higher US aversiveness than low fearful conditioners (p=<.001).

Table 2. Characteristics of participants assigned to the classes on fearfulness ratings to the CS+

	Normal conditioners	**Low fearful conditioners**	**Poor extinguishers**		
	***(n*=170)**	***(n*=95)**	***(n*=35)**	**Test Statistic**	***V/η²***
Gender	**(%total)**	**(%total)**	**(%total)**		
Men	37.80%	52.40%	9.80%	$\chi^2(2) = 22.81$, p=<.001*	.276
Women	63.80%	23.90%	12.40%		
	M (SD)	***M (SD)***	***M (SD)***		
Age	21.95 (2.60)	22.08 (4.27)	21.86 (1.96)	$F(2,299) = 00.08$, p=.920	.00
STAI-state	11.40 (2.86)	10.00 (2.35)	12.34 (3.40)	$F(2, 299) = 11.92$, p=<.001*	.26
STAI-trait	37.65 (8.95)	34.20 (7.72)	41.11 (12.09)	$F(2, 299) = 08.68$, p=<.001*	.24
Mean aversiveness	80.41 (15.70)	58.21 (23.65)	91.19 (10.15)	$F(2, 299) = 62.13$, p=<.001*	.54
Mean certainty	76.51 (13.51)	80.02 (14.83)	78.02 (12.91)	$F(2,299) = 01.96$, p=.143	.01

STAI-state = State-Trait Anxiety Inventory, state subscale, STAI-trait = State-Trait Anxiety Inventory, trait subscale, Mean aversiveness = mean aversiveness rating across all blocks, *significant with a p=<.05.

3.2. Classes based on the subjective fearfulness ratings to the CS-

LCGA yielded three distinct classes on fearfulness rating to the CS-, see Fig. 3. This was based on a substantial drop in BIC and AIC score from the 2 to 3-class model, which was present to a much lesser extent from the 3 to 4-class model, see Table 3. The entropy score did increase slightly when including more classes, but contained too few participants (2 or 3) with patterns that did not add meaningful information (the 4-class

model included an extreme generalization pattern and the 5-class model extreme safety ambiguity). The largest class of the 3-class model (77%, *N*=232) labeled as 'non-generalizers' was characterized by a low fearfulness score for the CS- during all phases. The second largest class (18%, *N*=53) was labeled 'safety ambiguous' and characterized by a strong decrease in fear during both the acquisition and extinction instructed blocks, and a strong increase in fear during extinction uninstructed blocks, suggesting that participants in this class reacted with generalization of fear primarily when the contingencies were somewhat ambiguous. The third class 'generalizers' (5%, *N*=15) was characterized by high fear scores for CS- throughout the experiment.

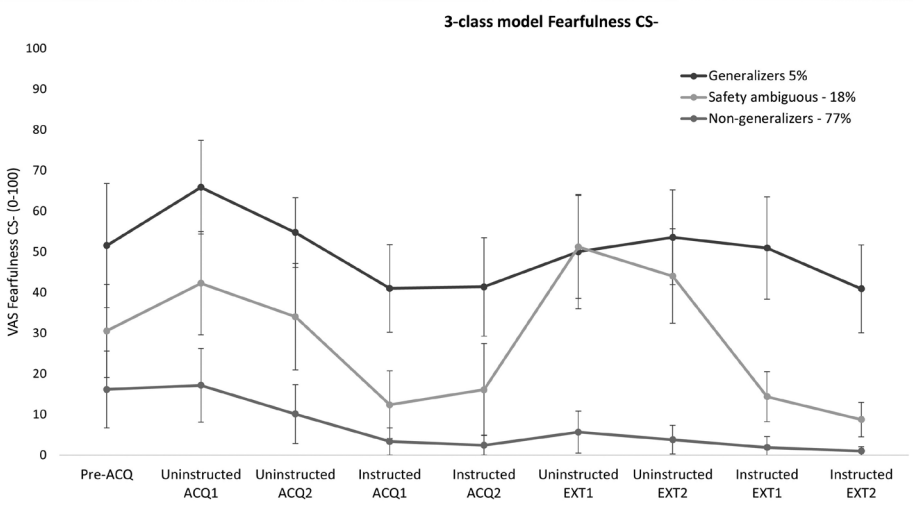

Fig. 3. Estimated means and standard deviation of the final model on fearfulness ratings to the CS- in the 3-class model. ACQ1 = acquisition block 1; ACQ2 = acquisition block 2; EXT1 = extinction block 1; EXT2 = extinction block 2.

Table 3. Fit indices for one-to six class Latent Growth Models based on subjective fearfulness ratings on the CS-.

No. of classes	*AIC*	*BIC*	*Entropy*	*Sample size per class based on most likely class membership*
1	23287	23354	NA	300
2	-994	-957	.968	254/46
3	**-402**	**-365**	**.974**	**232/53/15**
4	-198	-161	.977	2/229/51/18
5	-217	-181	.986	20/234/3/7/36
6	-176	-138	.956	50/200/26/16/3/5

AIC = Akaike Information Criterion; BIC = Bayesian Information Criterion; NA = not applicable. AIC and BIC values for 2 classes and up are expressed as reduction with respect to the model with one class less. Best fitting model values are displayed in bold.

3.2.1. Group characteristics fearfulness CS- classes

Classes differed in state and trait anxiety, US aversiveness and certainty, see Table 4. Non-generalizers had a lower score on state anxiety than the safety ambiguous class (p=.017) and generalizers (p=.012). Non-generalizers also had a significantly lower rating on trait anxiety than generalizers (p=.021). Non-generalizers rated the US as less aversive than generalizers (p=.028) and were more certain about their answers given than the safety ambiguous class (p=.001).

Table 4. Characteristics of participants assigned to the classes on fearfulness ratings to the CS-

	Non-generalizers	**Safety ambiguous**	**Generalizers**		
	***(n*=232)**	***(n*=52)**	***(n*=15)**	**Test Statistic**	***V/η²***
Gender	**(%total)**	**(%total)**	**(%total)**		
Men	81.70%	14.60%	3.70%	$\chi^2(2) = 1.27$, p=.529	.065
Women	75.70%	18.80%	5.50%		
	M (SD)	***M (SD)***	***M (SD)***		
Age	22.04 (3.32)	21.58 (2.70)	22.53 (2.03)	$F(2,299) = 0.68$, p=.507	.00
STAI-state	10.75 (2.64)	11.94 (3.22)	12.93 (3.92)	$F(2, 299) = 7.36$, p=.001*	.05
STAI-trait	36.09 (9.01)	39.13 (8.86)	42.67 (11.20)	$F(2, 299) = 5.51$, p=.004*	.04
Mean aversiveness	72.54 (22.30)	80.26 (16.47)	87.29 (17.99)	$F(2,299) = 5.67$, p=.004*	.04
Mean certainty	79.40 (14.18)	71.72 (11.66)	74.44 (10.98)	$F(2,299) = 7.34$, p=.001*	.05

STAI-state = State-Trait Anxiety Inventory, state subscale, STAI-trait = State-Trait Anxiety Inventory, trait subscale, Mean aversiveness = mean aversiveness rating across all blocks, Mean certainty = mean certainty rating across all blocks, * ANOVA significant with a p=<.05.

3.3. Classes based on the US expectancy ratings to the CS+

LCGA demonstrated two distinct classes in US expectancy ratings to the CS+, see Fig. 4. The selection was based on a combination of a more substantial drop in BIC and AIC from 1 to 2-class than from the 2 to 3-class, and peak in entropy score, see Table 5. The largest class 'normal conditioners' (88%, n=263) showed a fear conditioning trajectory characterized by an increase in US expectancy ratings during acquisition and a decrease during extinction. The second class 'poor extinguishers' (12%, n=37) showed an increase

in US expectancy rating comparable to the 'normal conditioners' during acquisition but a smaller decrease in US expectancy ratings during extinction blocks.

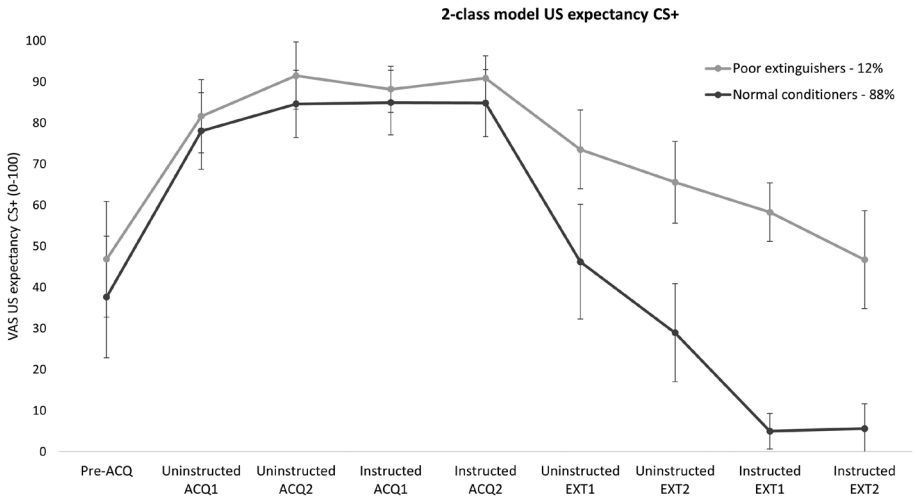

Fig. 4. Estimated means and standard deviation of the final model on US expectancy ratings to the CS+ in the 2-class model. ACQ1 = acquisition block 1; ACQ2 = acquisition block 2; EXT1 = extinction block 1; EXT2 = extinction block 2.

Table 5. Fit indices for one-to six class Latent Growth Models based on Expectancy ratings on the CS+.

No. of classes	*AIC*	*BIC*	*Entropy*	*Sample size per class based on most likely class membership*
1	24009	24076	NA	300
2	**-517**	**-480**	**.975**	**263/37**
3	-225	-188	.883	110/153/37
4	-198	-162	.927	104/37/16/143
5	-169	-131	.932	79/31/37/137/16
6	-112	-75	.932	76/131/6/35/15/37

AIC = Akaike Information Criterion; BIC = Bayesian Information Criterion; NA = not applicable. AIC and BIC values for 2 classes and up are expressed as reduction with respect to the model with one class less. Best fitting model values are displayed in bold.

3.3.1. Group characteristics US expectancy CS + classes

We found no significant association between group characteristics and US expectancy CS + classes, see supplementary data.

3.4. Classes based on the US expectancy ratings to the CS-

LCGA demonstrated two distinct classes in the ratings on US expectancy for the CS- based on a combination of a substantial drop in BIC and AIC and a perfect entropy score of 1, see Fig. 5 and Table 6. BIC and AIC scores also showed a relatively big drop from a 2 to 3-class model, but the third class contained 7 individuals with variable patterns characterized by high expectancy at the end of extinction. The then second largest group contained more individuals than with two classes (49 as opposed to 18), with a pattern similar to the safety ambiguous class for CS- fearfulness ratings. However, we proceed with the 2-class model in our further analyses because of the clear peak in entropy. The largest class (94%, *N*=282) showed a fear conditioning trajectory that we labeled as 'non-generalizers', characterized by a low US expectancy during the CS- which was never paired with the US. The second class (6%, *N*=18) was labeled as 'generalizers' and showed an enhanced US expectancy rating during the CS- throughout the experiment.

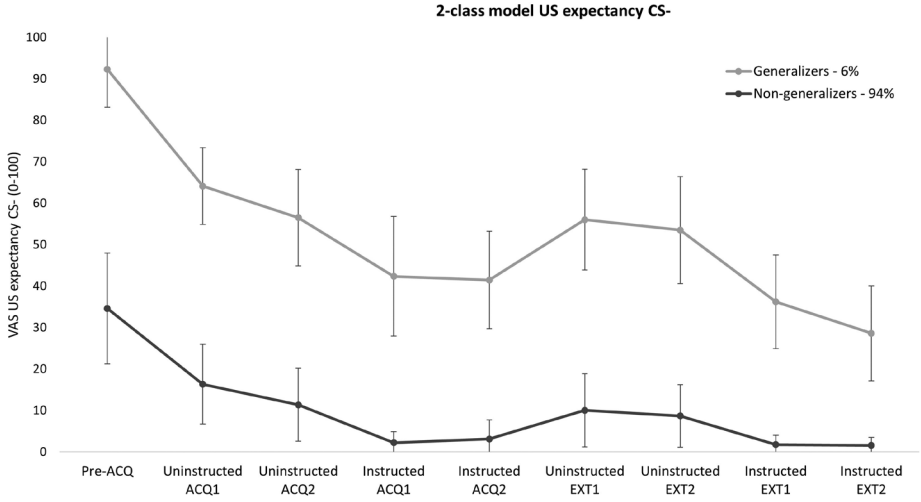

Fig. 5. Estimated means and standard deviation of the final model on US expectancy ratings to the CS- in the 2-class model. ACQ1 = acquisition block 1; ACQ2 = acquisition block 2; EXT1 = extinction block 1; EXT2 = extinction block 2.

Table 6. Fit indices for one-to six class Latent Growth Models based on US expectancy ratings on the CS-.

No. of classes	*AIC*	*BIC*	*Entropy*	*Sample size per class based on most likely class membership*
1	22624	22690	NA	300
2	**-867**	**-829**	**1**	**282/18**
3	-457	-420	.979	49/244/7
4	-333	-296	.983	1/243/45/11
5	-237	-200	.990	1/242/9/41/7
6	-176	-139	.989	35/222/10/25/6/2

AIC = Akaike Information Criterion; BIC = Bayesian Information Criterion; NA = not applicable. AIC and BIC values for 2 classes and up are expressed as reduction with respect to the model with one class less. Best fitting model values are displayed in bold.

3.4.1. Group characteristics US expectancy CS- classes

Generalizers had a significant higher state anxiety score, rated the US as more aversive and were less certain about their answers during the experiment than non-generalizers, see Table 7.

Table 7. Characteristics of participants assigned to the classes on US expectancy ratings to the CS-

	Non-generalizers (*n*=282)	**Generalizers (*n*=18)**	**Test Statistic**	φ /η^2
Gender	**(%total)**	**(%total)**		
Men	95.10%	4.90%	$\chi 2(1) = 0.25$, *p*=.616	.029
Women	93.60%	6.40%		
	M (SD)	***M (SD)***		
Age	21.95(3.21)	22.44(2.43)	F(1, 299) = 00.41, *p*=.525	.00
STAI-state	10.95(2.38)	12.94(3.08)	F(1, 299) = 08.36, *p*=.004*	.17
STAI-trait	36.75(9.21)	40.22(9.26)	F(1, 299) = 02.40, *p*=.122	.00
Mean aversiveness	73.83(21.81)	87.33(13.48)	F(1, 299) = 06.78, *p*=.010*	.14
Mean certainty	78.37(13.91	68.79(10.83)	F(1, 299) = 08.22, *p*=.004*	.03

STAI-state = State-Trait Anxiety Inventory, state subscale, STAI-trait = State-Trait Anxiety Inventory, trait subscale, Mean aversiveness = mean aversiveness rating across all blocks, Mean certainty = mean certainty rating across all blocks, * ANOVA significant with a *p*=<.05.

3.5. Follow-up measurements

Response rates on the follow-ups were respectively 94% (week 1) and 56% (week 6). Analysis revealed that attrition did not introduce bias (no a priori differences between (non)responders), see supplementary data.

3.5.1. Intrusions as a function of subjective fearfulness and US expectancy classes

Individuals belonging to the maladaptive fear learning patterns of poor extinguishers (fearfulness CS + classes) and generalizers (fearfulness and US expectancy CS- classes) experienced more intrusions 1 week after completion of the experiment, see Table 8. During the 6 week follow-up only generalizers (US expectancy CS- classes) reported more intrusions than non-generalizers.

Table 8. Statistics for intrusions experienced at 1 and 6 weeks follow-up for the classes based on subjective fearfulness ratings to the CS+ and CS-, and on ratings of US expectancy to the CS+ and CS- classes.

Intrusions week 1 (*n*=281)	Classes	*M (SD)*	*p*	η^2	Bonferroni posthoc statistics	*p*
CS+ fearfulness	Normal conditioners	1.35(2.08)	<.001*	.08	norm vs. low	.015*
	Low fearful conditioners	0.60(1.28)			norm vs. poor	.011*
	Poor extinguishers	2.43(2.76)			low vs. poor	<.001*
CS- fearfulness	Non-generalizers	1.10(1.93)	.020*	.03	non-gen vs. safe	.408
	Safety Ambigiuous	1.57(2.18)			non-gen vs. gen	.035*
	Generalizers	2.47(2.75)			safe vs. gen	.395
CS+ US expectancy	Normal conditioners	1.23(2.04)	.615	.00		
	Poor extinguishers	1.42(2.06)				
CS- US expectancy	Non-generalizers	1.17(1.91)	.005*	.03		
	Generalizers	2.59(3.34)				

Table 8. Statistics for intrusions experienced at 1 and 6 weeks follow-up for the classes based on subjective fearfulness ratings to the CS+ and CS-, and on ratings of US expectancy to the CS+ and CS- classes. (continued)

Intrusions week 1 (*n*=281)	Classes	*M (SD)*	*p*	η^2	Bonferroni posthoc statistics	*p*
Intrusions week 6 (*n*=169)	Classes	*M (SD)*	*p*	η^2		
CS+ fearfulness	Normal conditioners	0.70(1.50)	.570	.00		
	Low fearful conditioners	0.65(1.59)				
	Poor extinguishers	1.05(1.53)				
CS- fearfulness	Non-generalizers	0.64(1.48)	.202	.02		
	Safety Ambigiuous	0.84(1.46)				
	Generalizers	1.56(2.19)				
CS+ US expectancy	Normal conditioners	0.73(1.57)	.946	.00		
	Poor extinguishers	0.71(1.23)				
CS- US expectancy	Non-generalizers	0.66(1.45)	.041*	.02		
	Generalizers	1.64(2.25)				

'norm'= normal conditioners, 'gen' = generalisers, "safe' = safety ambiguous.
* ANOVA significant with a p=<.05.

3.5.2. Subjective fearfulness ratings as a function of subjective fearfulness classes

In order to interpret group differences at follow-up in the light of already existing differences during the experiment, the group differences in subjective fearfulness at the end of extinction were also analyzed and included, see Table 9. Poor extinguishers (fearfulness CS + classes) experienced the highest fearfulness to the CS+ during both the experiment and follow-ups. For the generalizers (fearfulness CS- classes) differences were restricted to the experiment and 1 week follow-up on fearfulness CS- ratings.

Table 9. Statistics for subjective fearfulness ratings during extinction, 1 and 6 weeks follow-up for the classes based on subjective fearfulness ratings to the CS+ and CS- separately.

At the end of extinction (*n*=300)						
	Classes	*M (SD)*	*p*	η^2	Bonferroni posthoc statistics	*p*
CS+ fearfulness	Normal conditioners	10.51(10.52)	<.001*	.71	norm vs. low	<.001*
ratings	Low fearful conditioners	2.70(5.47)			norm vs. poor	<.001*
	Poor extinguishers	56.73(17.23)			low vs. poor	<.001*
CS- fearfulness	Non-generalizers	1.46(3.18)	<.001*	.72	non-gen vs. safe	<.001*
ratings	Safety Ambigiuous	11.56(8.71)			non-gen vs. gen	<.001*
	Generalizers	45.90(20.16)			safe vs. gen	<.001*
week 1 (*n*=281)	Classes	*M (SD)*	*p*	η^2		
CS+ fearfulness	Normal conditioners	48.38(20.80)	<.001*	.10	norm vs. low	<.001*
ratings	Low fearful conditioners	35.35(22.53)			norm vs. poor	.089
	Poor extinguishers	57.14(22.17)			low vs. poor	<.001*
CS- fearfulness	Non-generalizers	30.37(22.73)	.002*	.04	non-gen vs. safe	.092
ratings	Safety Ambigiuous	37.84(20.23)			non-gen vs. gen	.006*
	Generalizers	48.67(17.67)			safe vs. gen	.288
week 6 (*n*=169)	Classes	*M (SD)*	*p*	η^2		
CS+ fearfulness	Normal conditioners	36.88(24.05)	.001*	.08	norm vs. low	1.000
ratings	Low fearful conditioners	36.11(22.77)			norm vs. poor	.001*
	Poor extinguishers	56.82(23.58)			low vs. poor	.002*
CS- fearfulness	Non-generalizers	33.02(24.39)	.657	.01		

Table 9. Statistics for subjective fearfulness ratings during extinction, 1 and 6 weeks follow-up for the classes based on subjective fearfulness ratings to the CS+ and CS- separately. (continued)

At the end of extinction (*n*=300)						
	Classes	*M (SD)*	*p*	η^2	Bonferroni posthoc statistics	*p*
ratings	Safety Ambigiuous	31.61(24.51)				
	Generalizers	40.00(21.79)				

'norm'= normal conditioners, 'gen' = generalisers, "safe' = safety ambiguous. * ANOVA significant with a p=<.05.

3.5.3. Subjective US expectancy ratings as a function of subjective US expectancy classes

During the experiment both maladaptive patterns of poor extinguishers (CS + US expectancy classes) and generalizers (CS– US expectancy classes) were associated with higher US expectancy ratings, see Table 10. At the 1 week follow-up this difference was only significant for the generalizers (CS– US expectancy classes) who reported higher US expectancy ratings to the CS-.

Table 10. Differences in US expectancy ratings during extinction, 1 and 6 weeks follow-up for the US expectancy CS+ and US expectancy CS- classes.

At the end of extinction (*n*=300)				
	Classes	*M (SD)*	*p*	η^2
CS+ US expectancy	Normal conditioners	5.32(8.55)	<.001*	.71
ratings	Poor extinguishers	52.51(17.10)		
CS- US expectancy	Non-generalizers	1.67(3.55)	<.001*	1.17
ratings	Generalizers	30.74(22.59)		
week 1 (*n*=281)				
	Classes	*M (SD)*	*p*	η^2
CS+ US expectancy	Normal conditioners	48.24(30.09)	.744	.00
ratings	Poor extinguishers	50.00(29.57)		
CS- US expectancy	Non-generalizers	17.92(21.36)	.008*	.02
ratings	Generalizers	32.35(25.62)		

Table 10. Differences in US expectancy ratings during extinction, 1 and 6 weeks follow-up for the US expectancy CS+ and US expectancy CS- classes. (continued)

At the end of extinction (*n*=300)				
week 6 (*n*=169)				
	Classes	*M (SD)*	*p*	η^2
CS+ US expectancy	Normal conditioners	37.79(31.08)	.567	.00
ratings	Poor extinguishers	41.67(27.77)		
CS- US expectancy	Non-generalizers	20.51(24.36)	.366	.00
ratings	Generalizers	27.27(15.55)		

* ANOVA significant with a p=<.05.

4. DISCUSSION

The current study aimed to replicate individual fear learning trajectories with the use of an easy to administer fear conditioning task based on the task used by Duits et al. (2021)[19] and investigate whether specific individual characteristics measured during testing, return of fear and intrusions tested at follow-up would be associated with these trajectories. To test this 300 healthy subjects completed a fear conditioning task, multiple questionnaires and a one and six week follow-up.

Our study replicated the fear trajectories found by Duits et al. (2021)[19] with a much shorter fear conditioning task (±15 min). The fear trajectories are in line with a previous study[18]. In contrast to earlier studies, this task focused on only subjective measures. With respect to the fearfulness ratings to the CS + we observed classes of normal conditioners (56%), low fearful conditioners (32%) and poor extinguishers (12%). Duits et al. (2021)[19] found the same trajectories in a sample of anxiety patients and healthy controls, but with a much longer version of the task. The replication of these trajectories with a shorter, easier to administer task will benefit the implementation of this task in a clinical setting as a possible screening tool for maladaptive fear learning patterns.

With regard to fearfulness ratings to the CS- the trajectories of fear generalizers and non-generalizers were replicated[19]. Additionally, a third class was observed, which was not found by Duits et al. (2021)[19]. We labeled this class 'safety ambiguous' (18%), as it was characterized by a strong decrease in fear to the CS- after instructions were provided in both the acquisition and extinction phase, whereas fear was strongly increased during both uninstructed blocks. This pattern suggests that individuals in this class reacted with generalization of fear primarily when the contingencies were somewhat ambiguous. The safety ambiguous class also reported more uncertainty toward their given answers than non-generalizers. Literature indicate the influence of (intolerance of) uncertainty

on fear generalization[36,37], i.e. with uncertainty about whether the aversive stimuli will come, expectancy increases. The fact that the safety ambiguous class was not observed by Duits et al. (2021)[19] may be due to a smaller sample size and/or differences in the population (a mix of anxiety patients and healthy controls). Another explanation is that even though the same general principles were applied, selecting models based on a combination of different criteria contains an element of subjectivity[34,35]. The question of how the safety ambiguous class may be represented in anxiety patients remains open.

Classes based on US expectancy ratings were also replicated[19]. The different classes based on fearfulness and US expectancy ratings yielded groups of people that significantly differed on several characteristics. Both normal conditioners and poor extinguishers reported higher state and trait anxiety than low fearful conditioners. Individuals in the generalizers group reported higher state and trait anxiety. Also US aversiveness was reported to be higher in comparison to other classes for both poor extinguishers and generalizers. Considering that the scream (US) was of a constant loudness, this suggests a possible lower sensitivity threshold to aversiveness of stimuli in individuals in maladaptive learning trajectories. The classes based on US expectancy ratings did not differentiate with regard to the different characteristics. The US expectancy ratings may evoke a more cognitively mediated risk assessment, as opposed to the more emotionally mediated measure of fearfulness. Classes based on fearfulness ratings may more strongly distinguish participants on different characteristics relating to anxiety and aversion because the expectancy of an aversive stimulus does not necessarily go along with a fearful or nervous feeling in all participants. Indeed, our data demonstrated that only 48,6% of participants who were ascribed to the poor extinguishers class (fearfulness CS + ratings) also belonged to the poor extinction class (US expectancy CS + ratings). In the generalizers class (fearfulness CS-), 66,7% of participant also belonged to the generalization class (US expectancy CS-).

In the sample of Duits et al. (2021)[19] anxiety patients were overrepresented in all of the maladaptive fear learning trajectories. Since fear generalization and failure to extinguish fear are core deficits seen in people that develop an anxiety disorder, the fear learning profiles assessed with this task may allow identifying ‘at risk’ groups of people who may be prone to lasting anxiety because of a reduced capacity to extinguish fear and/or to inhibit fear to stimuli that are not associated with danger (fear generalization)[13]. In addition, the analysis of whether maladaptive learning profiles could predict success of subsequent cognitive behavior therapy in the patients in the study by Duits et al. (2021)[19] yielded tentative indications that indeed markers of maladaptive fear learning may be useful to assess in advance which patients are likely to benefit from behavioral treatment.

With respect to the prediction of intrusions, individuals in the maladaptive classes from both the CS+ (poor extinguishers, fearfulness ratings) and CS- (generalizers, fearfulness

and expectancy ratings) experienced more intrusions 1 week after the experiment. This is in line with research showing that people with higher fear during extinction experience more aversive memories[38]. Only the higher levels of intrusions for generalizers in the expectancy measure persisted at the week 6 follow-up. Interestingly, although individuals from the maladaptive fear learning classes retained the scored higher on fearfulness and US expectancy rating during the experiment and at the follow-ups, most other classes that scored low during the experiment showed an increase in fearfulness and US expectancy during both follow-ups. This return of fear can be simply due to the passage of time (regression to the mean because of forgetting). Or, although AAB renewal is often much weaker that ABC or ABA renewal, renewal may have occurred because people completed the follow-ups in a different context[39,40]. Longer follow-ups are necessary to entangle how this pattern develops over time and to investigate the influence of context.

To conclude, the identification of distinct fear learning profiles was replicated with a short fear conditioning task and LCGA in healthy participants. Additionally, groups of individuals assigned to different patterns of fear learning display significant differences in characteristics associated with increased risk in the development of an anxiety disorder. The task also gives insight in the development of intrusions and return of fear. This shorter and easier to administer task allows to further study fear learning trajectories in larger samples of anxiety patients, for example at baseline before the start of behavioral treatment to assess predictive value for treatment success. Additional studies may elucidate the possible predictive value of the poor extinguishers and/or fear generalizers class with respect to future the development of the anxiety disorder in anxiety patients. This may, in turn, help the development of more personalized treatment.

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Author statement

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Declaration of competing interest

The authors declare no conflict of interest.

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APPENDIX A. SUPPLEMENTARY DATA

1. Differences between the different trajectories based on the different phases.

Table S1. Fearfulness rating to the CS+ and difference between the classes per phase

Phase	Classes	*M(SD)*	*p*	*η2*	Bonferroni posthoc statistics	*p*
Pre-acquisition	Low fearful conditioners (n=95)	13.97 (19.03)	<.001*	.09	low vs. norm	.004*
	Normal conditioners (n=170)	23.97 (24.07)			low vs. poor	<.001*
	Poor extinguishers (n=35)	39.48 (33.13)			norm vs. poor	.002*
Acquisition uninstructed part 1	Low fearful conditioners	34.38 (24.72)	<.001*	.38	low vs. norm	<.001*
	Normal conditioners	68.13 (20.78)			low vs. poor	<.001*
	Poor extinguishers	79.53 (16.15)			norm vs. poor	.015*
Acquisition uninstructed part 2	Low fearful conditioners	28.57 (22.89)	<.001*	.61	low vs. norm	<.001*
	Normal conditioners	76.66 (17.06)			low vs. poor	<.001*
	Poor extinguishers	84.83 (13.66)			norm vs. poor	.059
Acquisition instructed part 1	Low fearful conditioners	21.93 (16.73)	<.001*	.68	low vs. norm	<.001*

Table S1. Fearfulness rating to the CS+ and difference between the classes per phase (continued)

Phase	Classes	*M(SD)*	*p*	*η2*	Bonferroni posthoc statistics	*p*
	Normal conditioners	73.20 (19.10)			low vs. poor	<.001*
	Poor extinguishers	87.21 (11.38)			norm vs. poor	<.001*
Acquisition instructed part 2	Low fearful conditioners	14.89 (13.22)	<.001*	.73	low vs. norm	<.001*
	Normal conditioners	72.43 (19.86)			low vs. poor	<.001*
	Poor extinguishers	86.68 (12.43)			norm vs. poor	<.001*
Extinction uninstructed part 1	Low fearful conditioners	14.79 (15.30)	<.001*	.62	low vs. norm	<.001*
	Normal conditioners	61.29 (21.99)			low vs. poor	<.001*
	Poor extinguishers	84.31 (12.82)			norm vs. poor	<.001*
Extinction uninstructed part 2	Low fearful conditioners	8.06 (11.81)	<.001*	.51	low vs. norm	<.001*
	Normal conditioners	39.46 (24.26)			low vs. poor	<.001*
	Poor extinguishers	73.50 (15.42)			norm vs. poor	<.001*
Extinction instructed part 1	Low fearful conditioners	2.71 (7.07)	<.001*	.59	low vs. norm	<.001*

Table S1. Fearfulness rating to the CS+ and difference between the classes per phase (continued)

Phase	Classes	*M(SD)*	*p*	*η2*	Bonferroni posthoc statistics	*p*
	Normal conditioners	12.97 (13.61)			low vs. poor	<.001*
	Poor extinguishers	57.74 (23.54)			norm vs. poor	<.001*
Extinction instructed part 2	Low fearful conditioners	2.68 (5.96)	<.001*	.67	low vs. norm	<.001*
	Normal conditioners	8.04 (9.13)			low vs. poor	<.001*
	Poor extinguishers	55.72 (22.73)			norm vs. poor	<.001*

low' = low fearful conditioners, 'norm' = normal conditioners, 'poor' = poor extinguishers. *ANOVA significant with a p=<.05.

Table S2. Fearfulness rating to the CS- and difference between the classes per phase

Phase	Classes	*M(SD)*	*p*	*η2*	Bonferroni posthoc statistics	*p*
Pre-acquisition	Non-generalizers (n=232)	16.15 (18.98)	<.001*	.16	non-gen vs. safety ambiguous	<.001*
	Safety ambiguous (n=53)	30.55 (23.11)			non-gen vs. generalizers	<.001*
	Generalizers (n=15)	51.54 (31.50)			safety ambiguous vs. generalizers	.002*
Acquisition uninstructed part 1	Non-generalizer	17.15 (18.12)	<.001*	.32	non-gen vs. safety ambiguous	<.001*
	Safety ambiguous	42.25 (25.61)			non-gen vs. generalizers	<.001*
	Generalizers	65.86 (23.90)			safety ambiguous vs. generalizers	<.001*
Acquisition uninstructed part 2	Non-generalizer	10.10 (14.50)	<.001*	.35	non-gen vs. safety ambiguous	<.001*
	Safety ambiguous	34.01 (26.40)			non-gen vs. generalizers	<.001*
	Generalizers	54.73 (17.76)			safety ambiguous vs. generalizers	<.001*
Acquisition instructed part 1	Non-generalizer	3.35 (6.64)	<.001*	.41	non-gen vs. safety ambiguous	<.001*
	Safety ambiguous	16.77 (2.30)			non-gen vs. generalizers	<.001*
	Generalizers	40.98 (22.39)			safety ambiguous vs. generalizers	<.001*
Acquisition instructed part 2	Non-generalizer	2.44 (4.87)	<.001*	.40	non-gen vs. safety ambiguous	<.001*
	Safety ambiguous	16.12 (22.77)			non-gen vs. generalizers	<.001*
	Generalizers	41.38 (25.06)			safety ambiguous vs. generalizers	<.001*
Extinction uninstructed part 1	Non-generalizer	5.64 (10.30)	<.001*	.61	non-gen vs. safety ambiguous	<.001*
	Safety ambiguous	51.20 (25.57)			non-gen vs. generalizers	.001*
	Generalizers	50.02 (29.09)			safety ambiguous vs. generalizers	1
Extinction uninstructed part 2	Non-generalizer	3.79 (7.08)	<.001*	.66	non-gen vs. safety ambiguous	<.001*

Table S2. Fearfulness rating to the CS- and difference between the classes per phase (continued)

Phase	Classes	*M(SD)*	*p*	*η2*	Bonferroni posthoc statistics	*p*
	Safety ambiguous	44.02 (23.39)			non-gen vs. generalizers	<.001*
	Generalizers	53.54 (24.09)			safety ambiguous vs. generalizers	.033*
Extinction instructed part 1	Non-generalizer	1.90 (5.38)	<.001*	.61	non-gen vs. safety ambiguous	<.001*
	Safety ambiguous	14.37 (12.35)			non-gen vs. generalizers	<.001*
	Generalizers	50.92 (26.09)			safety ambiguous vs. generalizers	<.001*
Extinction instructed part 2	Non-generalizer	1.01 (2.14)	<.001*	.67	non-gen vs. safety ambiguous	<.001*
	Safety ambiguous	8.76 (8.56)			non-gen vs. generalizers	<.001*
	Generalizers	40.86 (22.41)			safety ambiguous vs. generalizers	<.001*

non-gen' = non-generalizers. *ANOVA significant with a p=<.05.

Table S3. US expectancy rating to the CS+ and difference between the classes per phase

Phase	Classes	*M(SD)*	*p*	*η2*
Pre-acquisition	Normal conditioners (n=263)	37.64 (29.61)	.077	.01
	Poor extinguishers (n=37)	46.83 (28.52)		
Acquisition uninstructed part 1	Normal conditioners	78.06 (18.68)	.275	.00
	Poor extinguishers	81.63 (18.05)		
Acquisition uninstructed part 2	Normal conditioners	84.60 (16.35)	.017*	.02
	Poor extinguishers	91.48 (16.61)		
Acquisition instructed part 1	Normal conditioners	84.95 (15.77)	.228	.00
	Poor extinguishers	88.19 (11.36)		
Acquisition instructed part 2	Normal conditioners	84.84 (16.38)	.031*	.02
	Poor extinguishers	90.87 (11.07)		
Extinction uninstructed part 1	Normal conditioners	46.22 (27.93)	<.001*	.10
	Poor extinguishers	73.52 (19.43)		
Extinction uninstructed part 2	Normal conditioners	28.96 (23.88)	<.001*	.21
	Poor extinguishers	65.57 (20.17)		
Extinction instructed part 1	Normal conditioners	5.00 (8.69)	<.001*	.77
	Poor extinguishers	58.29 (14.48)		
Extinction instructed part 2	Normal conditioners	5.64 (12.11)	<.001*	.27
	Poor extinguishers	46.71 (24.17)		

*ANOVA significant with a p=<.05.

Table S4. US expectancy rating to the CS- and difference between the classes per phase

Phase	Classes	*M(SD)*	*p*	*η2*
Pre-acquisition	Non-generalizers (n=282)	34.59 (26.83)	<.001*	.04
	Generalizers (n=18)	57.70 (18.92)		
Acquisition uninstructed part 1	Non-generalizers	16.33 (19.30)	<.001*	.13
	Generalizers	47.79 (22.41)		
Acquisition uninstructed part 2	Non-generalizers	11.36 (17.61)	<.001*	.17
	Generalizers	45.15 (23.91)		
Acquisition instructed part 1	Non-generalizers	2.23 (5.32)	<.001*	.51
	Generalizers	40.11 (29.66)		
Acquisition instructed part 2	Non-generalizers	3.11 (9.20)	<.001*	.38
	Generalizers	38.33 (24.19)		
Extinction uninstructed part 1	Non-generalizers	10.03 (17.65)	<.001*	.18
	Generalizers	45.99 (25.02)		
Extinction uninstructed part 2	Non-generalizers	8.68 (15.10)	<.001*	.22
	Generalizers	44.81 (26.58)		
Extinction instructed part 1	Non-generalizers	1.77 (4.57)	<.001*	.55
	Generalizers	34.45 (23.23)		
Extinction instructed part 2	Non-generalizers	1.57 (3.82)	<.001*	.45
	Generalizers	27.04 (23.60)		

*ANOVA significant with a p=<.05.

2. Group characteristics US expectancy CS+ classes

We found no significant association between group characteristics and US expectancy CS+ classes, see Table S5.

Table S5. Characteristics of participants assigned to the classes on US expectancy ratings to the CS+

	Normal conditioners (*N*=263)	Poor extinguishers (*N*=37)	Test Statistic	φ /η^2
Gender	(%total)	(%total)		
Men	85.40%	14.60%	χ2(1) = 0.55, p=.457	.043
Women	88.50%	11.50%		
	M (SD)	*M (SD)*		
Age	22.02(3.28)	21.76(2.24)	F(1, 299) = .216, *p*=.643	.00
STAI-state	11.03(2.89)	11.35(2.80)	F(1, 299) = .412, *p*=.521	.00
STAI-trait	36.83(9.05)	37.89(10.57)	F(1, 299) = .429, *p*=.513	.00
Mean aversiveness	74.25(21.39)	77.37(22.66)	F(1, 299) = .677, *p*=.411	.00
Mean certainty	78.08(14.01)	75.79(13.92)	F(1, 299) = .882, *p*=.348	.00

STAI-state = State-Trait Anxiety Inventory, state subscale, STAI-trait = State-Trait Anxiety Inventory, trait subscale, Mean aversiveness = mean aversiveness rating across all blocks, Mean certainty = mean certainty rating across all blocks, * ANOVA significant with a *p*=<.05.

3. Supplementary data on follow-up measures

3. 1. Responders and Non-responders

The response rates on the follow-up assessments were respectively 94% for the 1 week follow-up and 56% for the 6 week follow-up. No differences were found between responders and non-responders on the 6 week follow-up questionnaires. Classes (fearfulness CS+ $\chi^2(2, n=300) = .799$, $p=.671$, $V=.052$, fearfulness CS- $\chi^2(2, n=300) = .232$, $p=.890$, $V=.028$, US expectancy CS+ $\chi^2(1, n=300) = 1.249$, $p=.264$, $\varphi=.065$, US expectancy CS- $\chi^2(1, n=300) = .178$, $p=.673$, $\varphi=.024$), state $F(1,299) = 1.459$, $p=.228$, $\eta^2 =.00$ and trait anxiety $F(1,299) = .381$, $p=.538$, $\eta^2 =.00$,gender $\chi^2(1, n=300) = .989$, $p=.320$, $\varphi=.057$ or US aversiveness $F(1,299) = 1.715$, $p=.191$, $\eta^2 =.01$.

3. 2. Intrusions Time x Class interactions

Repeated measures ANOVA's where conducted to investigate possible Time x Class interactions. Analysis were conducted with Time (intrusion scores at week 1 and 6) as

within subject factor and the different classes as (for Fearfulness CS+/CS- and US expectancy CS+/CS- classes) as between subject factor. These analyses were conducted with the participants that completed both the 1 and 6 week follow-up (n=169). For the Fearfulness CS+ classes repeated measures ANOVA demonstrated a significant Class x Time interaction (F(2,163) = 4.400, p=.014, partial η^2 = .05). Simple effects analyses revealed that for low fearful conditioners the difference in intrusions between 1 and 6 weeks was not significant, F(1,90) = 19.21, p=.176. Normal conditioners showed a reduction in intrusion from week 1 (1.62*±2.17) to week 6* (0.71*±1.51), F*(1,163) = 17.803, p=<.001, partial η^2 = .10. Poor extinguishers also displayed a reduction on intrusions between week 1 (2.36*±2.44)* and 6 (1.05*±1.53)*, F(1,21) = 19.114, p=.002, partial η^2 = .36, see figure S1. For the CS- fearfulness classes only a main effect of Time (F(1,163) = 12.090, p=.001, partial η^2 = .07) and Class (F(1,163) = 3.166, p=.045, partial η^2 = .04) was demonstrated. Simple main effect analysis revealed that the main effect of Class was no longer significant after post hoc testing. The effect of Time remained significant, i.e. during week 1 intrusions were higher than during week 6, see Figure S2. In sum, intrusions showed a reduction in almost all classes from the 1 to 6 week follow-up.

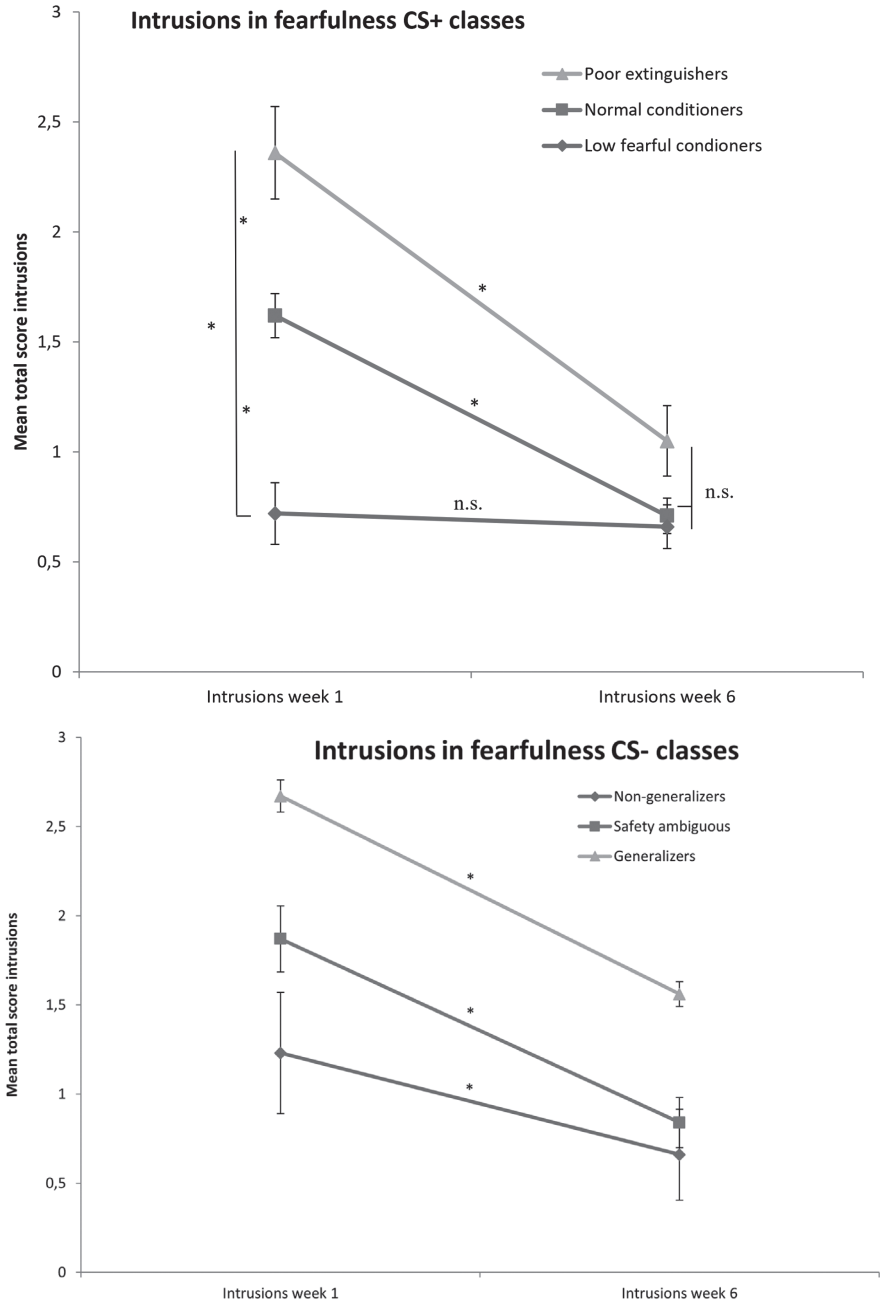

Figure S1 / S2. Mean and S.E.M. of the total intrusions experienced per CS+ fearfulness (top) and CS- fearfulness (bottom) class 1 and 6 weeks after the experiment. n.s. = not significant. * Significant with p=<.005.

6.3. Time x Class interactions for the fearfulness and US expectancy classes

Repeated measure ANOVA's where conducted with Time (scores during instructed extinction, week 1 and 6) as within subject factor and the Class (for fearfulness CS+/CS- and US expectancy CS+/CS-) as between subject factor. The repeated measure ANOVA's were conducted with the participants that completed both the 1 and 6 week follow-up (*N*=169).

6.3.1. Fearfulness CS+

Repeated measures ANOVA's for the CS+ fearfulness trajectories demonstrated a significant Class x Time interaction on subjective fearfulness CS+ ratings, (F(4,326) = 14.715, p=<.001, partial η^2 = .15). Simple effects analyses revealed that the subjective fear ratings on CS+ fearfulness of poor extinguishers did not significantly differ, F(2,42) = 0.132, p=.877. Normal conditioners differed on all time points, F(2,180) = 108.924, p=<.001, partial η^2 = .55. Lastly low fearful conditioners also showed differences (F(2,104) = 61.119, p=<.001, partial η^2 = .55), but only between extinction and week 1 and extinction and week 6 (p=<.001), see figure S3.

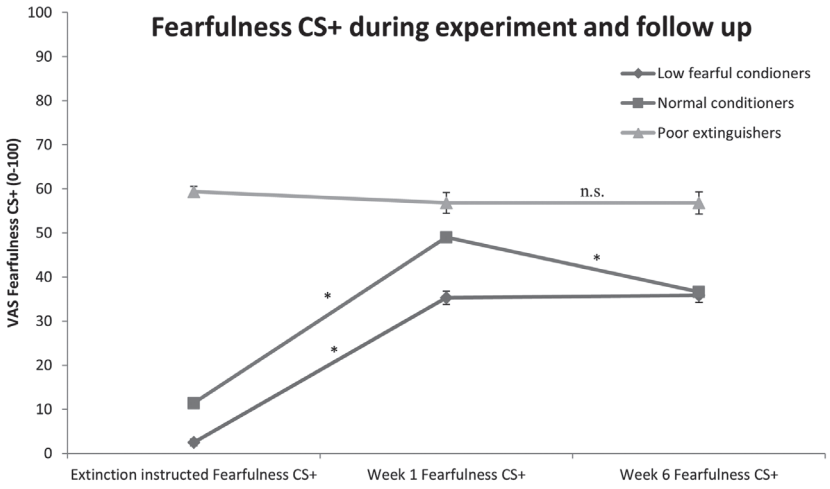

Figure S3. Mean and S.E.M. on subjective fearfulness rating to the CS+ as a function of fearfulness CS+ classes during the extinction instructed blocks, the 1 week and 6 weeks follow up. n.s. = not significant. * Significant with p=<.005

6.3.2. Fearfulness CS-

For the subjective fearfulness rating on the CS- as a function of the CS- classes there was also a significant Class x Time interaction ($F(4,326) = 5.371$, p=<.001, partial η^2 = .06). Simple effects analyses revealed that generalizers did not differ significantly, $F(2,16) = 1.173$, p=.335. Non-generalizers did differed, $F(2,250) = 124.142$, p=<.001, partial η^2 = .50. Non-generalizers differed between the extinction blocks and week 1 (p=<.001) and extinction and week 6 (p=<.001). The safety ambiguous class displayed the same pattern ($F(2,60) = 21.362$, p=<.001, partial η^2 = .42), see Figure S4.

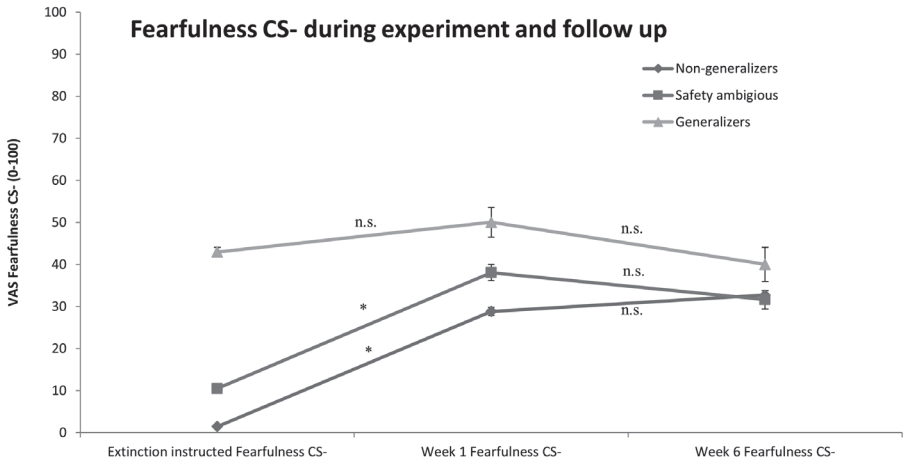

Figure S4. Mean and S.E.M. on subjective fearfulness rating to the CS- as a function of fearfulness CS- classes during the extinction instructed blocks, the 1 week and 6 weeks follow up. n.s. = not significant. * Significant with p=<.005

6.3.3. US Expectancy CS+

Repeated measures ANOVA on US expectancy ratings to the CS+ as function of US expectancy CS+ classes revealed Time x Class interaction effects ($F(2,328) = 30.882$, p=<.001, partial η^2 = .16). Simple effect analysis revealed that for the poor extinguishers the difference between week 1 and 6 (p=.019) was significant, F(2,46) = 3.513, p=.038, partial η^2 = .13. Normal conditioners differed on all time points (all with p=<.001), F(2,282) = 150.760, p=<.001, partial η^2= .52, see Figure S5.

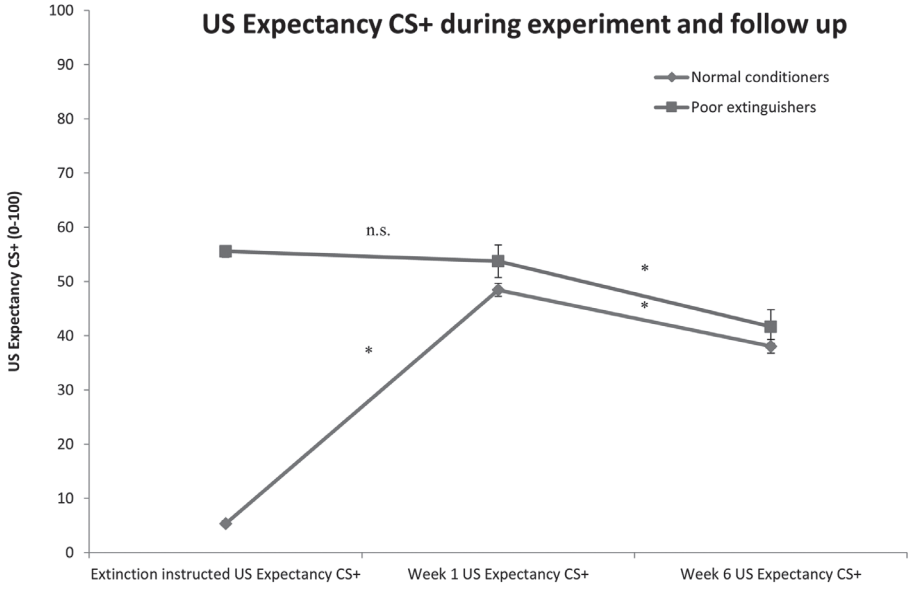

Figure S5. Mean and S.E.M. on subjective US expectancy rating to the CS+ as a function of US expectancy CS+ classes during the extinction instructed blocks, the 1 week and 6 weeks follow up. n.s. = not significant. * Significant with p=<.005

6.3.4. US Expectancy CS-

For the US expectancy CS- classes a significant Class x Time interaction (F(2,328) = 7.049, p=.001, partial η^2 = .04) was demonstrated. Simple effects analyses revealed a significant differences for the non-generalizers F(2,308) = 60.185, p=<.001, partial η^2 = .28. That is, differences between extinction and week 1 (p=<.001) were significant. Also the difference between extinction and week 6 (p=<.001) differed significantly. Simple effects analyses revealed no significant effect for time in the generalizers class, F(2,20) = 1.208, p=.320, see Figure S6.

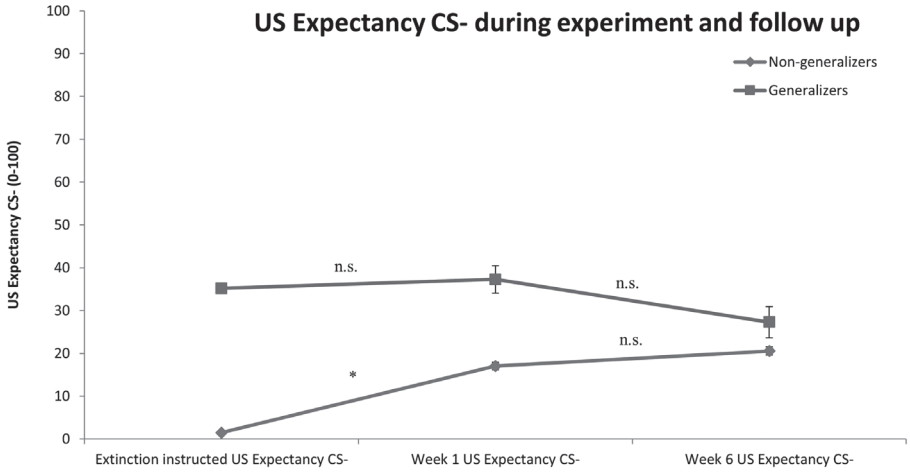

Figure S6. Mean and S.E.M. on subjective US expectancy rating to the CS- as a function of US expectancy CS- classes during the extinction instructed blocks, the 1 week and 6 weeks follow up. n.s. = not significant. * Significant with p=<.005

CHAPTER 3

Fear learning classes and treatment outcome in anxiety-related disorders: a preliminary report of fear learning classes in clinical practice

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MANUSCRIPT IN PREPARATION:
THIS CHAPTER DESCRIBES PRELIMINARY RESULTS FROM AN ONGOING STUDY.

ABSTRACT

Background: Fear conditioning and extinction paradigms are widely used to investigate (psycho)physiological processes underlying the acquisition and extinction of fear. Patients with anxiety-related disorders are more likely to show dysfunctional patterns of fear learning as evidenced by latent class growth analyses (LGCA). Because fear extinction is thought to underlie the beneficial effects of exposure therapy for anxiety disorders, we investigated whether underlying fear learning classes may be associated with treatment outcome.

Methods: Patients with various anxiety-related disorders (*N*=122) completed a fear conditioning and extinction task with both uninstructed and instructed phases. Two faces were used as conditioned stimuli, of which one (CS+) was paired with a loud scream (US) and the other one was not (CS-). Subjective fear and US expectancy were used as outcome measures. The Brief Symptoms Inventory (BSI) was used to measure clinical symptoms at baseline and twelve weeks after start of the anxiety related treatment. In addition, data with regard to the treatment was collected (e.g. primary diagnosis, kind of treatment, number of treatment sessions).

Results: Fear learning classes replicated those found previously: classes of normal conditioning, low fear and poor extinction were found for the CS+ for both the fear and US expectancy ratings. For ratings of the CS-, fear and US expectancy classes of generalizers and non-generalizers were demonstrated. Fear learning classes were not associated with symptom reduction twelve weeks after starting treatment. However, individuals belonging to the dysfunctional generalization classes reported higher fear and expectancy when the pictures used in the experiment were shown again.

Conclusion: Fear learning classes are associated with experimental fear and US expectancy twelve weeks later but not with reductions in anxiety and overall symptoms as a result of treatment. Relations between fear learning classes and treatment characteristics must be investigated in a larger sample due to small sample size and a small group of individuals belonging to the poor extinguishers classes.

1. INTRODUCTION

The fear conditioning and extinction model is widely used as an experimental paradigm to investigate (psycho)physiological processes underlying the acquisition and extinction of fear memories[1]. The model is also used to gain more insight into the development, maintenance and treatment of anxiety, trauma- and stressor related disorders[2]. However, it is still largely unknown why some individuals develop these disorders and what characteristics are related to individuals that respond to the current state of the art treatment[3]. Therefore, there is a need to focus on individual markers to differentiate individuals on underlying mechanisms that are related to the development and treatment of anxiety, trauma- and stressor related disorders [4].

Fear conditioning research has mainly focused on differences in fear learning responses between predefined groups, e.g. the difference between anxiety patients and healthy controls. In the fear conditioning paradigm a neutral cue (CS), e.g. a picture, is paired with an unconditioned aversive stimulus (US), e.g. a shock or loud noise[5,6]. This will then form an associative memory between the CS and the US. Subsequent fear extinction training, by presenting the CS without the US, leads to diminishment or extinction of the fear response[7]. Meta-analyses demonstrated that when comparing anxiety patients with healthy controls, anxiety patients have a higher fear response to the CS+ during both fear acquisition and fear extinction[8,9]. Additionally, patients demonstrated a higher response to the CS- during fear acquisition[9]. This suggests two potential mechanisms for developing anxiety disorders: First, a failure to extinguish a fear response that is no longer predictive of an aversive outcome[2,10,11] and second fear generalization to stimuli that were present during fear acquisition but were never paired with the US[12]. It has been suggested that both a failure to extinguish fear and fear generalization may be associated with poorer treatment outcome in anxiety patients[13].

Differences in fear conditioning at group level are reported commonly. However, this does not allow conclusions at the level of the individual, and therefore the focus is shifting from comparisons between predefined groups towards focusing on individual differences[14]. Several studies have investigated the existence of individual differences during a fear conditioning and extinction paradigm with the use of latent class growth analysis (LCGA)[14–19]. LCGA is a data-driven approach to investigate latent homogenous classes within a larger heterogeneous sample based upon similar patterns over time[20,21]. Two studies in patients with post-traumatic stress disorder (PTSD) and in rodents demonstrated three different classes based on freezing behavior and startle response[16,19]. Using LGCA, a differentiation was demonstrated between rapid extinguishers, slow extinguishers and a class that demonstrated a failure to extinguish fear. These two studies point to the importance of studying individual differences in fear extinction learning especially because a failure to extinguish fear is one of the core features in PTSD and anxiety disorders[2,10,11]. Exposure treatment is based on the fear conditioning model in which people

are repeatedly exposed to their feared stimuli in order to extinguish their fear response. The ability to differentiate individuals based on their fear learning class might help to indicate who will respond to the current state of the art treatments[22].

Another study tried to implement the fear conditioning and extinction model in a group of patients with different anxiety-related disorders and matched healthy controls[15]. In this study, different fear learning classes were not found based on potentiated startle response but rather on subjective measures of fear and US expectancy to the CS+ and CS-. Because of the post-hoc nature the statistical tests were at most explorative, but this study gave a preliminary indication that patients within the maladaptive fear classes of poor extinction and generalization showed an impaired treatment response[15]. Since the different classes in this specific study with anxiety patients were only found on subjective measures, we decided to modify the task that was used in this study into a short and 'easy to implement in clinical practice' version with a total duration of 15 minutes[15,17]. The proof-of-concept test in a sample of healthy subjects (*N*=300) showed good replicability of the fear learning classes[17]. In addition, follow-up measures indicated higher fear and US expectancy ratings one week after participation in the study to pictures of the CS+ and CS- for the maladaptive classes of poor extinction and generalization.

So far previous studies have mainly focused on identifying fear learning classes as a response to a fear conditioning and extinction task. This study takes these endeavors a step further by studying the associations between these fear learning classes and treatment outcome. Therefore, we made use of our previously developed fear conditioning task[17] in patients with various anxiety disorders and patients with PTSD. Patients with various anxiety-related disorders were recruited at different treatment facilities and subjected to the fear learning task prior to the start of their treatment. The primary aim was to replicate classes described in previous research[15–18]. The secondary aim was to investigate whether patients in these classes differed with regard to reduction in anxiety and overall psychological symptoms twelve weeks after starting initial treatment. Additionally, we investigated differences between classes in characteristics of the treatment, such as primary diagnosis, kind of treatment, and number of treatment sessions.

2. METHOD

2.1. Participants

Patients with various anxiety-related disorders (*N*=122), from the Military Mental Health Organisation (MGGZ), Altrecht Academic Anxiety Center (Altrecht), and special healthcare clinics for severe dental anxiety and dental phobia in Zwolle, Rijnmond and Utrecht (The Netherlands), were included in the study. Dental phobia a specific phobia according to the DSM-5 and treated with CBT, exposure therapy and EMDR by a dentist specialized in the treatment of patients with dentist anxiety[23–27]. Patients from all clinics were included if they received a primary diagnosis after intake for an anxiety disorder or trauma and

stressor related disorder according to DSM-5 criteria as determined by their therapist. Exclusion criteria included self-reported cardiovascular disorders and hearing problems. Additionally, individuals with a comorbidity of severe depression, bipolar disorder and psychoses were excluded from participation. The study was approved by the Ethics Review Board of the Faculty of Social Sciences of Utrecht University, The Netherlands (19-226).

2.2. Procedure

All patients had followed an intake procedure at one of the participating outpatient clinics with the aim to receive treatment for their anxiety symptoms. All participants were informed about the study and gave verbal and written informed consent. On the day before starting outpatient treatment, patients first participated in the fear conditioning procedure. During the first research session participants completed the Brief Symptom Inventory (BSI) and a fear conditioning task on a computer[28,29]. Twelve and twenty-four weeks after the initial visit participants completed an online follow-up questionnaire. The follow-up questionnaire consisted of the BSI and they were asked to rate 'fearfulness' and 'US expectancy' as in the experiment when showing the two pictures from the experiment again, see Figure 1; outcome ratings.

2.3. Fear conditioning task

The fear conditioning task was previously used and described[29]. In short, the conditioned stimuli (CS's) were two female faces with a neutral facial expression displayed in either a blue or green color against a black background. There were two different versions of the task with a fixed order that was counterbalanced. A female scream (85-95 dB(A), duration 1s) was used as unconditioned stimulus (US, adapted from[30]) and presented through over-ear headphones. The task consisted of 5 phases namely pre-conditioning, acquisition (uninstructed and instructed) and extinction (uninstructed and instructed). Except the pre-conditioning phase, all phases consisted of 2 blocks, see Figure 1. During pre-conditioning and extinction phases, the CS's were presented in the absence of the US. In both acquisition phases, one of the two faces (CS+) was followed by the scream (6 out of 8 trials). Prior to each phase, a text screen was displayed. In the uninstructed phases, the text screen contained a reminder to pay attention to the faces on the screen, but no explicit information about the CS-US relation. In the instructed phases the text screen included instructions about the CS-US contingency. Halfway the acquisition phase (after two acquisition uninstructed blocks) the participants were presented with the CS+ face and the text "The scream is only played during the presentation of the image presented above". Halfway the extinction phase the instruction was "You will no longer hear the scream in the next phase". After each block participants rated questions on a computerized VAS scales (range 0–100) for both faces (CS+ and CS-): How anxious/nervous the participants were in the displayed condition (fearfulness CS+ and fearfulness CS-), whether they thought the occurrence of a scream was very unlikely/likely (US expectancy CS+ and US expectancy CS-), how aversive they rated the scream (US) and

how certain they were of their answers. The task was programmed using OpenSesame version 26[31].

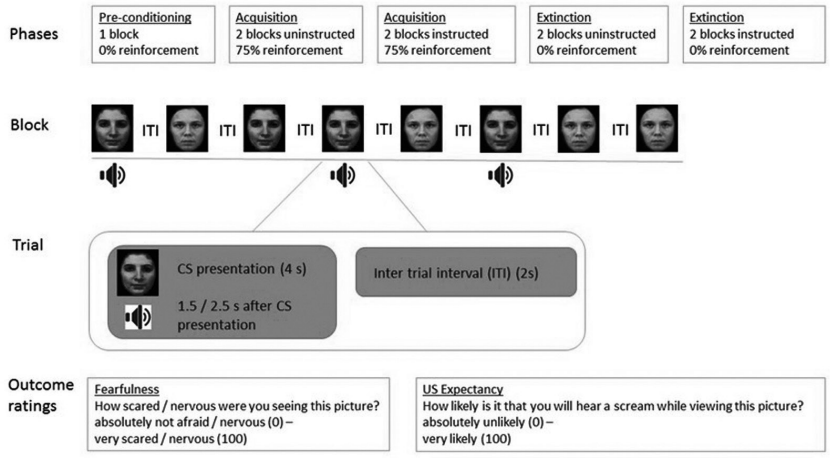

Figure 1. Overview of the fear conditioning task.

2.4. Treatment characteristics

Twelve and twenty-four weeks after the start of the initial treatment the therapist provided the following information about the participants in the study: diagnosis, comorbidity, type of treatment, number of treatment sessions, number of treatment sessions containing exposure and current medication use. For patients from Altrecht this information was collected from the electronic health record by one of the researchers.

2.5. Data analysis

Latent class growth analyses (LCGA) was conducted in MPlus (version 6.12)[32]. LCGA is a data-driven approach to investigate latent homogenous classes within a larger heterogeneous sample based upon similar patterns over time [20,21]. LCGA was conducted with one data point per block from the fear conditioning task for pre-conditioning and two for acquisition (uninstructed and instructed) and extinction (uninstructed and instructed). Analyses were conducted per outcome measure and stimulus type (fearfulness CS+/CS- and US expectancy CS+/CS-). The reliability of the loglikelihood estimation random sets starting value was set at 800 and the number of final optimizations at 200[33]. In case of convergence problems, the LRTSTARTS option was used, starting at LRTSTARTS = 0 0 100 20 [34]. These options can resolve potential convergence issues by increasing the number of random starts [34]. The number of LRTSTARTS was increased until convergence was reached[34].

Model fit for a nested model with a linear slope and both quadratic and cubic parameters were compared between models with 1–5 classes. Model selection was based on four criteria: 1) Apparent drops in Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC)[35]; 2) The Lo-Mendel-Rubin likelihood ratio test (LMR-LRT), the bootstrapped likelihood ratio test (BLRT), and the Vuong-Lo-Mendell-Rubin test (VLMR), 3) A large entropy score and 4) The smallest number of classes that would still be theoretically meaningful[36]. This was based on previous research that investigated different classes on fear conditioning paradigms[15–19]. Participants were assigned to the class for which they had obtained the highest probability according to the best fitting model. These classes were saved to IBM SPSS Statistics (version 27) for further analysis. Fit indices (BIC and AIC), parameter estimates for LCGA identified classes, and estimated means and observed individual values per class are presented in the Supplementary data, part 3.2-3.5.

Because the study is still ongoing only the twelve-week follow-up data were analyzed. Chi-square tests of Independence and one-way ANOVAs were applied to determine differences in characteristics between individuals assigned to the different classes (location, kind of treatment, medication use, expectancy and fear at the twelve-week follow-up, US aversiveness, baseline total symptoms, baseline anxiety symptoms, and number of treatment sessions). *P*-values were considered statistically significant at <0.05 and post-hoc Bonferroni correction was used to test the differences between classes in case of more than two classes.

3. RESULTS

3.1. Participants

The clinical characteristics of the included sample (*N*=122), with a mean age of 35.38 (*SD*=12.34) and a *N*=53/69 (43/57%) male/female ratio, are displayed in Table 1. General demographic characteristics are displayed in Table S1 in the Supplementary data. Mean total score at baseline on the BSI questionnaire was 1.04 (*SD*=0.69). On the anxiety subscale of the BSI the mean baseline score was 1.43 (*SD*=0.89). Mean number of treatment session between baseline and the twelve-week follow-up was 8.52 (*SD*=8.14).

Table 1. Participants clinical characteristics.

	N	%
Location		
Dental anxiety clinics	54	44.3
Altrecht	35	28.6
MGGZ	33	27.1
Diagnosis		

Table 1. Participants clinical characteristics. (continued)

	N	%
Dental Phobia (Specific Phobia)	61	50
Obsessive Compulsive Disorder	20	16.4
Panic Disorder	14	11.5
Post-traumatic Stress Disorder	7	5.7
Other specified anxiety disorder	3	2.5
Other specified trauma and stressor related disorder	3	2.5
Other Specific Phobias	2	1.6
Adjustment disorder	2	1.6
Illness anxiety disorder	1	0.8
Social Phobia	1	0.8
Treatment*		
Exposure therapy	62	50.8
Weekly sessions	32	26.2
Intensive sessions	30	24.6
Eye Movement Desensitization and Reprocessing (EMDR)	20	16.4
Cognitive behavioural therapy (CBT)	24	19.7
Other**	11	9.0
Medication*	22	18.0
Selective serotonin reuptake inhibitors (SSRIs)	19	15.6
Benzodiazepines (BENZOs)	4	3.3

*Individuals could follow more than 1 type of treatment or type of medication.
**This included different group treatments and expressive therapies.
Sample sizes might not add up to total participants due to missing data.

3.2. Classes based on the subjective fearfulness ratings to the CS+

LCGA demonstrated three distinct classes on the subjective fear rating to the CS+, see also Table 2 and Figure 2. The 3-class model met all the model-based selection criteria. The largest class (63%, *N*=77) reported low fear during all phases of the experiment and was labeled 'low fearful conditioners.' The second largest class (30%, *N*=37) demonstrated an increase in fear during the acquisition phases and a decrease in anxiety during extinction phases was labeled 'normal conditioners.' Lastly, a small group (7%, *N*=8) that was labeled 'poor extinguishers' showed an increase in fear during acquisition phases that sustained during extinction phases.

Table 2. Fit indices for one-to-five Latent Growth Models based on fearfulness ratings to the CS+.

No. Of classes	AIC	BIC	BLRT	VLMR	LMR-LRT	Entropy	Sample Size Per Class Based on Most Likely Class Membership	Class probabilities
1	10554	10591	-	-	-	-	122	1
2[a]	9875	9926	*p*=<.001	*p*=<.001	*p*=<.001	.980	77/45	.999/.989
3[b]	**9609**	**9673**	***p*=<.001**	***p*=.006**	***p*=.007**	**.987**	**77/37/8**	**.993/.999/1**
4[b]	9439	9518	*p*=<.001	*p*=.092	*p*=.103	.977	73/26/16/7	.996/.962/.990/1
5[b]	9357	9450	*p*=<.001	*p*=.212	*p*=.223	.955	53/24/22/15/8	.969/.959/.975/.994/.999

[a] LRTSTARTS = 0 0 100 20; [b] LRTSTARTS = 0 0 1000 180. AIC = Akaike information criterion; BIC = Bayesian information criterion; BLRT = bootstrap likelihood ratio test; VLMR = Vuong-Lo-Mendell-Rubin test. Best fitting model values are displayed in bold.

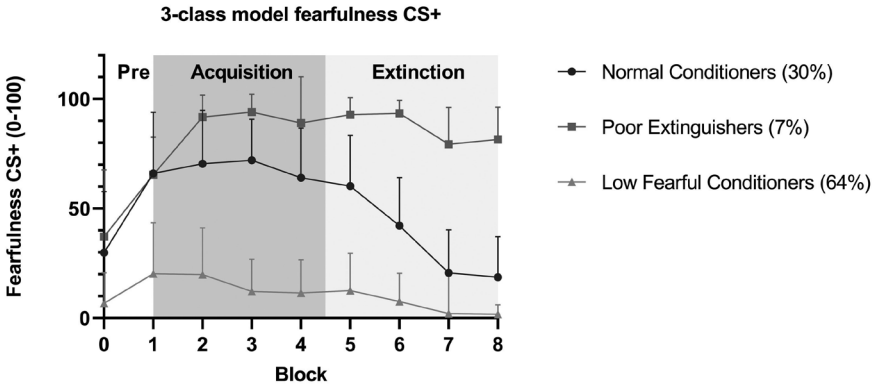

Figure 2. Estimated means and standard deviation of the final model on fearfulness ratings to the CS+. Blocks 1,2,5 and 6 are uninstructed blocks and blocks 3,4,7 and 8 are instructed blocks; Pre = pre-conditioning block, see also figure 1.

3.3. Classes based on the subjective fearfulness ratings to the CS-

For the fear rating to the CS- LCGA demonstrated two classes, see also Table 3 and Figure 3. The 2-class model also met all the model-based selection criteria. The first and largest class was labeled as 'non-generalizers' (81%, *N*=99) and demonstrated low fearfulness scores throughout all phases of the experiment. The second class 'generalizers' (19%, *N*=23) was characterized by higher fear scores during all the phases of the experiment.

Table 3. Fit indices for one-to-five Latent Growth Models based on fearfulness ratings to the CS-.

No. Of classes	AIC	BIC	BLRT	VLMR	LMR-LRT	Entropy	Sample Size Per Class Based on Most Likely Class Membership	Class probabilities
1	10084	10121	-	-	-	-	122	1
2	**9525**	**9576**	***p*=<.001**	***p*=.023**	***p*=.026**	**.986**	**99/23**	**.999/.984**
3[a]	9312	9377	*p*=<.001	*p*=.319	*p*=.309	.982	92/23/7	.994/.980/.999
4[a]	9228	9307	*p*=<.001	*p*=.547	*p*=.555	.979	87/17/12/6	.998/.947/.974/1
5[a]	9163	9256	*p*=<.001	*p*=.448	*p*=.452	.971	83 / 17 / 11 / 7 / 4	.982 / .955 / .996 / 1 / 1

[a] LRTSTARTS = 0 0 2000 360. AIC = Akaike information criterion; BIC = Bayesian information criterion; BLRT = bootstrap likelihood ratio test; VLMR = Vuong-Lo-Mendell-Rubin test. Best fitting model values are displayed in bold.

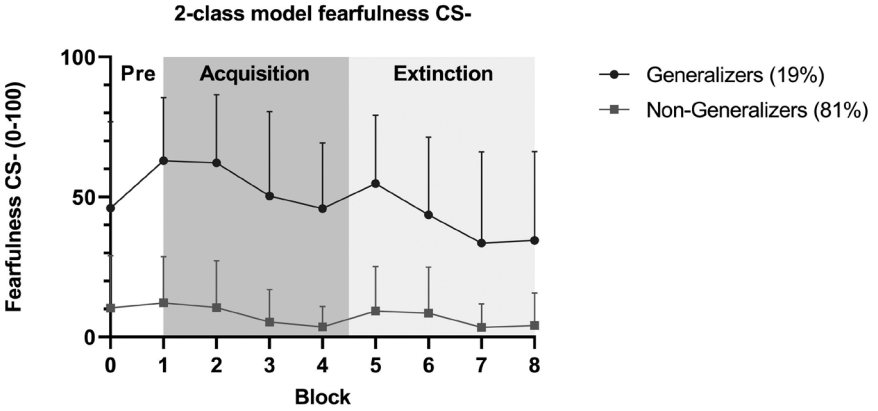

Figure 3. Estimated means and standard deviation of the final model on fearfulness ratings to the CS+. Blocks 1,2,5 and 6 are uninstructed blocks and blocks 3,4,7 and 8 are instructed blocks; Pre = pre-conditioning block, see also figure 1.

3.4. Classes based on the subjective US expectancy ratings to the CS+

To decide on the number of classes on US expectancy rating to the CS+ both the 2- and 3-class model yielded results that comply with the model-based selection criteria. Although both VLMR and LMR-LRT tests approached significance in the 3-class model we decided to choose this latter model. The 3-class model was favored because additional selection criteria were superior compared to the 2-class model, see also Table 4. More specific, both BIC and entropy score favored the 3-class model and these two criteria are preferred and decisive in model (class) selection[35]. Lastly, the added third class of poor extinguishers was theoretically relevant with regard to previous research[15–19], see also the Supplementary data part 3.4.1 for the estimated means and observed individual values for the 2-class model. In the 3-class model the largest class 'normal conditioners' (65%, *N*=79) demonstrated an increased US expectancy during the acquisition phases and a decrease in expectancy during extinction phases. The other two classes 'poor extinguishers' (18%, *N*=22) and 'low fearful conditioners' (17%, *N*=21) were of equal size. 'Poor extinguishers' demonstrated high US expectancy during all phases of the experiment and 'low fearful conditioners' low expectancy during all phases of the experiment, see also Figure 4.

Table 4. Fit indices for one-to-five Latent Growth Models based on US expectancy ratings to the CS+.

No. Of classes	AIC	BIC	BLRT	VLMR	LMR-LRT	Entropy	Sample Size Per Class Based on Most Likely Class Membership	Class probabilities
1	10703	10740	-	-	-	-	122	1
2	10420	10470	<.001	*p*=.007	*p*=.009	.962	100 / 22	.993 / .975
3[a]	**10244**	**10309**	**<.001**	***p*=.055**	***p*=.061**	**.969**	**79 / 22 /21**	**.995 / .974 / .979**
4[b]	10180	10258	<.001	*p*=.296	*p*=.310	.969	69 / 20 / 18 / 15	.992 / .968 / .959 / .997
5	10144	10237	<.001	*p*=.209	*p*=.219	.976	65 / 19 / 15 / 12 / 11	.994 / .970 / 1 / .940 / .989

[a] LRTSTARTS = 0 0 1000 180; [b] LRTSTARTS = 0 0 2000 360. AIC = Akaike information criterion; BIC = Bayesian information criterion; BLRT = bootstrap likelihood ratio test; VLMR = Vuong-Lo-Mendell-Rubin test. Best fitting model values are displayed in bold.

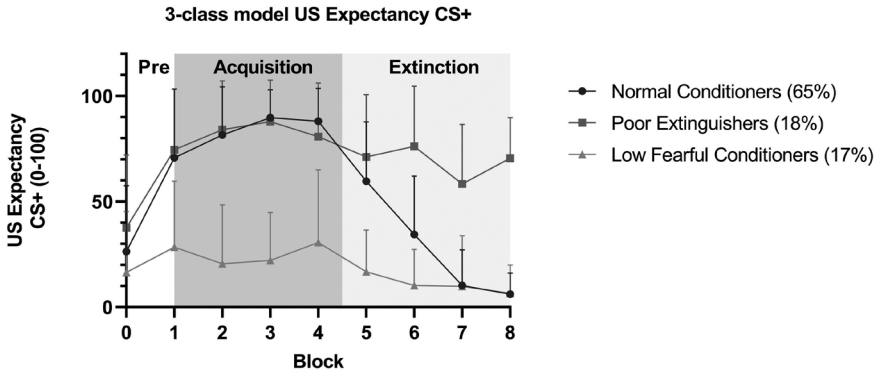

Figure 4. Estimated means and standard deviation of the final model on fearfulness ratings to the CS+. Blocks 1,2,5 and 6 are uninstructed blocks and blocks 3,4,7 and 8 are instructed blocks; Pre = pre-conditioning block, see also figure 1.

3.5. Classes based on the subjective US expectancy ratings to the CS-

For US expectancy rating to the CS- LCGA demonstrated two, see also Table 5 and Figure 5. The largest class 'non-generalizers' (78%, *n*=95) demonstrated low US expectancy during all phases of the experiment. 'Generalizers' (22%, *n*=27) reported higher expectancy of the US during all phases of the experiment.

Table 5. Fit indices for one-to-five Latent Growth Models based on US expectancy ratings to the CS-.

No. Of classes	AIC	BIC	BLRT	VLMR	LMR-LRT	Entropy	Sample Size Per Class Based on Most Likely Class Membership	Class probabilities
1	10439	10475	-	-	-	-	122	1
2	**9964**	**10014**	***p*=<.001**	***p*=.003**	***p*=.004**	**.976**	**95 / 27**	**.996 / .989**
3[a]	9795	9860	*p*=<.001	*p*=.558	*p*=.569	.978	92 / 19 / 11	.993 / .980 / .997
4[a]	9684	9763	*p*=<.001	*p*=.281	*p*=.290	.990	91 / 15 / 10 / 6	1 / .969 / .996 / 1
5[a]	9614	9706	*p*=<.001	*p*=.533	*p*=.542	.957	77 / 22 / 9 / 8 / 6	.973 / .950 / .969 / .991 / 1

[a] LRTSTARTS = 0 0 4000 720. AIC = Akaike information criterion; BIC = Bayesian information criterion; BLRT = bootstrap likelihood ratio test; VLMR = Vuong-Lo-Mendell-Rubin test. Best fitting model values are displayed in bold.

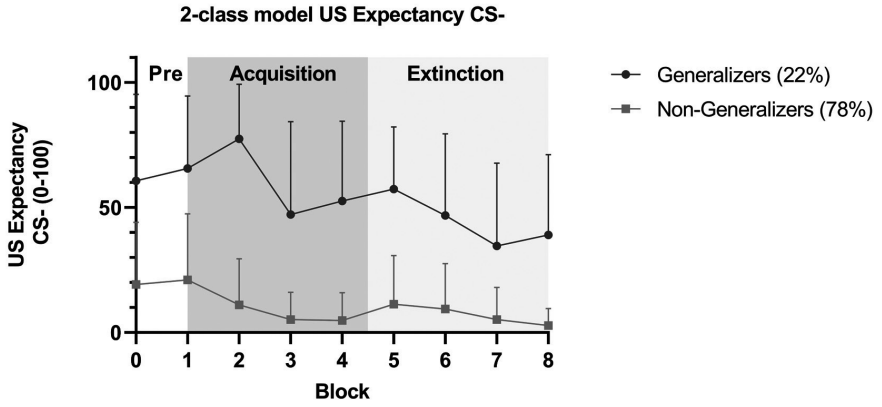

Figure 5. Estimated means and standard deviation of the final model on fearfulness ratings to the CS+. Blocks 1,2,5 and 6 are uninstructed blocks and blocks 3,4,7 and 8 are instructed blocks; Pre = pre-conditioning block, see also figure 1.

3.6. Clinical Symptoms

From the total sample size of *N*=122, 35.2% (*N*=43) did not completed the follow-up measures and 4.1% (*N*=5) still needed to complete the twelve-week follow-up. Responding participants did not differ from non-responding patients with regard to baseline BSI total scores ($F(1,116=.036$, $p=.850$) and anxiety scores ($F(1,116=.124$, $p=.725$). Therefore, the remaining sample size of *N*=74 was used to analyze the reduction of clinical symptoms over time with regard to the different fear learning classes. This resulted in no significant reduction in total clinical symptoms and anxiety symptoms between the different fear learning classes, see also Table 6 and Figure 6.

3.7. Baseline differences

Baseline symptoms (total symptoms and anxiety symptoms) were higher for normal conditioners than for low fearful conditioners (fearfulness CS+ classes) and higher for generalizers than non-generalizers (US expectancy CS- classes), see table 7 and 8. With regard to the aversiveness of the scream (US) all classes demonstrated significant differences. For the fearfulness CS+ (Table 7), low fearful conditioners had lower aversiveness to the scream than normal conditioners and poor extinguishers (Bonferroni corrected). For the US expectancy CS+ (Table 7), the class of low fearful conditioners had lower aversiveness to the scream than poor extinguishers. Generalizers from both the CS- classes (fearfulness and US expectancy) demonstrated higher aversiveness ratings to the US than non-generalizers.

3.8. Treatment characteristics

Chi-squares tests were used to investigate differences in the distribution of nominal variables medication and type of treatment across different classes. The statistics are

presented in Table 9 and all data in Table S2-S5. There were several significant differences regarding treatment parameters. In the Fearfulness CS- class the group that received intensive exposure treatment consisted of more generalizers (36.7%) than non-generalizers (63.3%). This was also the case for the US expectancy CS- class where more generalizers (standardized residual 5.9; 40%) than non-generalizers (60%) followed this type of treatment. In addition, the fearfulness CS+ classes, normal conditioners received more treatment sessions than low fearful conditioners (Table 10). Additionally, with respect to both the fear and US expectancy CS- classes generalizers received more treatment sessions than non-generalizers. Lastly, the US expectancy CS+ classes demonstrated differences with regard to the group of individuals that received exposure treatment (weekly sessions). That is, more low fearful conditioners received exposure treatment (34.4%), than poor extinguishers (9.4%), normal conditioners (56.3%).

3.9. Treatment location

The treatment locations (MGGZ, dentist anxiety clinics, Altrecht) differed in respect to the distributions of the US expectancy CS- classes. The statistics are presented in Table 9 and Table S2-S5. At the dental anxiety clinics there were more non-generalizers (80.3%) than generalizers (19.7%), at the MGGZ more non-generalizers (92.3%) than generalizers (7.7%), and at Altrecht more generalizers (37.1%) than non-generalizers (62.9%).

3.10. Twelve-week follow-up

Finally, ANOVAs were used to investigate the association between the fear learning classes based on fear (Table 11) and US expectancy (Table 12) and scores on the fear and US expectancy ratings for the CS+ and CS- pictures shown again twelve weeks after completion of the initial experiment. The class of normal conditioners based on subjective fear to the CS+ showed higher fear than the low fear class twelve weeks after the experiment to both the pictures that were used as CS+ and CS-. The class of generalizers in subjective fear to the CS- demonstrated higher fear to the CS+ and CS- picture and higher US expectancy to the CS- picture (Table 11) compared to the non-generalizers. Lastly, for the US expectancy CS- classes, generalizers scored higher than non-generalizers on both fear and US expectancy measures for both CS+ and CS- pictures (Table 12).

Table 6. Statistical comparison of the percentage reduction in total clinical symptoms and anxiety symptoms between the different fearfulness (CS+ and CS-) and US Expectancy (CS+ and CS-) classes.

		Test statistic	*p*-value
Total Symptoms			
	Fearfulness CS+	*F*=.845	.434
	Fearfulness CS-	*F*=.234	.630
	US Expectancy CS+	*F*=.809	.449
	US Expectancy CS-	*F*=.117	.733
Anxiety Symptoms			
	Fearfulness CS+	*F*=.153	.858
	Fearfulness CS-	*F*=.584	.447
	US Expectancy CS+	*F*=.184	.833
	US Expectancy CS-	*F*=1.239	.269

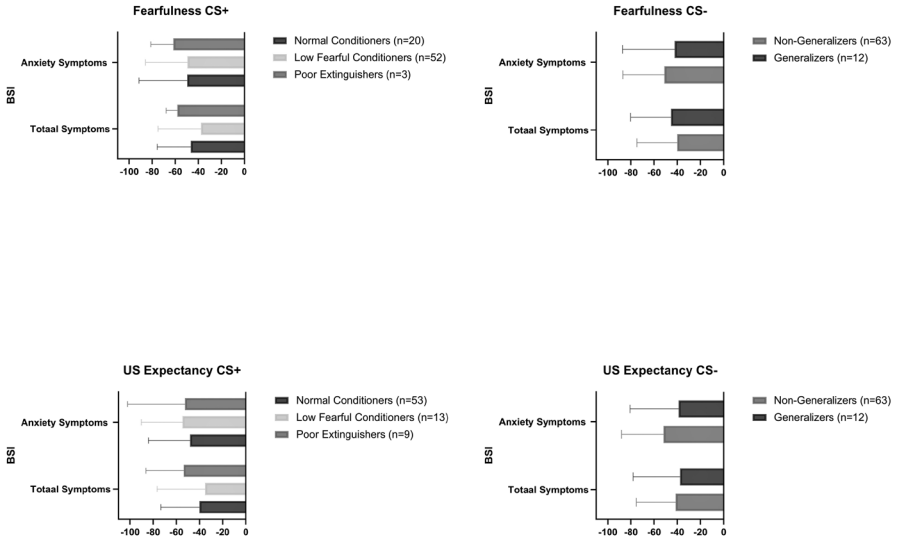

Figure 6. Mean percentage improvement and standard deviation on the BSI (total score and anxiety subscale), displayed per model and class.

Table 7. Statistics and mean differences in aversiveness and clinical symptoms for the subjective fear CS+ and CS- classes.

Classes Fearfulness CS+	Low fearful conditioners (*N*=77)		Normal conditioners (*N*=37)		Poor Extinguishers (*N*=8)		Test Statistic	p-value	Cohen's *d*
	M	*SD*	*M*	*SD*	*M*	*SD*			
Aversiveness Scream	51.17^	29.40	80.04^	15.36	95.99^	6.08	*F*=24.160	<.001*	.64
Total Symptoms	0.89#	0.67	1.27#	0.63	1.44	0.77	*F*=5.584	.005*	.31
Anxiety Symptoms	1.23#	0.84	1.74#	0.93	1.83	0.71	*F*=5.363	.006*	.30
Classes Fearfulness CS-	Non-generalizers (*N*=99)		Generalizers (*N*=23)				Test Statistic	p-value	Cohen's *d*
	M	*SD*	*M*	*SD*					
Aversiveness Scream	58.18	29.92	83.03	15.68			*F*=14.848	<.001*	.35
Total Symptoms	1.01	0.69	1.21	0.68			*F*=1.672	.199	NA
Anxiety Symptoms	1.36	0.86	1.72	0.99			*F*=3.230	.075	NA

*ANOVA significant with a *p*=<.05; #These classes differ significantly after Bonferroni correction; ^ Low fearful conditioners differed from both normal conditioners and poor extinguishers after Bonferroni correction.

Table 8. Statistics and mean differences in aversiveness and clinical symptoms for the US Expectancy CS+ and CS- classes.

Classes US Expectancy CS+	Low fearful conditioners (*N*=21)		Normal conditioners (*N*=79)		Poor Extinguishers (*N*=22)		Test Statistic	p-value	Cohen's *d*
	M	*SD*	*M*	*SD*	*M*	*SD*			
Aversiveness Scream	46.65#	30.79	62.50	26.43	79.65#	30.46	*F*=7.510	.001*	.36
Total Symptoms	0.80	0.77	1.05	0.67	1.28	0.64	*F*=2.712	.071	NA
Anxiety Symptoms	1.29	1.05	1.39	0.89	1.67	0.71	*F*=1.164	.316	NA
Classes US Expectancy CS-	Non-generalizers (*N*=95)		Generalizers (*N*=27)				Test Statistic	p-value	Cohen's *d*
	M	*SD*	*M*	*SD*					
Aversiveness Scream	59.94	28.89	73.15	29.46			*F*=4.358	.039*	.19
Total Symptoms	0.98	0.67	1.29	0.72			*F*=4.484	.036*	.19
Anxiety Symptoms	1.31	0.83	1.83	1.00			*F*=7.617	.007*	.25

*ANOVA significant with a *p*=<.05; #These classes differ significantly after Bonferroni correction.

Table 9. Statistics for treatment characteristics shown separately for the different classes based on subjective fear ratings to the CS+ and CS-, and on ratings of US expectancy to the CS+ and CS-.

	Fearfulness CS+			Fearfulness CS-			US Expectancy CS+			US Expectancy CS-		
	Test Statistic	p-value	φ/*V*	Test Statistic	p-value	φ/*V*	Test Statistic	p-value	φ/*V*	Test Statistic	p-value	φ/*V*
Location	$\chi^2(4, N=122)=12.421$	.014*	.23	$\chi^2(2, N=122)=10.733$	.005*	.30	$\chi^2(4, N=122)=14.727$	.005*	.25	**$\chi^2(2, N=122)=7.936$**	**.019*#**	**.26**
Medication use												
SSRI	$\chi^2(2, N=104)=10.162$	.006*	.31	$\chi^2(1, N=104)=1.008$	.333	NA	$\chi^2(2, N=104)=4.124$	.127	NA	$\chi^2(1, N=104)=.371$	.544	NA
Benzo	$\chi^2(2, N=104)=1.060$	.589	NA	$\chi^2(1, N=104)=.126$	.560	NA	$\chi^2(2, N=104)=.974$	.614	NA	$\chi^2(1, N=104)=2.075$	.196	NA
Treatment												
Exposure	$\chi^2(2, N=93)=9.219$	.010*	.32	$\chi^2(1, N=93)=3.113$	.100#	NA	**$\chi^2(2, N=93)=10.535$**	**.005*#**	**.34**	$\chi^2(1, N=93)=.693$	.589#	NA
Exposure Intensive	$\chi^2(2, N=93)=7.926$	.019*	.29	**$\chi^2(1, N=93)=8.503$**	**.005*#**	**.30**	$\chi^2(2, N=93)=1.821$	.402	NA	**$\chi^2(1, N=93)=10.433$**	**.002*#**	**.34**
EMDR	$\chi^2(2, N=93)=2.472$	.291	NA	$\chi^2(1, N=93)=.310$	.754	NA	$\chi^2(2, N=93)=1.657$	.437	NA	$\chi^2(1, N=93)=1.705$	.346	NA
CBT	$\chi^2(2, N=93)=2.413$	.299	NA	$\chi^2(1, N=93)=2.517$	.142	NA	$\chi^2(2, N=93)=4.808$	.090	NA	$\chi^2(1, N=93)=2.912$	.140	NA
Other	$\chi^2(2, N=93)=1.836$	.399	NA	$\chi^2(1, N=93)=2.312$	.214	NA	$\chi^2(2, N=93)=2.609$	.271	NA	$\chi^2(1, N=93)=.359$	.690	NA

*Chi-square significant with a $p=<.05$; # In these analyses all cells had a minimum value of 5. Other chi-square test should be interpreted with caution since expected counts are less than 5. Only the ones that pass this criterion and that are significant (highlighted in **bold**) are discussed in the text. Distributions across the different classes are shown in the Supplementary data Table S2-S5.

Table 10. Number of treatment sessions for the subjective fear (CS+ and CS-) and US expectancy (CS+ and CS-) classes.

	M	*SD*	Test Statistic	p-value	Cohen's *d*
Classes Fearfulness CS+			*F*=3.560	.032*	.28
Low fearful conditioners (*n*=59)	6.98#	7.95			
Normal conditioners (*n*=26)	11.96#	7.82			
Poor extinguishers (*n*=6)	8.67	8.02			
Classes Fearfulness CS-			*F*=7.407	.008*	.29
Non-generalizers (*n*=75)	7.48	7.61			
Generalizers (*n*=16)	13.38	9.02			
Classes US expectancy CS+			*F*=2.696	.073	NA
Low fearful conditioners (*n*=16)	4.31	5.72			
Normal conditioners (*n*=61)	9.48	8.71			
Poor extinguishers (*n*=14)	9.14	6.66			
Classes US expectancy CS-			*F*=6.557	.012*	.27
Non-generalizers (*n*=74)	7.50	7.54			
Generalizers (*n*=17)	12.94	9.37			

*ANOVA significant with a *p*=<.05; #These classes differ significantly after Bonferroni correction.

Table 11. Differences in fear ratings during the twelve-week follow-up for the different fear CS+ and CS- classes (*n*=74).

Classes Fearfulness CS+	Low fearful conditioners (*N*=51)		Normal conditioners (*N*=20)		Poor Extinguishers (*N*=3)		Test Statistic	p-value	Cohen's *d*
	M	*SD*	*M*	*SD*	*M*	*SD*			
Fearfulness CS+	13.35#	20.78	29.80#	25.70	39.33	19.40	*F*(2,73)=5.288	.007*	.39
Fearfulness CS-	12.41#	21.63	27.00#	21.98	33.00	21.52	*F*(2,73)=4.068	.021*	.34
US Expectancy CS+	20.04	30.62	32.20	28.06	37.67	24.58	*F*(2,73)=1.523	.225	NA
US Expectancy CS-	15.75	23.48	24.35	23.40	48.33	27.54	*F*(2,73)=3.312	.042*	.31
Classes Fearfulness CS-	Non-generalizers (*N*=62)		Generalizers (*N*=12)				Test Statistic	p-value	Cohen's *d*
	M	*SD*	*M*	*SD*					
Fearfulness CS+	15.68	20.72	35.25	30.21			*F*(1,73)=7.653	.007*	.33
Fearfulness CS-	13.90	19.03	34.17	31.79			*F*(1,73)=8.950	.004*	.35
US Expectancy CS+	21.82	29.52	35.50	31.23			*F*(1,73)=2.120	.150	NA
US Expectancy CS-	16.74	23.02	33.08	27.22			*F*(1,73)=4.776	.032*	.26

*ANOVA significant with a *p*=<.05; #These classes differ significantly after Bonferroni correction.

Table 12. Differences in US expectancy ratings during the twelve-week follow-up for the different US Expectancy CS+ and CS- classes (*n*=74).

Classes US Expectancy CS+	Low fearful conditioners (*N*=13)		Normal conditioners (*N*=52)		Poor Extinguishers (*N*=9)		Test Statistic	p-value	Cohen's *d*
	M	*SD*	*M*	*SD*	*M*	*SD*			
Fearfulness CS+	20.62	28.84	17.08	22.87	26.56	25.66	*F*(2,73)=.666	.517	NA
Fearfulness CS-	21.31	25.40	15.94	22.81	18.44	18.54	*F*(2,73)=.303	.740	NA
US Expectancy CS+	23.15	25.41	23.06	31.10	31.00	31.96	*F*(2,73)=.270	.764	NA
US Expectancy CS-	31.00	30.75	14.45	20.88	30.56	26.23	*F*(2,73)=3.715	.029*	.32
Classes US Expectancy CS-	Non-generalizers (*N*=62)		Generalizers (*N*=12)				Test Statistic	p-value	Cohen's *d*
	M	*SD*	*M*	*SD*					
Fearfulness CS+	15.61	21.07	35.58	28.59			*F*(1,73)=8.002	.006*	.33
Fearfulness CS-	13.82	19.89	34.58	28.40			*F*(1,73)=9.454	.003*	.36
US Expectancy CS+	20.82	29.58	40.67	27.70			*F*(1,73)=4.613	.035*	.25
US Expectancy CS-	15.15	22.08	41.33	24.36			*F*(1,73)=13.689	<.001*	.44

*ANOVA significant with a *p*=<.05.

4. DISCUSSION

The current study aimed to investigate different fear learning classes in patients (*N*=122) with various anxiety-related disorders. We investigated additionally whether the different fear learning classes differed with regard to symptom reduction twelve weeks after the start of the initial anxiety related treatment. Lastly, we investigated differences in treatment related variables (kind of treatment, number of sessions, and medication use) and other related characteristics between the different fear learning classes.

4.1. Fearfulness fear learning classes

Our study replicated fear learning classes found in previous studies[15–17,19]. However, our focus has been on subjective measures as in two previous studies[15,17]. To that end, we used a fear conditioning task that we developed that is short and easy to implement in clinical practice in patients with various anxiety-related disorders[17]. Other studies found similar fear learning classes when using fear potentiated startle (FPS) measures[16,19]. However, measuring fear classes with the use of FPS or other physiological measure required special equipment and a longer duration of the test because of the physiological measures, and was therefore more difficult to implement in clinical situations[37]. For our first outcome measure, the subjective fear response to the CS+, we could differentiate between three classes, similar as in previous studies[15–19]. These classes were normal conditioners (30%), low fearful conditioners (64%) and poor extinguishers (7%). Surprisingly, we found a smaller class of poor extinguishers and a larger group of low fearful conditioners in comparison to our study in healthy students (*N*=300; respectively 56% normal conditioners, 32% low fearful conditioners, and 12% poor extinguishers)[17]. This was also dissimilar to the study by Duits et al. (2021) which combined patients with various anxiety-related disorders (*N*=104) and healthy comparison subjects (*N*=93)[15]. In that study the overall distribution was 50% normal conditioners, 32% low fearful conditioners and 18% poor extinguishers, whereas the patients were specifically overrepresented in the poor extinguishers class (27%). However, our results do correspond with the study of Galatzer-Levy et al. (2017) in individuals with PTSD (*N*=724) who used FPS as an outcome measure[16]. In this study the distribution was 14.8% high FPS extinguishers (corresponding to our normal conditioner class), 78.9% modal FPS responders (corresponding to our low fearfulness class) and 6.3% high FPS non-extinguishers (responding to our poor extinguishers class)[16]. However, there are a couple of important differences between the current study and other studies in patients that may account for these differences[15,16].

The variability in the anxiety diagnosis that was included differed. Our sample mainly consisted of individuals with dental phobia (50%) and obsessive compulsive disorder (16.4%). In other studies the majority of patients had PTSD or social anxiety disorder (29%) and, panic disorder and/or agoraphobia (25.8%)[15,16]. Although a failure to extinguish fear is a general feature of individuals with anxiety disorders this could also be

different across patients with anxiety disorders[2,8,9,38–40]. To our knowledge this was never investigated in individuals with dental anxiety which accounts for half of our sample.

Another factor is the unconditioned stimulus (US), which was an annoying stimulus (a loud scream) but not a painful stimulus such as the electric shock used in the previous studies[15,16]. A comparison between extinction classes of US expectancy with an aversive versus a non-aversive US indicated that assignment to classes differed depending on the US, but also showed quite substantial overlap[18]. However, US expectancy is related to a more factual estimate of occurrence, and it is likely that fear ratings are more susceptible to the aversiveness of the US[41]. In addition, the shock intensity is usually set at the individual level that is perceived as uncomfortable[3]. However, to our knowledge, this is less common when loud noises are used as US. In our study it was also demonstrated that low fearful conditioners and non-generalizers rated the US as less aversive. In order to equalize scream aversiveness across individuals, an individual adjustment to set the intensity of the scream may be considered.

Lastly, differences might be due to differences in background of the sample and/or the different context in which the task was completed. Military personnel are possibly less fearful of the relatively innocuous scream (US). This may explain the high percentage of military patients that were characterized as low fearful (58%). Military personnel has been selected for specific personality traits to perform well under pressure and dealing with unpredictable and uncontrollable factors during operations[42]. The high percentage of patients who reported low fear induced by the scream in the dental anxiety clinics may be due to the task being executed in a context that is specifically fearful for them: the treatment room, right next to the dentist chair. In addition, the task was assessed by the dentist itself and not by a researcher as on the other locations. Being already exposed to the context of their phobia may have overshadowed the minor fear induced by the scream, which may explain their high proportion of low fear individuals (77%). An alternative potential explanation is the relatively high percentage of individuals with alexithymia, for whom subjective outcome measures may not be sensitive to fear[43].

Finally, the percentage of patients with low fear in the sample from Altrecht (43%) was still higher than in the study by Duits et al. (2021)[29], even though this sample was drawn from a very similar population. Hence, we cannot rule out that the less aversive US led to a larger proportion of patients being characterized as low fearful. Additionally, at all three locations the portion of poor extinguishers was relatively small (respectively 3.8%, 4.9% and 11.4%), which may also be related to the relatively mild US. All these different choices with regard to the design and context of the experiment may have influenced the outcomes and should be again considered in future studies[3,44,45].

With regard to the fear ratings to the CS-, the observed generalizers (19%) and non-generalizers (81%) classes have been reported before[15,17]. In our previous study in healthy

subjects we also found a third class of 'safety ambiguous'[17]. This 'safety-ambiguous' class was characterized by an increase in fear to the CS- during uninstructed blocks and a decrease in fear during the instructed blocks. We interpreted this as an increase of fear generalization of fear when contingencies were somewhat ambiguous. The fact that the safety-ambiguous class was not observed in the current study may be due to a smaller sample size. It could also be related to differences in the population (anxiety patients in comparison to healthy controls), because the model with two classes in line with our previous study in anxiety patients[15].

4.2. US expectancy fear learning classes

For the US expectancy ratings to the CS+ we found beside the normal conditioners (65%) and low fearful conditioners (17%) an additional class of poor extinguishers (18%) that was not reported in previous studies[15,29]. The US expectancy rating is a more cognitive measure of fear in that it asks to report a contingency in the environment rather than a subjective state in response to these contingencies[41]. Nevertheless, it provides indication to the extent of which participants expect an aversive outcome and grasp changes in beliefs that are crucial to fear learning[45]. High US expectancy to the CS+ is also seen in people with PTSD. That is, they show higher US expectancy rating to the CS+ during extinction in comparison to healthy controls.[46,47] This can be interpreted as a failure to retain observed evidence, even when the contingencies have been attended to. In addition, memory impairment and processing negative information more quickly than neutral or positive stimuli is characteristic for PTSD[48,49]. How this is related to US expectancy ratings and if this particular class may be especially relevant in PTSD and potentially is related to treatment outcome still needs to be elucidated. Finally, for the US expectancy rating to the CS- we again replicated classes of generalizers (22%) and non-generalizers (78%) that were found in previous studies[15,29].

4.3. Treatment outcome

None of the four outcome measures and their classes were associated with improvement on the BSI (total symptoms and anxiety symptoms) twelve weeks after starting treatment. This in is contrast with other studies that give indications for the association between fear extinction learning and treatment outcome[15,50,51]. Although the other study in patients by Duits et al. (2021)[15] found indications of a treatment effect, future studies might also benefit from incorporating more disorder specific questionnaires. Half of our sample consisted of dental anxiety patients and the Dental Anxiety Scale (DAS) and Dental Fear Survey (DFS) are questionnaires developed especially for this disorder[52,53]. Although the BSI measures general anxiety and can also be used in dental anxiety, more disorder specific questionnaires are possibly better to detect treatment success at follow-up[54]. In addition, 40% of individuals with dental phobia also experience comorbid diagnoses and psychiatric symptoms which may account for the stability in BSI symptoms.[55]

An important limitation in this respect in our study is that the groups were relatively small, especially of the dysfunctional poor extinction (*n*=3 for the fearfulness CS+ class and *n*=9 for the US expectancy CS+ class) and generalization classes (*n*=12 for the US expectancy in both CS+ and CS- classes). This may have precluded significant differences between this class and the other classes in the model. This reflects the preliminary nature of the current analysis. Future studies with larger sample sizes will allow more definitive conclusions regarding the predictive nature of dysfunctional fear learning classes on therapy outcomes. Interestingly, classes did differ in the ratings to the former CS+/CS- pictures at follow-up. Classes of normal conditioners compared to low fear participants, and generalizers compared to non-generalizers scored higher on fear and expectancy to the pictures that were used in the experiment, twelve weeks after completion of the initial experiment. This finding was similar to what was found in our previous study and point to the validity of the task[17]. However, the question remains to what extent this can be extended to ecological validity in relation to treatment outcome[56].

4.4. Other characteristics

Overall, the classes that can be qualified as dysfunctional were associated with higher aversiveness toward the scream. Considering that the scream (US) was of a constant loudness, this suggests a possible lower sensitivity threshold to aversiveness of stimuli in these individuals[57]. For the Fearfulness CS+ classes, normal conditioners received more treatment sessions than low fearful conditioners. Additionally, with respect to the fearfulness and US expectancy CS- classes generalizers received more treatment session than non-generalizers. Suggesting more treatment sessions are needed is these classes to treat anxiety symptoms[58]. Lastly, at baseline total and anxiety symptoms were higher for normal conditioners than for low fearful conditioners (Fearfulness CS+ classes) and higher for generalizers than non-generalizers (US expectancy CS- classes). For generalizers versus non-generalizers, the trend is for the maladaptive fear class of generalizers to experience higher symptoms at baseline and for them to subsequently need more treatment sessions.

5. CONCLUSION

The identification of different fear learning classes was investigated in a sample of patients with various anxiety-related disorders. Although fear learning classes were replicated from previous studies, we were not able to predict treatment success. Because these are preliminary results and the study is still ongoing, analysis with a larger sample size should give more insight into the relationship between fear classes, symptoms reduction and treatment characteristics. More insight into the relation between fear learning classes and treatment outcome might lead to a better comprehension of the development and treatment of anxiety disorders. In the future this may help to develop a more personalized treatment with the use of screening tools, like our fear conditioning task, that is short and easy to implement in clinical practice.

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Declaration of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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SUPPLEMENTARY DATA

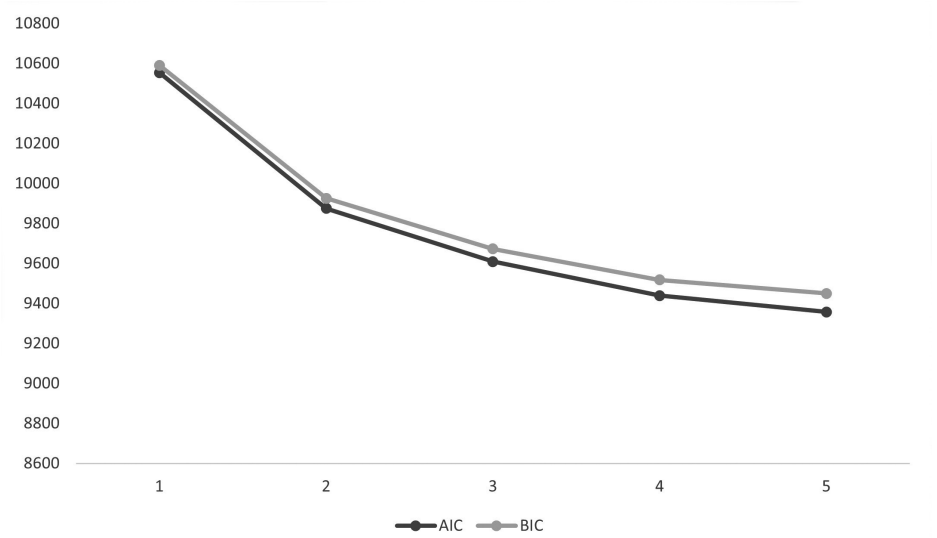
3.1. Participants

Table S1. Participants demographic characteristics.

	N	%
Education*		
Low	19	15.5
Middle	69	56.6
High	32	26.2
Marital status		
Married or living with a partner	58	47.5
Widowed	4	3.3
Divorced	6	4.9
Separated	1	0.8
Never married	53	43.4
Living situation		
With spouse/partner	27	22.1
With family/children	64	52.5
Alone	21	17.2
Other	10	8.2

*Education (International Standard Classification of Education levels): Low = primary and lower secondary education; Moderate = upper secondary, postsecondary non-tertiary and short cycle tertiary education; High = bachelor, master and doctoral education. Sample sizes might not add up to total participants due to missing data.

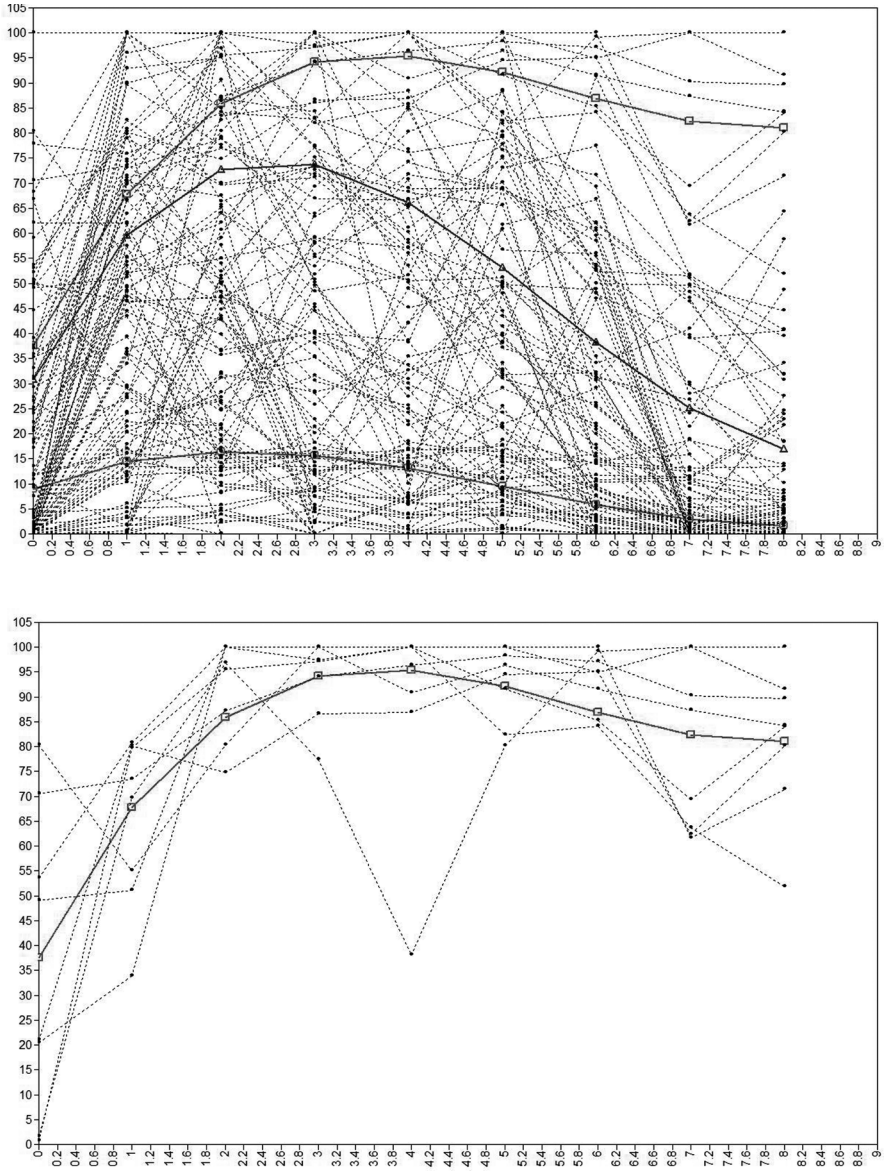
3.2. Classes based on the subjective fearfulness ratings to the CS+

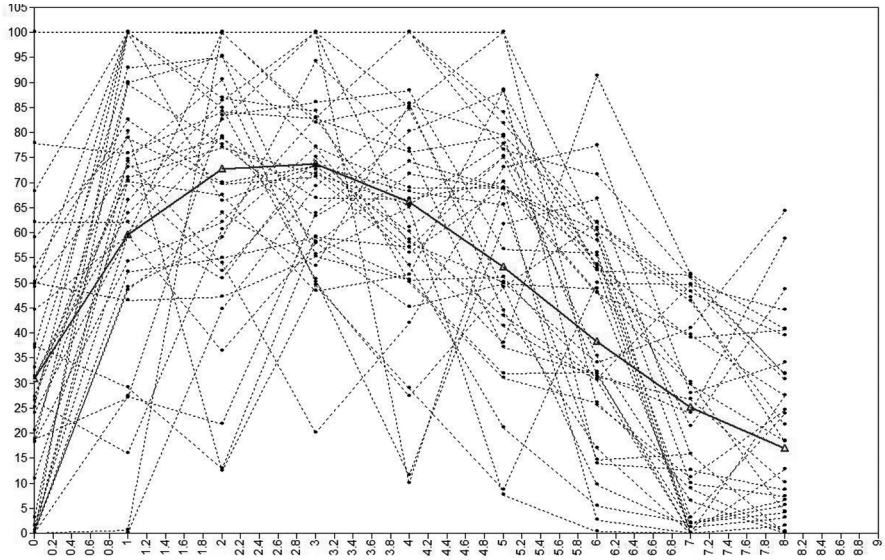


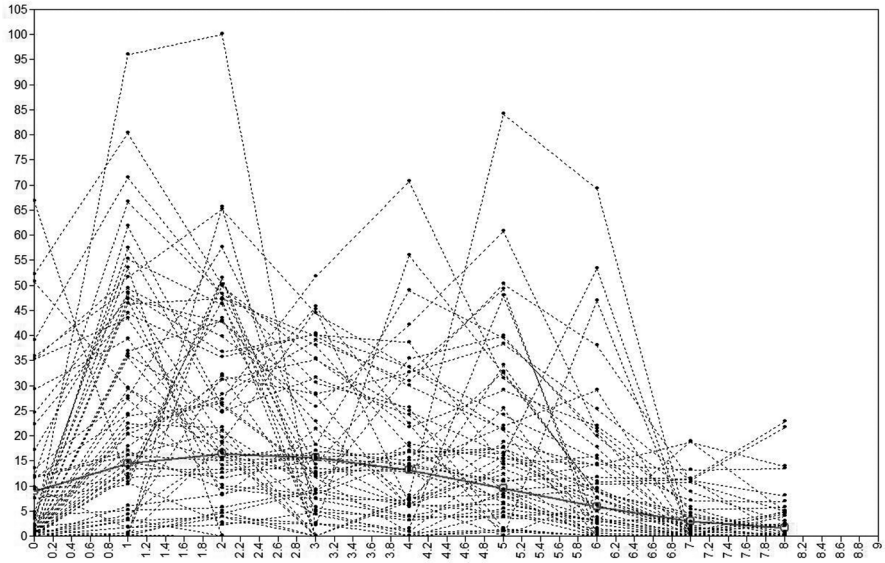
	Intercept			Slope			Quadratic			Cubic		
Class	EST	SE	*p*	EST	SE	*p*	EST	SE	*p*	EST	SE	*p*
Normal Conditioners	30,83	4,78	<.001	37,7	4,3	<.001	-9,51	1,2	<.001	0,57	0,09	<.001
Low Fearful Conditioners	8,9	1,83	<.001	7,63	1,78	<.001	-2,23	0,46	<.001	0,15	0,03	<.001
Poor extinguishers	37,47	8,4	<.001	37,36	7	<.001	-7,45	1,69	<.001	0,43	0,11	<.001

Note: Est = Estimate; *SE* = Standard Error of the Estimate; Estimated parameters for each latent class include the *intercept* representing measured scores on the y-axis
across trials; *slope* representing the degree of linear change across trials; Q*uadratic and Cubic* representing the degree of non-linear change across trials.

3.2.1. Estimated means and observed individual values 3-class model







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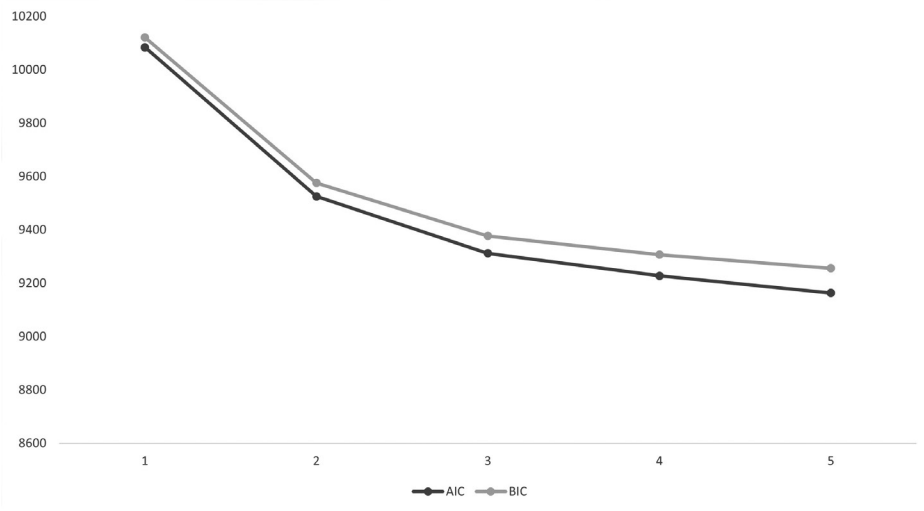

	Intercept			Slope			Quadratic			Cubic		
Class	EST	SE	*p*	EST	SE	*p*	EST	SE	*p*	EST	SE	*p*
Generalizers	51,77	5,88	<.001	7,7	6,09	.206	-2,72	1,77	.124	0,19	1,13	.162
Non-generalizers	12,76	2,12	<.001	-3,23	1,81	.074	0,53	0,49	.273	-0,03	0,04	.372

Note: Est = Estimate; *SE* = Standard Error of the Estimate; Estimated parameters for each latent class include the *intercept* representing measured scores on the y-axis
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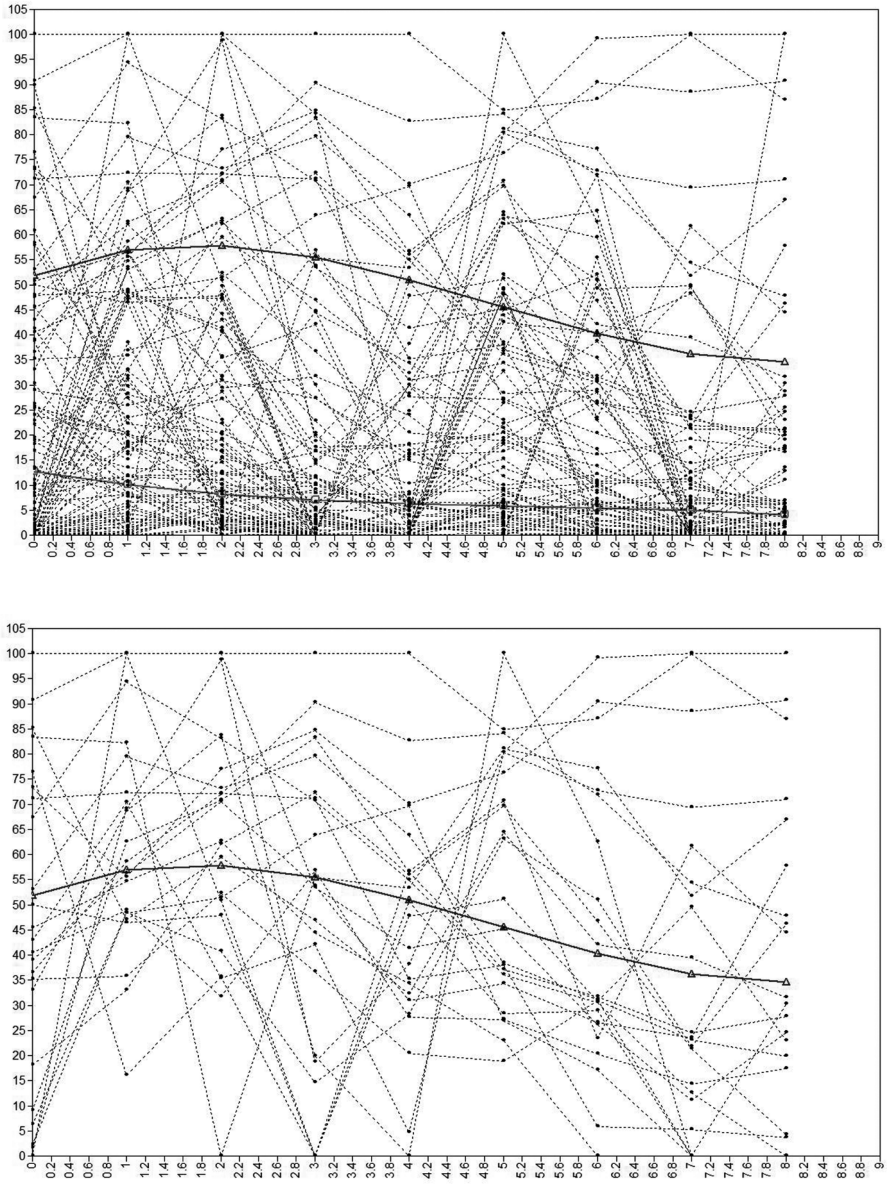
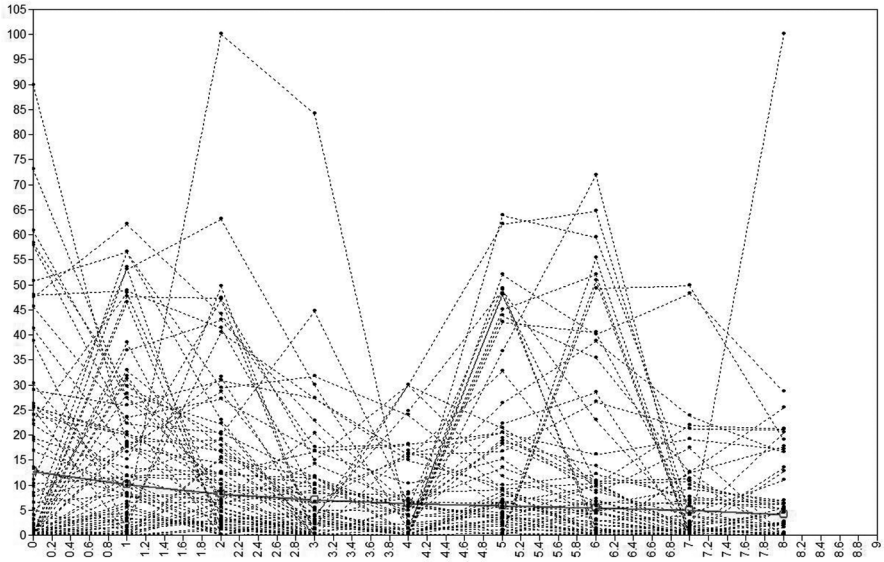
3.3.1. Estimated means and observed individual values 2-class model

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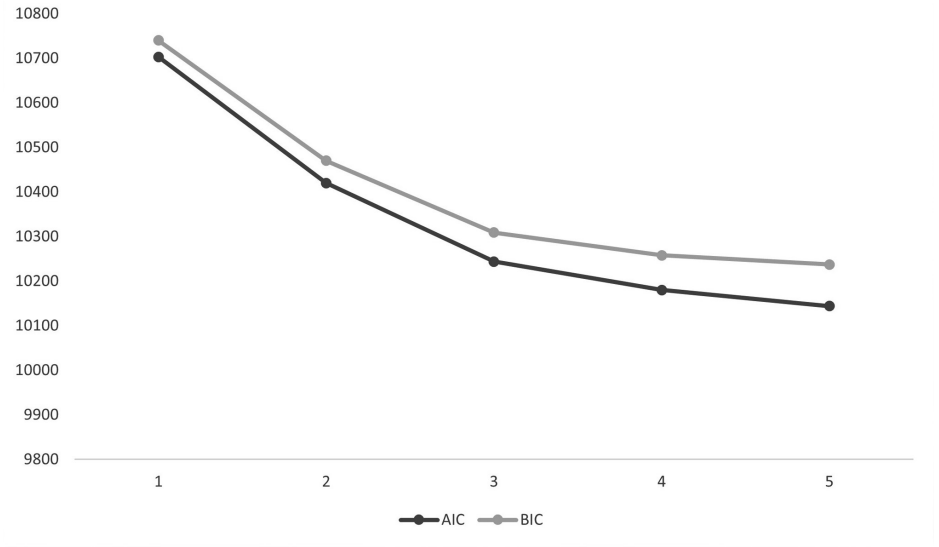
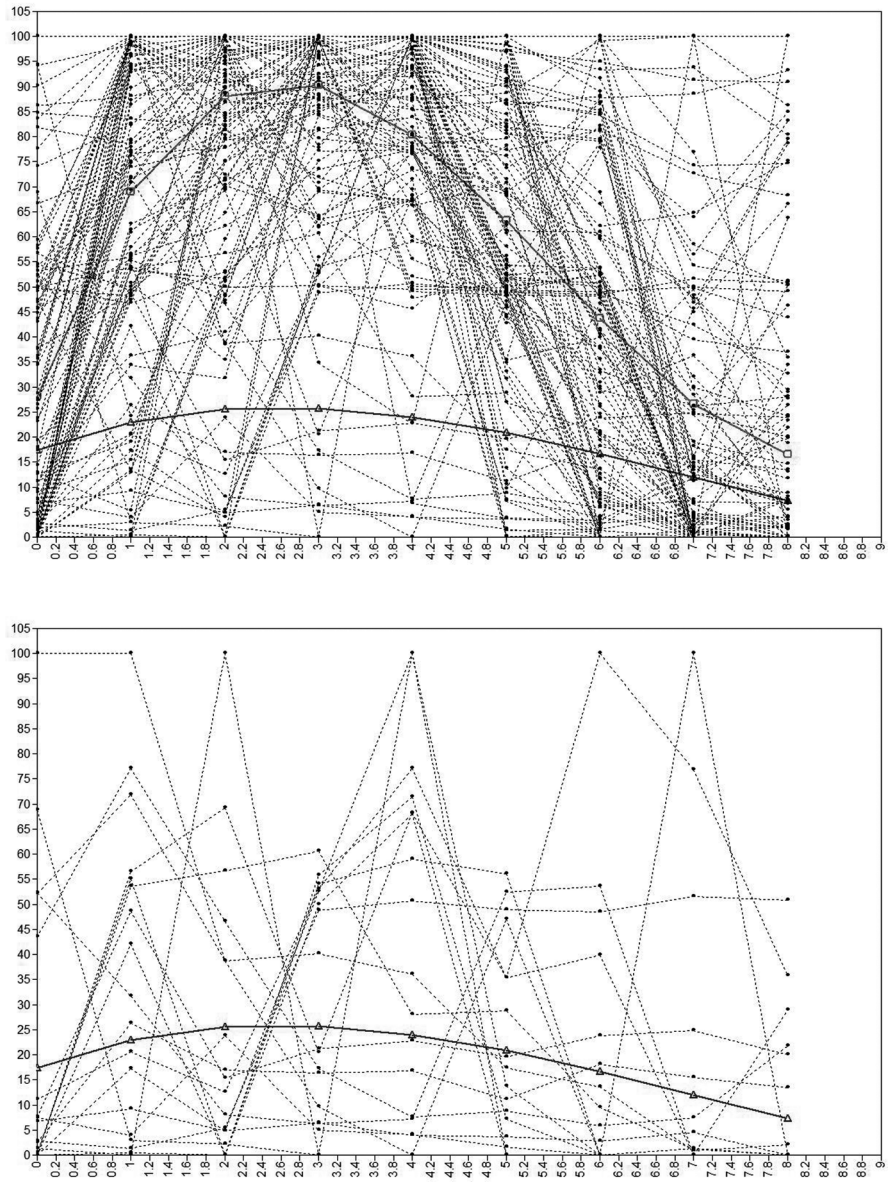
3.4. Classes based on the subjective US expectancy ratings to the CS+

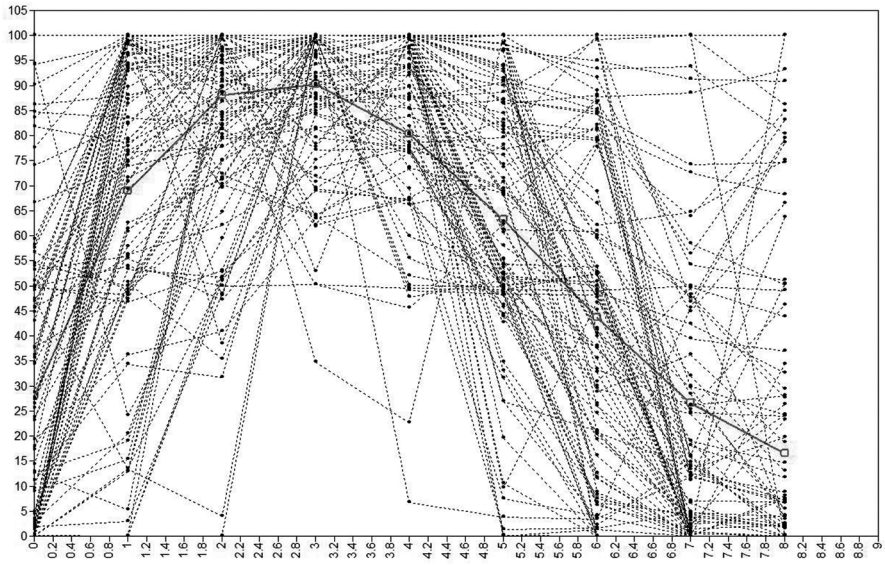


	Intercept			Slope			Quadratic			Cubic		
Class	EST	SE	*p*	EST	SE	*p*	EST	SE	*p*	EST	SE	*p*
Normal conditioners	23,86	3,26	<.001	58,83	3	<.001	-14,95	0,95	<.001	0,91	0,08	<.001
Low fearful condioners	17,68	7,23	.014	6,58	7,16	.358	-1,79	1,74	.303	0,1	0,12	.418
Poor extinguishers	38,97	8,33	<.001	40,66	8,4	<.001	-10,27	2,6	<.001	0,71	0,21	.001

Note: Est = Estimate; *SE* = Standard Error of the Estimate; Estimated parameters for each latent class include the *intercept* representing measured scores on the y-axis
across trials; *slope* representing the degree of linear change across trials; Q*uadratic and Cubic* representing the degree of non-linear change across trials.

3.4.1. Estimated means and observed individual values 2-class model

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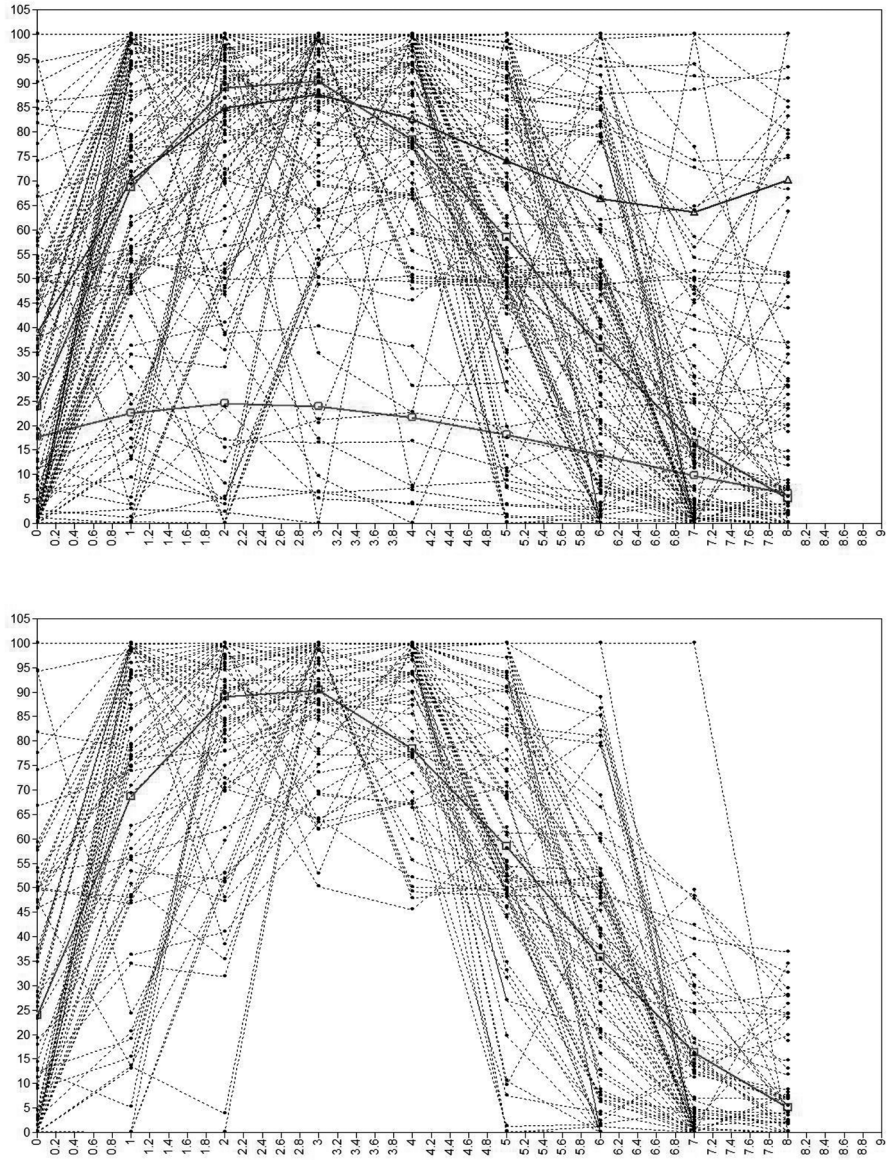
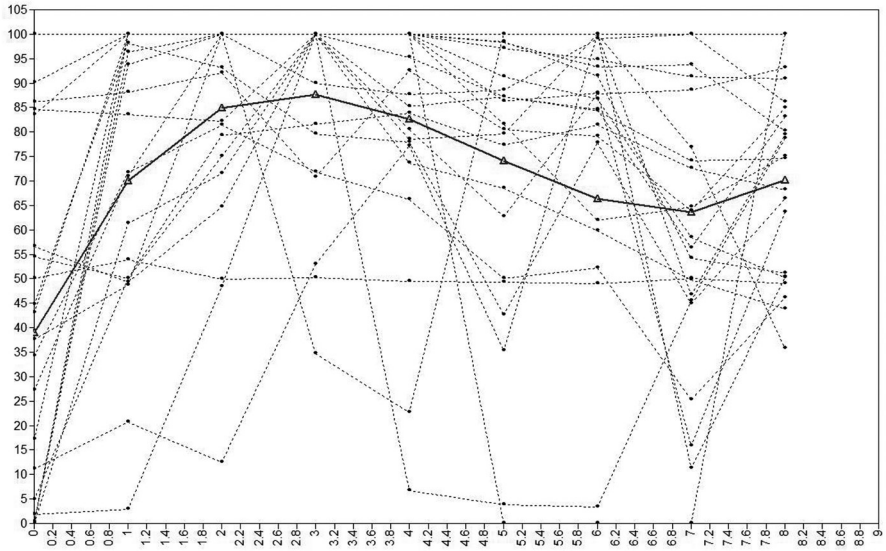
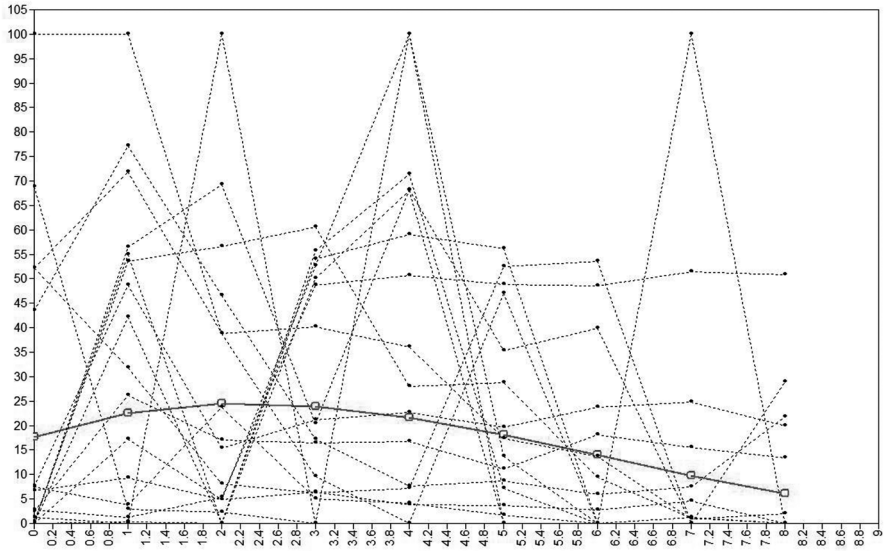
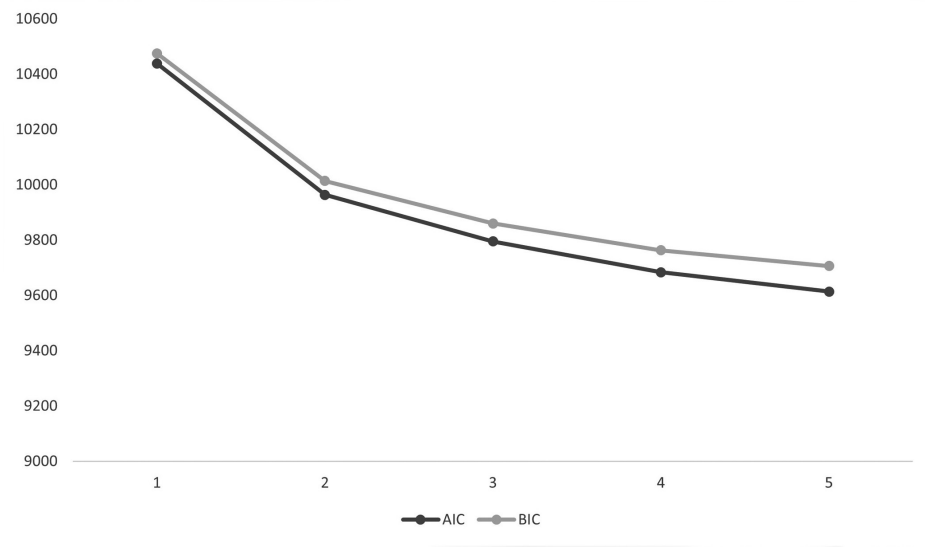
3.4.2. Estimated means and observed individual values 3-class model







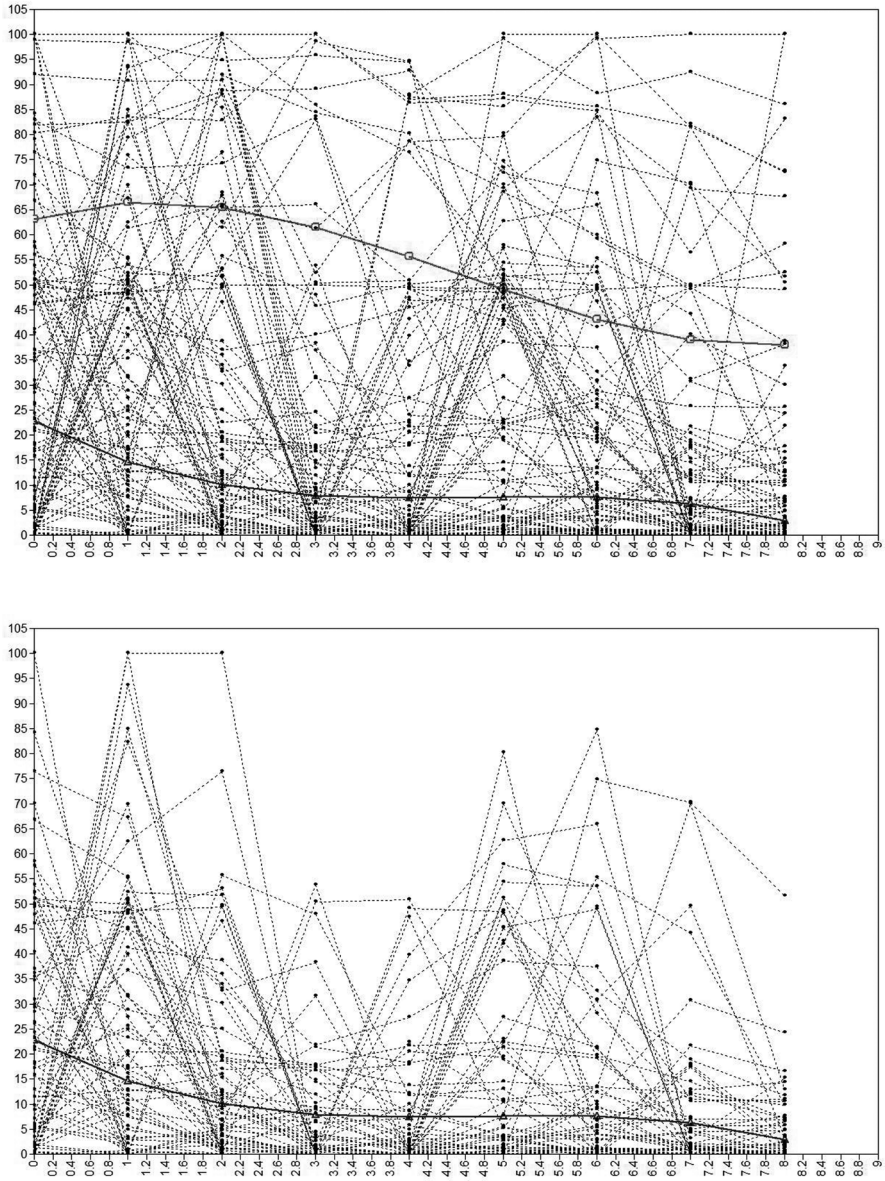
3.5. Classes based on the subjective US expectancy ratings to the CS-

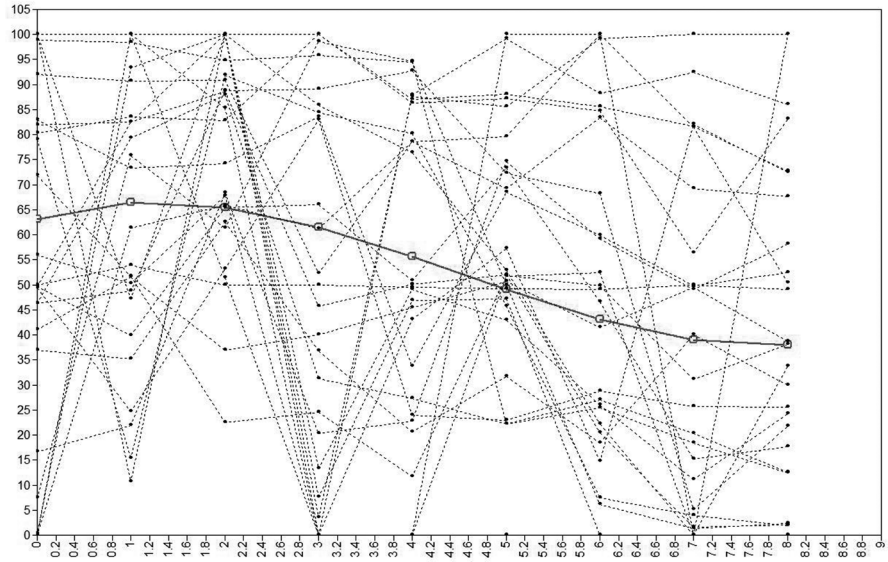


	Intercept			Slope			Quadratic			Cubic		
Class	EST	SE	*p*	EST	SE	*p*	EST	SE	*p*	EST	SE	*p*
Generalizers	63,04	6,15	<.001	5,93	7,39	.422	-2,76	2,14	.196	0,2	0,17	.218
Non-generalizers	22,75	2,87	<.001	-10,13	2,4	<.001	2,2	0,63	<.001	-0,16	0,05	.001

Note: Est = Estimate; *SE* = Standard Error of the Estimate; Estimated parameters for each latent class include the *intercept* representing measured scores on the y-axis
across trials; *slope* representing the degree of linear change across trials; Q*uadratic and Cubic* representing the degree of non-linear change across trials.

3.5.1. Estimated means and observed individual values 2-class model





3.7. Treatment and other characteristics

Table S2. Distribution of fear CS+ classes on location, medication use and type of treatment (*N*,%).

Classes Fearfulness CS+			
	Low fearful conditioners	Normal conditioners	Poor Extinguishers
Location			
Dental Anxiety Clinics	47 (77%)	11 (18%)	3 (4.9%)
MGGZ	15 (57.7%)	10 (38.5%)	1 (3.8%)
Altrecht	15 (42.9%)	16 (45.7%)	4 (11.4%)
Mediction use			
SSRIs	8 (42.1%)	11 (57.9%)	0 (0%)
BENZOs	2 (50%)	2 (50%)	0 (0%)
Treatment			
Exposure	27 (84.4%)	4 (9.3%)	1 (2.4%)
Exposure Intensive	13 (43.3%)	14 (46.7%)	3 (10%)
EMDR	15 (75%)	3 (15%)	2 (10%)
CBT	18 (75%)	4 (16.7%)	2 (8.3%)
Other	5 (45.5%)	5 (45.5%)	1 (9%)

Table S3. Distribution of fear CS- classes on location, medication use and type of treatment (*N*,%).

Classes Fearfulness CS-		
	Non-Generalizers	Generalizers
Location		
Dental Anxiety Clinics	54 (88.5%)	7 (11.5%)
MGGZ	23 (88.5%)	3 (11.5%)
Altrecht	22 (62.9%)	13 (37.1%)
Mediction use		
SSRIs	14 (73.7%)	5 (26.3%)
BENZOs	3 (75%)	1 (25%)
Treatment		
Exposure	29 (90.6%)	3 (9.4%)
Exposure Intensive	19 (63.3%)	11 (36.7%)
EMDR	17 (85%)	3 (15%)
CBT	22 (91.7%)	2 (8.3%)
Other	7 (63.6%)	4 (36.4%)

Table S4. Distribution of US Expectancy CS+ classes on location, medication use and type of treatment (*N*,%).

US Expectancy CS+			
	Low fearful conditioners	Normal conditioners	Poor Extinguishers
Location			
Dental Anxiety Clinics	18 (29.5%)	33 (54.1%)	10 (16.4%)
MGGZ	0 (0%)	22 (84.6%)	4 (15.4%)
Altrecht	3 (8.6%)	24 (68.6%)	8 (22.9%)
Mediction use			
SSRIs	1 (5.3%)	16 (84.2%)	2 (10.5%)
BENZOs	1 (25%)	3 (75%)	0 (0%)
Treatment			
Exposure	11 (34.4%)	18 (56.3%)	3 (9.4%)
Exposure Intensive	3 (10%)	21 (70%)	6 (20%)
EMDR	5 (25%)	11 (55%)	4 (20%)
CBT	1 (4.2%)	20 (83.3%)	3 (12.5%)
Other	0 (0%)	9 (81.8%)	2 (18.2%)

Table S5. Distribution of US Expectancy CS- classes on location, medication use and type of treatment (*N*,%).

US Expectancy CS-		
	Non-Generalizers	Generalizers
Location		
Dental Anxiety Clinics	49 (80.3%)	12 (19.7%)
MGGZ	24 (92.3%)	2 (7.7%)
Altrecht	22 (62.9%)	13 (37.1%)
Mediction use		
SSRIs	14 (73.7%)	5 (26.3%)
BENZOs	2 (50%)	2 (50%)
Treatment		
Exposure	27 (84.4%)	5 (15.6%)
Exposure Intensive	18 (60%)	12 (40%)
EMDR	18 (90%)	2 (10%)
CBT	22 (91.7%)	2 (8.3%)
Other	8 (72.7%)	3 (27.3%)

3

CHAPTER 4

Anxiolytic effects of endocannabinoid enhancing compounds: A systematic review and meta-analysis

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ABSTRACT

The endocannabinoid system is a promising candidate for anxiolytic therapy, but translation to the clinic has been lagging. We meta-analyzed the evidence for anxiety-reduction by compounds that facilitate endocannabinoid signaling in humans and animals. To identify areas of specific potential, effects of moderators were assessed. Literature was searched in Pubmed and Embase up to May 2021. A placebo/vehicle-control group was required and in human studies, randomization. We excluded studies that co-administered other substances. Risk of bias was assessed with SYRCLE's RoB tool and Cochrane RoB 2.0. We conducted three-level random effects meta-analyses and explored sources of heterogeneity using Bayesian regularized meta-regression (BRMA). The systematic review yielded 134 studies. We analyzed 120 studies (114 animal, 6 human) that investigated cannabidiol (CBD, 61), URB597 (39), PF-3845 (6) and AM404 (14). Pooled effects on conditioned and unconditioned anxiety in animals (with the exception of URB597 on unconditioned anxiety) and on experimentally induced anxiety in humans favored the investigational drugs over placebo/vehicle. Publication year was negatively associated with effects of CBD on unconditioned anxiety. Compared to approach avoidance tests, tests of repetitive-compulsive behavior were associated with larger effects of CBD and URB597, and the social interaction test with smaller effects of URB597. Larger effects of CBD on unconditioned anxiety were observed when anxiety pre-existed. Studies reported few side effects at therapeutic doses. The evidence quality was low with indications of publication bias. More clinical trials are needed to translate the overall positive results to clinical applications.

1. INTRODUCTION

Cannabis has long been considered to have therapeutic potential[1]. Research on the cannabis constituent Δ9-THC and cannabimimetic compounds led to the discovery of cannabinoid receptors and, subsequently, of endogenous cannabinoids N-arachidonoylethanolamide (AEA; anandamide)[2,3] and 2-arachidonoylglycerol (2-AG)[4–6]. Early studies with cannabidiol (CBD), a second major constituent of cannabis, demonstrated anxiolytic properties in animals and humans[7–10].

In subsequent years, preclinical data in rodents accumulated suggesting that disruptions in endocannabinoid tone in brain regions including the amygdala, hippocampus and prefrontal cortex contribute to anxiety-like behavior induced by acute or repeated stress (for narrative reviews see[11–14]). Several experiments in rodents used fear extinction, a widely used translational model for learning that takes place during exposure therapy[15–18]. It was shown that endocannabinoid signaling in the amygdala and hippocampus mediates the stress and glucocorticoid-induced enhancement of fear extinction and fear memory consolidation, and impairment of fear memory retrieval[13]. The clinical potential of this approach has spurred more mechanistic investigations in the endocannabinoid system (ECS) as a candidate target for anxiolytic drug development.

CBD is a prominent constituent of cannabis with a complex pharmacology, including as a mechanism of action inhibition of fatty acid amide hydrolase (FAAH), the primary metabolic enzyme of AEA. Although CBD's inhibition of FAAH is relatively weak subchronic CBD administration increased AEA levels in mouse hippocampal tissue and in serum of patients with acute schizophrenia[2,3,19,20].

In contrast to direct CB1R agonists such as Δ9-tetrahydrocannabinol (Δ9-THC), CBD does not induce psychomotor impairment or psychotomimetic effects or psychotomimetic effects[21,22]. Further, CBD does not induce a change in heart rate, and seems to attenuate the anxiogenic effect of Δ9-THC in healthy volunteers[21–23]. These data suggest that CBD may indirectly exert CB1R mediated therapeutic actions, while circumventing unwanted side effects.

To overcome the lack of target selectivity of CBD and aiming to optimize a fear extinction enhancing effect, several classes of more selective inhibitors of FAAH have been developed[2,3]. The O-aryl carbamate URB597 turned out to be a potent and irreversible inhibitor of FAAH[24]. The transport inhibitor AM404 selectively attenuates breakdown of AEA by inhibition of intracellular fatty acid binding proteins (FABS)[25–27]. The irreversible FAAH inhibitor PF-3845 is more potent, more selective, and has a longer duration of action than URB597[28]. URB597, PF-3845 and inhibitor of AEA cellular uptake AM404 are prototypical examples of the many compounds that were developed to increase CB1R activation by enhancing endocannabinoid levels[29].

To the best of our knowledge, numerous narrative but no systematic review on preclinical research into anxiolytic effects of ECS manipulations has been published so far[13,30,31]. One systematic review of animal studies of ECS manipulations including CBD, with a primary focus on inflammation and neurogenesis, included five studies that reported variable effects on anxiety outcomes[32].

A previous systematic review and meta-analysis summarized the limited available evidence from controlled studies conducted in human patients suffering from anxiety disorders, which included only two randomized controlled studies in patients[33]. This meta-analysis demonstrated no benefit of single doses of CBD (up to 600 mg) over placebo[33]. These preliminary findings in humans raise questions about the often discussed potential of pharmacological enhancement of AEA levels for treating anxiety symptoms. Clearly, there is a need for a systematic review and meta-analysis of the large body of mainly preclinical literature on this topic. This literature can provide an indication of clinical efficacy but is especially suitable for identifying potential moderators of clinical effects given the diversity in anxiety models used in these studies[30,34].

The primary aim of the present systematic review and meta-analysis was to investigate anxiolytic effects of inhibitors of FAAH and AEA transport, by synthesizing all evidence from animal, human, preclinical and clinical studies. Behavioral, physiological, and subjective effects were investigated. In addition, theoretically relevant moderators and sources of heterogeneity of drug effects were explored. Part of the current literature examines acute anxiolytic effects, but a more recent approach is to develop treatments that aim to work synergistically with psychotherapeutic approaches by supporting adaptive learning, particularly fear extinction[35]. As discussed above, modulators of brain endocannabinoid levels have been shown to exert an effect on fear extinction and related learning mechanisms (for narrative reviews see Lafenêtre[13,36,37]) and attempts have been made to translate these findings to potential use in psychotherapy[38]. We therefore conducted separate meta-analyses for tests of conditioned versus unconditioned anxiety[39]. Additionally, we explored whether drugs affected different aspects of fear conditioning and extinction, and investigated factors that are likely to moderate drug effects: 1) variables related to the drug regimen (single vs (sub)chronic administration, acute vs delayed effects); 2) species[40,41]; 3) the preexisting anxiety condition of the animal or human individual[42,43]; 4) type of anxiety test[43]; 5) sex differences with respect to the effects of AEA modulators, in light of the association between oestradiol and CB1 receptor density in amygdala and prefrontal cortex[44]; 6) Publication year[45].

For our secondary research aim we summarized any information that was available in included studies on drug safety and tolerability. Several reviews are available for CBD[41,46–48]. Previous preclinical research shows divergent results with respect to safety and tolerability of FAAH inhibitors[49]. We therefore evaluated adverse effects in included studies on a drug-by-drug basis.

2. METHOD

This review was preregistered with PROSPERO (CRD42021236572) and conducted in line with Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (see Supplemental Tables 1 and 2).

2.1. Search strategy

Studies were searched in the electronic databases PubMed and Embase using both free text and underlying terms (MeSH and Emtree, respectively) up to 19-05-2021. The search was aimed at evidence on modulation of fear expression, anxiety symptoms and fear memory or extinction learning, by AEA hydrolysis and transport inhibitors in humans and non-human mammals (see Supplemental Table 3). Only peer-reviewed articles were included. No restrictions were placed on publication year or language. Preregistered but as of yet unpublished studies were searched in the EU Clinical Trials Register, the Australian and New Zealand Clinical Trials Registry, Animal Study Registry (German Centre for the Protection of Laboratory Animals), ClinicalTrials.gov and Preclinicaltrials.eu, in order to get an indication of potential positive results bias.

2.2. In- and exclusion criteria

Table 1 lists in- and exclusion criteria for the selection of studies.

Table 1. Study in- and exclusion criteria

	Participants	Interventions	Comparison	Outcomes
Included				
	1. Healthy or anxious phenotype 2. Adult 3. Mammal	1. FAAH inhibitor or AEA transport inhibitor	1. Randomized placebo-controlled design	1. Fear expression, fear or extinction memory, extinction learning or anxiety disorder symptoms 2. Outcome domain behavioral physiological, or subjective 3. Data type continuous

Table 1. Study in- and exclusion criteria (continued)

	Participants	Interventions	Comparison	Outcomes
Excluded				
	1. Chronic users of cannabis compounds	1. Compounds with catabolic pathways for AEA other than FAAH hydrolysis 2. Dual FAAH/ monoacylglycerol lipase inhibitors (Fowler, 2021) 3. Intracerebral/ intracerebroventricular/ intravenous administration 4. Coadministration of other substances[b] 5. Time between drug administration and anxiety assay ≥ 24 h	1. Studies without control group 2. Non-randomization (studies in humans only)[a]	1. Acquisition of fear

Note: AEA: anandamide; FAAH: fatty acid amide hydrolase.
[a] The use of randomization is usually not reported in animal research and it had not been empirically demonstrated whether the use of randomization would influence outcomes[50]. Therefore, in animals we considered vehicle-controlled experiments without information about randomization and explicitly non-randomized but placebo/vehicle-controlled studies to be eligible as well.
[b] In humans, studies that allowed stable concomitant anxiolytic and/or antidepressant medication were included.

2.3. Study screening and selection

Titles and abstracts of articles retrieved using the search strategy were independently screened by a first (CK) and second reviewer (NL or RvdK) to identify studies that appeared to meet the inclusion criteria. They then independently screened the full text of these studies for eligibility. Disagreements were resolved through discussion, when no consensus was reached a third (LG) or fourth reviewer (JB) was consulted.

2.4. Data extraction

According to the PICO framework we recorded the details of the populations, interventions (including concomitant medication in human studies), and outcomes[51]. The comparison group was always placebo/vehicle.

2.4.1. Primary research aim

For our first research aim of drug effects on anxiety outcomes within behavioral, physiological, and subjective outcome domains (see Supplemental Table 4), parameters of interest were means (Ms) and standard deviations (SDs) of the anxiety outcome in vehicle/placebo and active drug conditions. We used these parameters to calculate

Hedge's g, an effect size that corrects for bias resulting from small sample sizes[52]. Higher scores on the effect size indicate an anxiolytic drug effect. Effect sizes were reverse-coded if higher values indicated less anxiety than lower values. Decision rules in case of unreported data, or multiple outcome measures or experimental drug-placebo comparisons are described in the Supplemental material, section 2.2. If parameters were not fully reported we estimated them from graphs in the paper or requested the information from the authors.

We extracted theoretically relevant moderators dose, type of anxiety test, selected outcome parameter, publication year, information on frequency of drug administration and timing of effect measurement, pre-existing anxiety condition, sex, and species (ten moderators in total), of which the first three were selected as theoretically most relevant for exploratory follow-up analysis. To standardize 'dosages' across species human equivalent dose (HED) was calculated by using allometric scaling factors[53]. This dose-normalization approach is common in systematic overviews of preclinical study results across different species[54]. Our semi-quantitative analyses on the relation between CBD dose and anxiety-reducing effects tentatively suggest an inverted U-shaped dose-response curve, modeled here with a quadratic trend for dose/HED[41].

2.4.2. Secondary research aim

The terms 'harm', 'adverse', 'side', 'unwanted', 'undesirable', 'safe*', 'toler*' were searched in included articles.

2.4.3. Procedure

The majority of the data were extracted by CK, the remainder by a second reviewer (NL or one of the collaborators on the project. When one of the authors was in doubt about (categorization of) the data to be extracted, the issue was resolved through discussion (with a third (LG) and fourth reviewer (JB) when necessary). Generally, the outcomes extracted by the first and second reviewer matched (see Supplemental material, section 2.3 for more information).

2.5. Data analysis

Meta-analyses were performed using R packages metafor and pema[55,56]. All models were three-level random effects models. A three-level random effects model accounts for three sources of variance: sampling error of the observed effect size (which is treated as known), within-experiment variance of true experiment-specific effect sizes, and variance of true experiment-specific effect sizes across experiments. Effect sizes from different papers were always categorized as independent; effect sizes from the same paper only if it was explicitly stated that effects were tested in independent experiments and/or independent sets of study subjects.

We conducted separate analyses per drug (within the class of AEA enhancing drugs), for unconditioned and conditioned anxiety in animals and experimentally induced anxiety in humans. Effect sizes per comparison and overall pooled effect size per meta-analysis were visualized in forest plots (Supplementary Figures 2-9).

Statistical heterogeneity was assessed using τ2 (a measure of between-study variance) and I2 (percent of variability in effect sizes not caused by sampling error)[34,57]. We conducted sensitivity analyses to examine whether substantiated conclusions would change by excluding studies with high risk of bias or atypical route of drug administration.

For categorical moderators, we used dummy coding, treating the largest category of each variable as the reference category. We standardized continuous predictors only and not dummy variables. This may have given dummy variables a slight advantage, leading them to become significant sooner than continuous ones.

The number of effect sizes was small relative to the number of moderators. This introduces risks of model-nonidentification, overfitting, and multicollinearity[58]. A novel technique called Bayesian regularized meta-regression (BRMA) overcomes these risks by imposing a regularizing horseshoe prior to shrink the regression coefficients of irrelevant moderators towards zero[56]. Thus, we used BRMA in all moderator analyses to select moderators that are important in predicting the effect size. The resulting regression coefficients are negatively biased by design, but simulation studies show that the estimate of residual heterogeneity τ2 is relatively unbiased[56]. Supplementary classic meta-regression with the maximum likelihood approach (Supplemental Tables 11, 13, 16, 18, 20, 22, 24, 28, 30, 32, 34, 36, 38, 40, 42) indeed evidenced model non-convergence and high variance inflation factors (VIF) confirmed the expected problems caused by the high ratio of moderators to effect sizes.

We decided a priori to only perform the planned quantitative syntheses for each meta-analysis (separate per drug and conditioned/unconditioned/experimentally induced anxiety for humans and animals) if the number of included effect sizes in the meta-analysis exceeded the number of moderator variables + 1, which we considered the minimum for model identification. In addition to planned moderator analyses which included all moderators, we conducted exploratory moderator analyses on potential interactions of drug dose with a smaller number of key moderators.

To interpret these interaction effects, see plots with posterior predictive distribution of drug effects per moderator category, conditional upon the observed effects (Figure 3). The Workflow for Open Reproducible Code in Science was used to make analyses reproducible[59]. A reproducible repository with all analysis codes and data are available at (doi:10.5281/zenodo.7829148).

2.6. Assessment of the quality of evidence

Assessment of the quality of the meta-analytic evidence with the Grading of Recommendations, Assessment, Development and Evaluation (GRADE) approach was done by CK and checked by NL[60]. GRADE criteria and are summarized in the Supplemental material, section 2.4.

3. RESULTS

3.1. Included studies and characteristics

A PRISMA flowchart is shown in Figure 1. Study characteristics of included studies are summarized in Table 2. The majority of included studies (*n*=114 out of a total of *n*=120 studies; 95%) were conducted in non-human mammals. Only *n*=6 studies (5%) were conducted in humans. Types of anxiety tests in included studies are provided in Supplemental Table 7.

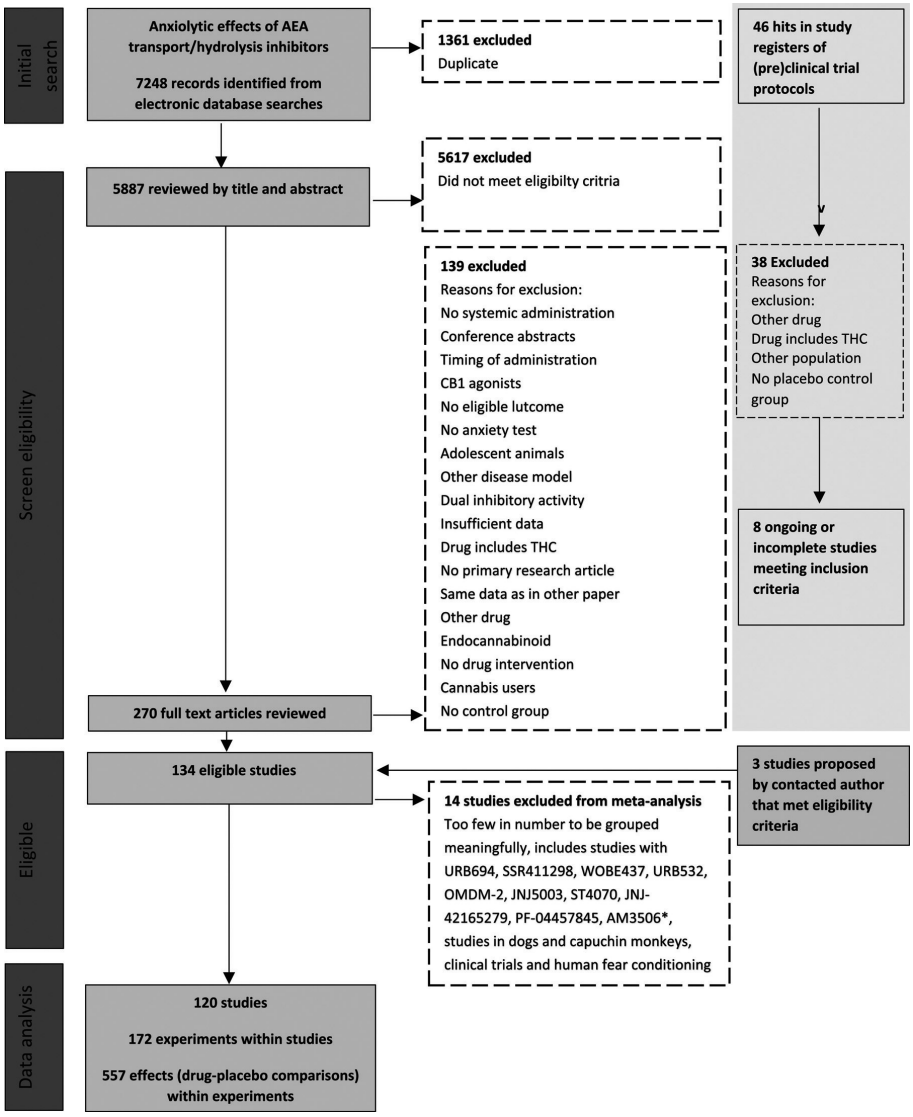

Figure 1. PRISMA flowchart.
Note: References of eligible studies are listed in Supplemental Table 5. Supplemental Table 6 describes ongoing or incomplete studies that meet inclusion criteria.

Table 2. Summary characteristics of included studies.

Population	
Publication year	1990-2021
Species	44% mouse, 50% rat, 5% human, 2% other
Pre-existing anxiety condition	in 17% of studies
Sample size per study*	88 (109)
Sample size per effect	20 (6)
Sex	90% male
Intervention	
Drug	52% CBD, 32% URB597, 11% AM404, 5% PF-3845
HED*60	90.08 (143.65)
Administration route	90% i.p., 10% oral
Frequency of administration	68% single dose
Timing of effect measurement	82% acute drug effects
Outcome	
Type of anxiety	71% unconditioned
Type of anxiety test	See Supplemental Table 7
Selected outcomes for tests of conditioned anxiety	See Supplemental Table 8 and Supplemental figure 1

Note: Numbers are mean (SD) or as otherwise stated.
* Sample sizes per tested effect can be found in the data files (doi:10.5281/zenodo.7829148).

In Supplemental Table 8 the distribution is shown of outcomes in tests of conditioned anxiety, selected from the studies according to a-priori definitions (see Supplemental Figure 1 for details). Outcomes were categorized as effects on fear memory reconsolidation when the drug was administered after memory retrieval, and as effects on extinction consolidation when administered after an extinction learning phase (before extinction retention was tested).

3.2. Effects of FAAH and AEA transport inhibitors on anxiety

3.2.1. Overall summary of findings regarding drug effects

Across meta-analyses, the pooled effect size estimates indicated a lower level of anxiety after treatment with the investigational drug than after placebo/vehicle treatment (Figure 2 and Table 3). This was true for all combinations of drug types and types of anxiety for humans and non-human mammals except one, the effect of URB597 on unconditioned

anxiety in animals. The size of these drug effects was moderate-to-large. Note that CBD was the only compound for which sufficient studies in humans were available to analyze meta-analytically. For PF-3845 only studies with tests of unconditioned anxiety in animals were available. The illustrations of effect sizes of all studies from which the pooled effect sizes were derived can be found in Supplemental Figures 2-9. For most analyses, both within- and between-experiment variance were significant, which indicates heterogeneity between effect sizes both within and across experiments (see section 3.2.2 and 3.2.5 for results of moderator analyses).

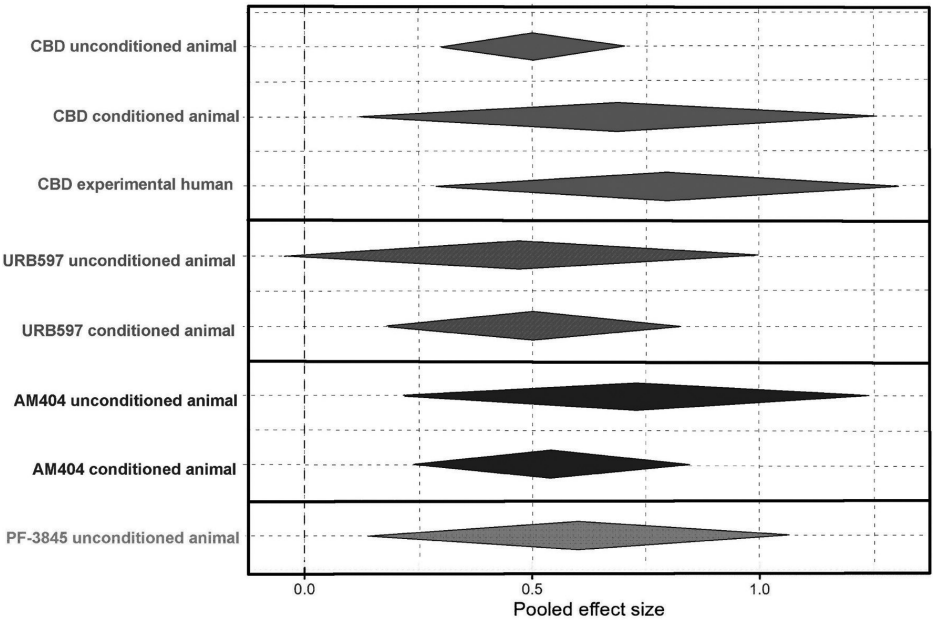

Figure 2. Pooled effects per drug for unconditioned and conditioned anxiety in animals and experimentally induced anxiety in humans.
Note: Diamonds illustrate point estimates plus 95% confidence intervals for each meta-analysis, see Table 3 for further details. Negative values indicate effects in favor of the placebo group; positive values indicate effects in favor of the experimental group that received the drug. Supplementary Figures 2-9 provide forest plots of the distributions of observed effect sizes.

Table 3. Summary of findings of anxiolytic effects of FAAH and AEA transport inhibitors.

Type of anxiety	Drug	Participants (experiments)	Hedge's G [95%CI]	σw², σb² [95%CI]	Iw², Ib²	Favors	QoE
Unconditioned in animals	Cannabidiol	4859 (61)	0.50 [0.29, 0.70]*	0.20 [0.12, 0.31] 0.44 [0.23, 0.82]	23.49 52.23	Cannabidiol	Low
	URB597	2153 (50)	0.47 [-0.06, 1.00]	0.12 [0.01, 0.31]* 3.31 [2.13, 5.35]	3.29 89.18	Neither	Low
	AM404	743 (12)	0.73 [0.21, 1.24]*	0.62 [0.27, 1.32]* 0.46 [0.08, 1.74]*	46.02 34.04	AM404	Low
	PF-3845	726.5 (7)	0.60 [0.13, 1.07]*	0.15 [0.00, 0.47]* 0.28 [0.01, 1.57]*	23.80 45.30	PF-3845	Low
Conditioned in animals	Cannabidiol	1125 (16)	0.68 [0.11, 1.26]*	<0.01 [<0.01, 0.10]* 1.22 [0.50, 3.17]	<0.01 83.57	Cannabidiol	Low
	URB597	787 (13)	0.50 [0.17, 0.83]*	0.01 [0.00, 0.23]* 0.23 [0.04, 0.83]	2.46 49.31	URB597	Low
	AM404	351 (7)	0.54 [0.24, 0.85]*	0.14 [0.00, 0.66] <0.01 [<0.01, 0.70]	47.40 <0.01	AM404	Low
Experimental in humans	Cannabidiol	442 (6)	0.79 [0.28, 1.31]*	<0.01 [<0.01,0.25]* 0.28 [0.03, 2.07]	0.09 60.62	Cannabidiol	Moderate

Note: QoE: Quality of evidence; σ_w^2, σ_b^2, I_w^2, I_b^2: heterogeneity statistics.
* $p < .05$.

3.2.2. Planned moderator analyses

Moderator analyses with theoretically relevant moderators were conducted to identify sources of heterogeneity of drug effects and to generate hypotheses on which circumstances and for whom the tested drugs could be beneficial. Supplemental Table 9 presents the applicable moderators per meta-analysis. Relevant predictors selected with BRMA are listed in the Supplemental Tables 10, 12, 14, 16, 18, 20, 22, 24. In the text below, only moderator effects whose 95% credible interval excluded zero are discussed. This interval contains the population effect size with 95% probability and is the Bayesian counterpart of statistical significance.

Publication year, presence or absence of a pre-existing anxiety condition, and anxiety test moderated CBD effects on unconditioned anxiety. Effects of CBD were larger in the presence of pre-existing anxiety (Figure 3, panel A) and in tests of repetitive compulsive-like behavior (RCLB) than in approach avoidance tests (Figure 3, panel B). Conversely, the effects of CBD were smaller in more recent compared to older publications. In URB597, anxiety test moderated drug effects on unconditioned anxiety. The social interaction test was associated with smaller anxiolytic effects compared to approach avoidance tests (Figure 3, panel C).

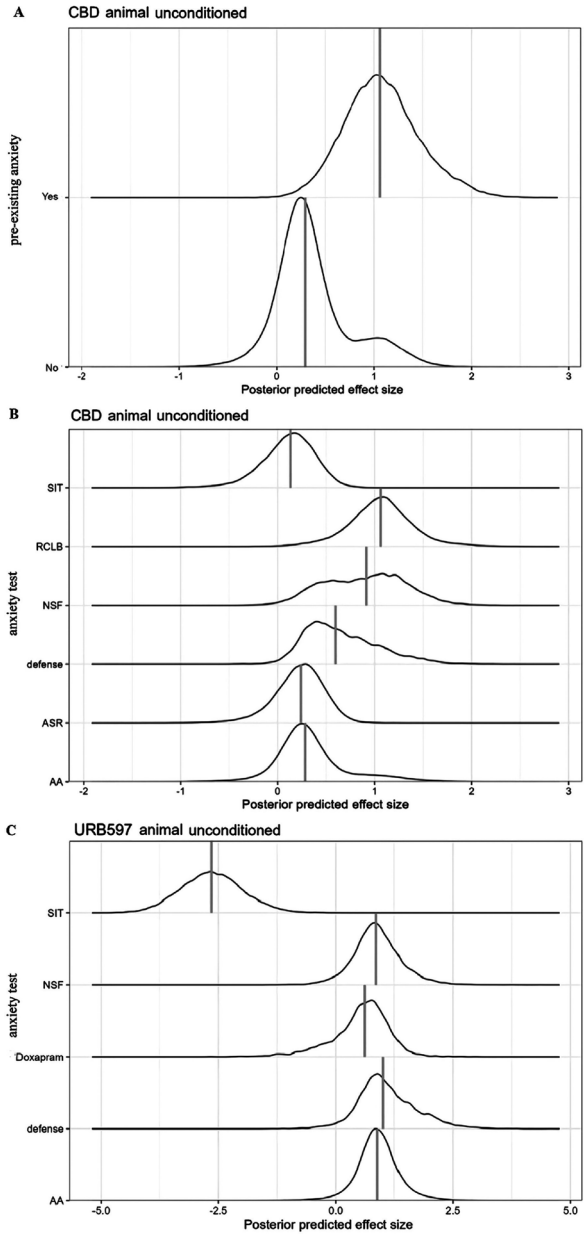

Figure 3. Plots of posterior predictive distributions of effect sizes for the levels of significant categorical moderator variables.
Note: Break-down is presented of the different levels of significant moderators of unconditioned anxiety in animals: pre-existing anxiety condition (Panel A, CBD) and anxiety test (Panel B, CBD; Panel C, URB597). Blue lines represent median effect sizes. CBD: Cannabidiol; SIT : social interaction test; RCLB: repetitive compulsive-like behavior; NSF: novelty suppressed feeding; ASR: acoustic startle response; AA: approach avoidance. Please note that all anxiety tests investigated per drug are plotted.

3.2.3. Quality of evidence

Assessments of the quality of evidence using the GRADE approach are summarized in Supplemental Table 25[60]. Risk of bias assessments for anxiety outcomes for individual studies are provided in Supplemental figure 10. Our ratings of quality of the body of evidence were low for all combinations of drug (CBD, URB597, AM404, PF-3845) in unconditioned and conditioned anxiety in animals and experimentally induced anxiety in humans. Quality of evidence was impacted negatively by:

1) Unclear to high risk of bias for reported effects. Risk of bias was considered serious across all animal studies due to underreporting of this information, and competing financial interests. Risk of bias was also considered serious for the effect of CBD on experimentally induced anxiety in humans, as 3 out of 6 studies were assessed as high risk of bias because of 1) increased mental sedation in the CBD condition and, as a potential consequence, unsuccessful blinding[61]; 2) highly variable CBD plasma concentrations (4.7 (7) and 17 (29) ng/mL 1 and 2 hours after administration), that led to concerns about failures in implementing the intervention[62]; 3) unclear bias due to missing outcome data and concerns about selective outcome reporting[10].

2) Publication bias which was (very) strongly suspected for all drugs and types of anxiety. Visual inspection of funnel plots (see Supplemental Figures 11-18) and significant results (ps ≤.02) on Egger's test for funnel plot asymmetry indicated an overrepresentation of publications with large and beneficial compared to smaller or adverse drug effects across smaller studies, relative to a more balanced mix of findings across larger studies[63];

3) Significant unexplained heterogeneity. High heterogeneity in our included animal studies renders interpretation of an overall effect rather difficult[34];

4) Indirect evidence by the use of healthy subjects and no pre-existing anxiety in most preclinical studies, which may lower the level of face and predictive validity, the use of conventional rather than ethological measures of anxiety, and test conditions that were not always optimized to measure anxiolytic effects[42,43,64,65];

5) Imprecision of URB597 effects on unconditioned anxiety, indicated by a large range of drug effects, from anxiolytic to anxiety increasing; Suspected risk due to points 3-5 are mitigated by the moderate to large overall effect sizes, despite the fact that within many studies (52%) different drug doses were tested.

3.2.4. Sensitivity analyses

The robustness of the findings regarding our primary research aim was evaluated in sensitivity analyses (see Supplemental Table 26 for excluded effects). Results of the sensitivity analyses are available in supplementary online material (doi:10.5281/

zenodo.7829148). After excluding studies with a high risk of bias, the pooled effect of CBD on human experimentally induced anxiety became smaller and non-significant, Hedge's g [95% CI] = 0.50 [-0.05, 1.05], p = 0.07. The pooled effect of URB597 on unconditioned anxiety became significant, Hedge's g [95% CI] = 0.55 [0.11, 1.00], p = 0.01, but direction and magnitude of the effect were unaltered. For the other compounds and types of anxiety, direction, magnitude, and significance of pooled effects remained unchanged in the sensitivity analyses. The moderators identified as having a non-zero effect with BRMA in the planned moderator analyses (section 3.2.2) remained the same in the sensitivity analyses. This indicates that the meta-analytic findings are largely robust to excluding studies assessed as high risk of bias or otherwise strongly affecting the overall results.

3.2.5. Exploratory moderator analyses

Exploratory moderator analyses were planned with a subset of theoretically most important study characteristics: anxiety test, drug dose (human equivalent dose (HED)*60 across drugs in included studies ranged between 0.05 and 900 mg) and type of outcome for tests of conditioned anxiety. See Supplemental Tables 27, 29, 31, 33, 35, 38, 40, 42 for all selected predictors with BRMA. Interaction effects between anxiety test and type of outcome, and dose and dose2 (or HED and HED2 for animal studies) were included in these models to explore dose-response relationships. The moderator analyses showed that tests of repetitive-compulsive behavior were associated with larger CBD effects and the social interaction test was associated with smaller URB597 effects compared to approach avoidance tests. Further, only effects of AM404 in tests of repetitive compulsive-like behavior were dependent on dose. Within the range of tested doses (HED 0.0081-1.62), higher HED was associated with larger drug effects (Supplemental Figure 19).

3.3. Safety and tolerability of FAAH hydrolysis and AEA transport inhibitors

Harm-related information was a secondary outcome, and our literature search did not include terms related to safety and tolerability. Our qualitative summary of harm-related information from the included studies with harm-related objectives (*n*=17) is therefore non-systematic.

3.3.1. Safety and tolerability of CBD

Included studies employing CBD, in which side effects were either noted when mentioned spontaneously by human participants or were monitored as part of the study in humans or dogs, reported no significant adverse events[62,66,67]. Self-rating of subjective states yielded no particularities, except from increased mental sedation in healthy individuals with 400 mg CBD, 60 and 75 min after oral drug intake, that was not observed in patients with social anxiety disorder[61,62,68]. This is in line with previous reviews[41,46–48].

No undesirable effects of the drug on learning and memory were observed when repeatedly administered in mice and rats[69–71]. Differential effects of repeated CBD administration, including no effect on motor activity in mice and rats and weight gain in rats and dogs underline the difficulties of interspecies translation[67,70–72].

3.3.2. Safety and tolerability of FAAH inhibitors

Sub-chronic treatment with irreversible FAAH inhibitors PF-04457845 and JNJ-42165279 in experimental studies with healthy human volunteers, and JNJ-42165279 in a clinical trial with patients with social anxiety disorder yielded no serious adverse events[73–75].

Doses of PF-3845 sufficient to induce an anxiolytic effects in acute and chronically stressed mice exerted no effect on working memory, locomotor activity, body temperature, and tests of learning and memory[76,77].

Six weeks of treatment with the irreversible FAAH inhibitor URB597 unexpectedly led to chemical alterations in the cingulate cortex in mice[78]. The reversible FAAH inhibitor SSR411298 elicited in mice hyperlocomotion, hypothermia, antinociception, and catalepsy at doses higher than needed to produce an anxiolytic effect[79].

3.3.3. Safety and tolerability of AEA transport inhibitors

The endocannabinoid transport inhibitor WOBE437 elicited in mice a full cannabinoid tetrad response at doses higher than needed to produce an anxiolytic effect[80].

3.3.4. Risk of bias for harm-related outcomes

All studies (*n*=17) with information on safety and tolerability were assessed as unclear risk of bias, see Supplemental material, section 3.6 for grading per criterion. Risk of bias for individual studies and summary risk of bias assessments are displayed in Supplemental Figure 20.

4. DISCUSSION

The endocannabinoid system has gathered a lot of interest in relation to its potential role in (the alleviation of) anxiety. The potential of pharmacological enhancement of AEA levels for treating anxiety symptoms has often been discussed. However, a comprehensive systematic review and meta-analysis into the effectiveness of this strategy, potential moderators, and side effects, had not yet been conducted, which was the aim of this paper.

4.1. Overall drug effects

Our results showed significant anxiety reduction across drugs for conditioned and unconditioned anxiety in rats, mice and Cricetidae, and for experimentally induced anxiety in humans, with moderate to large effect sizes (Hedge's g between 0.47-0.79) and

anxiety-reducing effects with all compounds (CBD, URB597, AM404, PF-3845). The only exception to these positive meta-analytic results was a lack of significant effect of the selective and irreversible FAAH inhibitor URB597 on unconditioned anxiety in animals. These findings provide broad evidence for the often discussed potential of AEA augmentation for treating symptoms of anxiety and related disorders.

4.2. Moderators of drug effects

We identified several moderators of drug effects on anxiety outcomes, as expected given the large diversity in study procedures. As explained in the introduction, a theoretical distinction can be made between unconditioned and conditioned anxiety. For animal studies we conducted meta-analyses for both classes of anxiety for CBD, URB597 and AM404. For PF-3845, only tests of unconditioned anxiety were available. Overall, the meta-analytic analyses demonstrated evidence of beneficial effects of CBD, AM404, and PF-3845 on unconditioned anxiety and of CBD, URB597 and AM404 on conditioned anxiety.

Moderators analyses were conducted using Bayesian regularized meta-regression (BRMA)[56]. Firstly, we found drug effects of CBD and URB597 on unconditioned anxiety to be dependent on type of anxiety test. More than half (56%) of the effects on anxiety outcomes in this meta-analysis were measured using approach avoidance tests in animals. Interestingly, approach avoidance tests yielded relatively low effect sizes, and in comparison larger beneficial effects of CBD were found in tests of repetitive compulsive-like behavior. The marble burying test is an established and often used model of repetitive behavior[81]. Attenuating effects of CBD on marble burying are not likely a consequence of sedation. Motor functioning was not affected by CBD in included studies that measured both marble burying and motor activity[82–84].

The dose effect-relation for AM404 on repetitive compulsive-like behavior, identified in exploratory moderator analyses, strengthens the evidence for beneficial effects of AEA enhancement for this type of behavior. However, beneficial effects of CBD and AM404 on repetitive compulsive-like behavior have mostly been demonstrated in studies using the marble burying test. Single test results have limited predictive validity for drug effects in patients. These preclinical findings therefore warrant more extensive testing in other models of repetitive behavior as well as in humans.

While URB597 was anxiolytic in other anxiety tests, our moderator analysis showed that overall, it decreased time in social interaction across studies[85–88]. An explanation for this finding may be that the social interaction test is not aversive enough to detect beneficial URB597 effects on anxiety[89,90]. Some effects in the opposite direction may result from a curvilinear relation between amygdalar AEA levels and time in social interaction[87]. That is, normal physiological AEA levels in the amygdala during the test were associated with maximum time in social interaction, and URB597 could only improve interaction

time in rats with pharmacologically reduced amygdalar AEA levels. Administration of URB597 to healthy animals increased AEA levels above the optimum and led to social withdrawal[87,88].

Next to type of anxiety test, a second moderator with respect to the effects of CBD on unconditioned anxiety in animals was pre-existing anxiety condition,, which increased effects compared to no such condition. Anxiety conditions were generated by exposure to a single stressor, or to chronic unpredictable stressors[19,91–94]. All procedures had in common that they induced anxiogenic behavior by stress, compared to control animals. From the stress literature it is known that the ECS acts mediates stress effects on behavior (for a review see[13]). Further, within single studies, anxiolytic effects of inhibitors of FAAH in rats seemed to depend on the stressfulness of experimental conditions[90,95].

A third moderator of CBD effects on unconditioned anxiety was publication year. Our sample was characterized by a large range in publication years (1990-2021). Effects of CBD were smaller in more recent compared to older publications. This result is in line with a phenomenon called the decline effect: over time, the number of controlled studies increases and scientifically discovered effects tend to become smaller[96]. Statistical power is very important to the decline effect: When studies are underpowered, the chance increases that positive (non-null) effects are not indicative of a true effect in the population, or are an overestimations of this true effect[97]. Unfortunately, most studies in neuroscience are grossly underpowered[97]. When initial underpowered studies that report an effect are being followed up, it is very likely to find smaller effects over time.

Further, differences between the sexes might partly explain the effect of publication year on effect size, given that only in publications from recent years the effect of CBD on female animals has been studied. Sex differences were not identified in the analysis. However, this might be a consequence of females still being poorly represented compared to males. Dependent on type of anxiety test, female animals show differences in anxiety-like behavior compared to their male counterparts[98]. In addition, differences in the endocannabinoid system in male and female rodents have been observed[99]. These differences may influence the effect of CBD in males and females.

No moderator effects related to different types of outcomes in conditioned anxiety tests in animals were identified. This may partly be due to the duration of drug effects that can overlap different phases unless they are carefully separated experimentally. No other moderators of drug effects on conditioned anxiety in animals, and of the effect of CBD on experimentally induced anxiety in humans were identified, whereas significant statistical heterogeneity suggests variation in effect sizes. Some categories in the moderator analyses included only few studies, and therefore these levels of moderator variables were relatively poorly represented.

4.3. Quality of the evidence

Notwithstanding our positive results, the quality of the evidence was assessed as low. Importantly, publication bias was strongly or very strongly suspected across all drug types and types of anxiety. To date no procedures are yet available to estimate the extent of this bias for multilevel meta-analysis. Nevertheless, we caution that the reported pooled effect sizes likely overestimate the true effect sizes. Furthermore, our findings provide only indirect evidence of clinical efficacy, since the vast majority of included studies (95%) was conducted in non-human mammals. Given the diversity in study procedures in preclinical research, the available body of evidence is suitable for identifying potential moderators of clinical effects, while conclusions about overall clinical efficacy are premature[34].

Our sensitivity analyses demonstrated lack of robustness of our findings with respect to the effect of URB597 on unconditioned anxiety in animals and of CBD on experimentally induced anxiety in humans. We excluded studies based on our assessment of bias that was, in retrospect, rather stringent. For example, concerns about blinding success given sedative effects of CBD led to a high risk of bias rating in one human study, while blinding may have been unsuccessful in other studies as well. However, this remains obscure because blinding success was rarely assessed across studies. Yet, the results of these sensitivity analyses indicate that more high quality evidence is paramount to further substantiate our findings regarding beneficial effects of AEA augmentation for treating symptoms of anxiety and related disorders[100].

4.4. Safety and tolerability

We described data from the *n*=17 included papers with harm-related objectives, each with unclear risk of bias for harm-related outcomes. In most of these studies no functional or behavioral side-effects were reported that could be attributed to the drugs under study. Side effects typically induced by CB1 receptor agonists were reported in two studies with drugs that were not studied enough to warrant meta-analysis (SSR411298 and WOBE437)[79,80]. In line with the overall favorable picture that emerges from previous reviews, the studies we reviewed reported no severe adverse events after CBD administration[41,46–48]. A systematic investigation of relations between drug concentrations and desirable and undesirable drug effects is needed to elucidate whether undesirable effects also occur at doses needed for anxiolytic effects. As we argue in Kwee et al. 2022[41], more studies that also include integrated pharmacokinetic and anxiety assessments are needed to answer this question for repeated CBD dosing.

4.5. Limitations of the review

A primary critical note concerns the assumption that the effects of the studied compounds are associated with an increase in AEA levels. Most studies have relied on single dosing, whereas available evidence with CBD suggests significant increases in AEA levels after continuous dosing during several weeks[20]. Moreover, some compounds exert

additional effects next to enhancement of AEA availability. Specifically, FAAH inhibitors do not only elevate AEA levels, but also elevate levels of oleoylethanolamide (OEA) and palmitoylethanolamide (PEA)[25,75,101]. Nevertheless, AM404, an AEA transport inhibitor that does not affect PEA and OEA levels also exerted beneficial effects on anxiety outcomes in our meta-analysis[25]. This strengthens the assumption that the anxiolytic effects of the drugs under study are set about via pharmacological enhancement of AEA levels. For CBD, the mechanistic route for anxiety reduction is even less clear. Although CBD is a weak inhibitor of FAAH its action may also be partly explained by its binding to intracellular AEA transporters[2,3]. In fact, 76 different molecular targets of CBD were identified, including ionotropic, non-cannabinoid targets[3].

Several methodological limitations affect the generalizability of our results. First, the number of studies in our meta-analysis did not allow testing a plethora of moderator variables. That is, although BRMA limits overfitting, generalizability can still be low if the sample of studies is small and idiosyncratic[56]. With this in mind, the data of different types of non-human mammals were analyzed together and we only investigated main effects of species. Although the 95% credible interval of species on itself included zero in our planned moderator analyses, an interaction between species and other variables, such as dose cannot be excluded[41].

Second, our findings regarding safety and tolerability of tested compounds do not result from a systematic literature search and evaluation of these parameters. For a translation of wanted and undesirable drug effects in preclinical models to substantiated and safe dose selection for clinical trials we recommend using the IB-de-risk tool[54]. See for example[41,102] for a dose response analysis of CBD. Such a structured approach for dose-rationale, as well as FAAH inhibition assays and measurement of AEA plasma concentrations are required to identify what constitutes unnecessarily high and perhaps riskful dosing[103].

Third, literature was searched up to May 2021 and at that time, only two clinical trials with inhibitors of FAAH and AEA transport were published. The first randomized controlled trial reported a positive effect of four weeks of 300 mg CBD in social anxiety disorder (*n*=37), whereas the second observed no effect of 12 weeks of JNJ-42165279 (*n*=134)[66,75]. More recent publications including two clinical trials were not included in this meta-analysis. The first entailed an open-label study in which 300 mg oral CBD plus standard care (*n*=61) was compared to standard care alone (*n*=59) in frontline health care professionals working with patients with COVID-19[68]. In this study, CBD induced anxiolytic effects. The second double-blind clinical, trial augmentation of eight therapist-assisted exposure in vivo sessions (weekly, outpatient) with 300 mg oral CBD yielded no differences in treatment outcome over time between CBD (n = 39) and placebo (n = 41)[38].

4.6. Recommendations for future work

This promising field of research has room for improvement. More systematic reporting of methods and study design can aid in interpreting each other's work and assessment of research quality. A structured approach to reporting for human research has been available in the form of the CONSORT statement[104]. Standards for reporting are now also available for animal research in the ARRIVE 2.0 guidelines[105]. More uniformity across anxiety tests in the parameters that are studied may aid in synthesizing findings from multiple studies. The definitions for outcomes of tests of conditioned anxiety that we established for this meta-analysis (see Supplemental Figure 1) may help specify (reporting of) endpoints in conditioned anxiety research.

In the past two decades FAAH inhibitors have been developed at a rapid pace. These compounds have greater selectivity than the 'old' FAAH inhibitor URB597, for example with respect to off-target carboxylesterases that may limit therapeutic applicability[106,107]. Keeping in mind the serious adverse events in the BIAL phase 1 trial and given the divergent results with respect to safety and tolerability of FAAH inhibitors, a structured approach for dose-rationale should be employed on a drug-by-drug basis before proceeding to first in-human trials[41,49,102,108].

5. CONCLUSION

This systematic review and meta-analysis provides extensive evidence for the beneficial effects of FAAH inhibitors and inhibitors of AEA transport in preclinical tests of anxiety. The beneficial drug effects on conditioned anxiety are especially relevant to clinical practice, because fear conditioning paradigms model the learning that takes place during psychotherapy. Furthermore, a pre-existing anxiety condition in animals predicted larger effects of CBD on unconditioned anxiety. It is therefore tempting to conclude from our meta-analytic results that effective application in patients is feasible. However, the quality of the evidence was low and human studies are still scarce. Therefore, definitive conclusions will have to await more high quality evidence. The analyses we present here indicate that anxiety-reducing effects of the studied compounds can be demonstrated across-the-board but may also depend on the specific facets of anxiety that are studied. They suggest that anxious animals and repetitive behavior seem most susceptible to pharmacological AEA enhancement. An increased focus on the specific aspects of stress and anxiety that that are under endocannabinoid control will narrow down potential clinical applications. At the same time, investigation of drug efficacy in patients remains paramount to allow the flow of information back and forth between preclinical and clinical research.

Role of the funding source

Only the team of researchers had a role in the design and execution of the study, the analyses and interpretation of the data, and decision to submit for publication. There was no influence from the funding organizations in any of these aspects.

Contributors

All authors contributed to the design of the study. CK, LG and JB were responsible for the study protocol. CK, NL and RvdK screened and selected studies, CK, NL and collaborators IdV and AM extracted data, and CK and NL graded the quality of the evidence, under the supervision of LG and JB. CvL analyzed the data. CK wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

The authors have no conflicting interests to declare.

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APPENDIX. SUPPLEMENTARY MATERIALS

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4

CHAPTER 5

The role of the endocannabinoids 2-AG and anandamide in clinical symptoms and treatment outcome in veterans with PTSD

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ABSTRACT

Background: Although current treatments for Post-Traumatic Stress Disorder (PTSD) in war veterans are effective, unfortunately 30–50% still do not benefit from these treatments. Trauma-focused therapies, eg exposure therapy, are primarily based on extinction processes in which the endocannabinoid system (ECS) plays a significant role. Therefore, it can be hypothesized that poor treatment response on trauma-focused therapy due to extinction deficits may be associated with a poorly functioning ECS. The present study examined whether the endocannabinoids anandamide (AEA) and 2-arachidonylglycerol (2-AG) are associated with post-treatment symptom reduction.

Methods: Blood plasma levels of AEA and 2-AG were determined in war veterans with a PTSD diagnosis (n = 54) and combat controls (n = 26) before and after a 6–8 month interval. During this period veterans with PTSD received trauma-focused therapy (eg cognitive behavioral therapy with exposure or eye-movement desensitization and reprocessing). Clinical symptoms were assessed before and after therapy with the Clinician Administered PTSD Scale (CAPS), State-Trait Anxiety Inventory (STAI) and Mood and Anxiety Symptom Questionnaire (MASQ).

Results: Regression analysis demonstrated that pretreatment endocannabinoid levels were not predictive of PTSD symptom reduction. Additionally, baseline endocannabinoid levels did not differ between either PTSD and combat controls or between combat controls, treatment responders, and non-responders. Only cortisol levels significantly decreased over time from pre- to posttreatment ($p = .041$). Endocannabinoid levels were significantly lower in individuals who reported cannabis use during their lifetime, independent of PTSD diagnosis. Furthermore, correlation analysis revealed that pretreatment 2-AG levels in PTSD were positively correlated with anxious arousal ($r = .354$, $p = .015$) and negatively with avoidance symptoms ($r = -.271$, $p = .048$). Both posttreatment AEA and 2-AG were positively correlated with trait anxiety (AEA $r = .459$, $p = .003$; 2-AG $r = .423$, $p = .006$), anxious arousal (AEA $r = .351$, $p = .024$; 2-AG $r = .311$, $p = .048$) and general distress depression symptoms (AEA $r = .414$, $p = .007$; 2-AG $r = .374$, $p = .016$).

Conclusion: Since endocannabinoids are mainly generated 'on demand', future work could benefit by investigating endocannabinoid circulation under both baseline and stressful conditions. In line with previous research cannabis use was associated with lower endocannabinoid levels. The correlation analysis between pre- and posttreatment endocannabinoid levels and pre- and posttreatment clinical symptomatology were exploratory analysis and should be replicated in future research.

1. INTRODUCTION

During military deployment exposure to traumatic situations can lead to the development of Post-Traumatic Stress Disorder (PTSD). PTSD is characterized by intrusive re-experiencing, avoiding reminders of the traumatic event, hyperarousal and alterations in mood and cognition[1]. European prevalence rates demonstrated that approximately 9% of soldiers develop PTSD symptomatology six months after returning from military deployment[2,3]. Although there is a large number of effective treatments for PTSD 30–50% of patients does not adequately benefit from these therapies[4–6]. Neurobiological mechanisms that are relevant to the etiology of PTSD are linked to the endocannabinoid system (ECS) and are suggested as a novel target for the development of pharmacological treatments besides current PTSD treatments[7,8].

The ECS is a lipid signaling system that plays an important role in the regulation of stress, depressive and anxiety like behavior[9]. It also acts as a feedback loop to inhibit responses of the HPA-axis to stressors[10,11]. The ECS consists of the CB1 and CB2 receptor which belong to the class of G protein-coupled receptors. The endogenous ligands that activate the CB1 and CB2 receptors are anandamide (AEA) and 2-arachidonylglycerol (2-AG)[12]. AEA and 2-AG, are generated 'on demand', eg in reaction to stressful situations, and act in a retrograde manner to regulate neurotransmitter release, primarily through inhibition of GABAergic and glutamatergic neurotransmission[13–16]. CB1 receptors are particularly of interest in relation to PTSD and anxiety disorders because they are expressed in most limbic structures in the brain, including the prefrontal cortex, amygdala and hippocampus, which are areas commonly reported as involved in PTSD and fear extinction learning[17]. CB2 receptors are located primarily in peripheral and immune tissues[18].

Preclinical research has demonstrated a crucial role for the ECS in the extinction of aversive memories[19]. Blocking or genetically deleting the CB1 receptor resulted in a failure to extinguish fear[19–21]. On the other hand, augmenting endocannabinoid signaling by CB1 agonists or the pharmacological blockade of the enzyme FAAH, that breaks down the endocannabinoid AEA, enhances extinction learning[22,23]. First line treatments for PTSD and anxiety disorders are often based on these (fear) extinction learning mechanisms, eg (imaginary) exposure therapy[24]. During treatment patients are repeatedly exposed to the feared stimuli without experiencing actual threat in order to achieve extinction of this fear response. Interestingly, PTSD patients often show an inability to inhibit the intense fear reaction to stimuli that reminds them of their trauma[17,25]. PTSD and anxiety disorders are therefore often described as a disorder of learning and memory because of these deficits in extinction and recall of the extinction memory[17,25]. Non-response to current treatments is possibly due through deficits in these fear extinction processes. Together these findings suggest that poor response to treatment due to an extinction deficit may be associated with a poorly functioning ECS.

Previous studies have demonstrated that alterations in circulating endocannabinoids levels were indeed associated with PTSD. In individuals who were exposed to the world trade center attacks, circulating 2-AG plasma levels measured 4–6 years after the event were reduced among those who had developed PTSD in comparison to those who did not[26]. Although no differences in plasma AEA levels were found, within the PTSD group AEA levels exhibited a negative relationship with the degree of intrusive symptoms experienced. Another study reported that PTSD was associated with reduced AEA levels accompanied by an upregulation of CB1 receptors within the amygdala-hippocampal-cortico-striatal neural circuit, compared with a trauma and healthy control group[27]. This might indicate a compensatory upregulation of CB1 receptors in PTSD due to low receptor occupancy by AEA. However, opposite or null effects were also reported on differences in AEA and 2-AG levels between PTSD patients and controls[14,28].

So far, studies have mainly focused on endocannabinoid levels in individuals with PTSD, and hardly on the predictive value of these levels on treatment outcome. Since preclinical studies suggests that extinction processes depend on endocannabinoid signaling, higher pretreatment endocannabinoid levels might be associated with better treatment outcome in PTSD. Insight into the predictive value of endocannabinoid levels on treatment outcome can give more insight into the potential role of augmenting this system (eg with cannabidiol or FAAH inhibitors), and hence inform the development of novel pharmacotherapy aimed at the ECS in the treatment of PTSD and anxiety related disorders.

In the current study blood plasma concentration were analyzed from war veterans with and without PTSD diagnosis. Veterans with a PTSD diagnosis who received trauma-focused therapy were assessed before and after 6–8 months of treatment (treatment as usual). This treatment consisted of cognitive behavioral therapy with exposure (tfCBT) and/or eye-movement desensitization and reprocessing (EMDR). The combat control group did not receive treatment during this interval. The primary aim of this study was to examine if pretreatment endocannabinoid levels in individuals with PTSD can predict treatment outcome. Additionally, we examined whether baseline endocannabinoid levels (AEA and 2-AG) differed between PTSD and combat controls to replicate finding from previous studies. We hypothesized that PTSD patients demonstrate lower baseline endocannabinoid levels compared to controls, and that higher pretreatment endocannabinoid levels are associated with larger PTSD symptom reduction within the patient sample. Our secondary aim was to explore if pre- and posttreatment endocannabinoid levels are associated with specific pre- and post-treatment PTSD symptom clusters (re-experiencing, avoidance and hyperarousal), state and trait anxiety, and anxiety and mood symptoms (depressive symptoms, anxious symptoms, mixed symptoms, anxious arousal and anhedonic depression).

2. METHOD

2.1. Participants

Subjects that were included in the study were part of a larger study on the neurobiological mechanism underlying the recovery of PTSD[29]. Individuals diagnosed with PTSD (*n* = 57) were recruited from one of the four outpatient clinics of the Military Mental Healthcare, Ministry of Defence, The Netherlands. Veterans without a current psychiatric illness (combat controls; *n* = 29) were recruited through advertisements. Subjects were included in the study if they were active duty military or veterans who in the past participated in a military deployment for a duration of at least 4 months and who were aged between 18 and 60 years at time of the study. Subjects were excluded in case of substance abuse and/or substance dependency during the study. All measurements were assessed twice within a 6–8 month interval. In between the two assessments PTSD patients received trauma-focused therapy (treatment as usual) consisting of cognitive behavioral therapy with exposure (tfCBT) and/or eye-movement desensitization and reprocessing (EMDR). The study was approved by the Medical Ethics Committee of the University Medical Center Utrecht (09/314) and conducted in accordance with the Declaration of Helsinki. All participants provided verbal and written informed consent before screening and participation in the study.

2.2. Clinical Evaluation

PTSD symptom severity was assessed using the Clinician Administered PTSD Scale for DSM-IV (CAPS-IV) which was administered by a trained researcher[30]. Participants with a PTSD diagnosis were included when the total score on the CAPS was 45 or above[31]. Controls were included if they had a total CAPS score of ≤15 and no current psychiatric disorder[30]. In addition, the Structural Clinical interview for DSM-IV axis I disorders (SCID-I)[32] was conducted to access comorbid psychiatric disorders. Additionally, in the SCID-I interview cannabis use was probed: 'Did you ever used cannabis during your lifetime.' The interview asked people to describe the period of the heaviest substance use (age, date and duration). This period could then be divided in "substance never used", "used substance less than 10 times a month" or "used substance at least 10 times a month or substance dependency." However, since the description of the period of use was not clearly documented in the interviews we chose to score this question on cannabis use during someone's lifetime as "yes" or "no".

2.3. Questionnaires

Questionnaires were assessed both pre- and posttreatment. State and trait anxiety were assessed with the State-Trait Anxiety Inventory (STAI-DY)[33]. The Early Trauma Inventory (ETI) was used to assess traumatic experiences during childhood[34]. Lastly, The Mood and Anxiety Symptom Questionnaire (MASQ) was used to measure depressive, anxious, and mixed symptoms symptomatology[35]. The questionnaire consists of three scales, one scale that measures general distress (depressive symptoms, anxious symptoms

and mixed symptoms), one scale that measures anxiety symptoms (anxious arousal) and one scale that measures depression symptoms (anhedonic depression). Additionally, data was collected about medication use (use during the time of the study visits) and cigarette use (average per week).

2.4. Endocannabinoid and Cortisol Measurements

Blood samples were taken both pre- and posttreatment between 08.00 and 11.00 AM All blood samples were collected between 2010–2013. After collection of the blood samples, they were immediately centrifuged to extract plasma and frozen at − 80 °C. In 2019 AEA and 2-AG levels were determined. Anandamide levels were determined using the AEA ELISA (abx258779, Abbexa Ltd, Cambridge, UK). The lower limit of detection was 3 ng/mL. Intra-assay variation at 60 ng/mL was 3.2%. Inter-assay variation at 60 ng/mL was 8.8%. Arachidonoylglycerol levels were determined using the 2-AG ELISA (abx258337, Abbexa Ltd, Cambridge, UK). The lower limit of detection was 4 ng/mL. Intra-assay variation at 95 ng/mL was 8.4%. Inter-assay variation at 95 ng/mL was 11.0%. Additionally, we determined cortisol levels because of the role of the ECS in mediating the actions of glucocorticoids[10]. Cortisol was measured in one batch using an electrochemiluminescence immunoassay on the Modular E411 (Cortisol II, Roche Diagnostics GmbH, D-68298 Mannheim, Germany). The lower limit of detection was 2 nmol/L and intra assay variation was <2.4% at 100–900 nmol/L respectively.

2.5. Statistical Analysis

Pre- and post-plasma levels of AEA, 2-AG and cortisol levels were first compared between individuals with PTSD and combat controls, using a mixed model analysis of variance (mixed ANOVA) for AEA, 2-AG and cortisol separately. In this analysis the factor Group (PTSD and combat controls) was the between-subjects factor and Time (pre- and post-plasma concentrations) the within-subject factor. Subsequently the PTSD subjects were divided into treatment responders and non-responders. Based on previous studies treatment responders were conceptualized to have a reduction in CAPS scores of ≥30% posttreatment[36,37]. To investigate differences in pre- and post-plasma concentration between combat controls, treatment responders, and non-responders we used a mixed ANOVA with a three level between-subject factor Group. The aforementioned analysis was also performed including age, comorbidity (depression and anxiety), medication use (SSRIs and Benzodiazepines), childhood trauma, cigarette use, units of alcohol the day before blood sampling, and cannabis as separate covariates to the model. It was not possible to analyze type of treatment (tfCBT and EMDR) since most participants had received a combination of these treatments. Linear regression analysis was used to test whether pretreatment endocannabinoids levels (AEA and 2-AG) and cortisol in the PTSD group could predict symptom reduction. Symptom reduction was conceptualized as percentage reduction in symptoms from pre- to posttreatment. Lastly, bivariate correlations were performed to explore the relationship between pretreatment plasma levels and pretreatment CAPS symptom clusters (re-experiencing, avoidance, hyperarousal);

state and trait anxiety; and the MASQ subscales (anxious arousal, anhedonic depression, depressive symptoms, anxious symptoms and mixed symptoms). The aforementioned bivariate correlation analyses were also performed to explore this relationship at post-treatment levels and clinical symptoms. The p-values from the bivariate correlations were corrected for multiple testing by using the Benjamini-Hochberg procedure[38]. All analyses were carried out with IBM SPSS Statistics (version 25) and $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Demographic and Clinical Characteristics

Clinical characteristics of the included sample (N = 80) are displayed in Table 1. Groups did not differ on any of these clinical characteristics, except, as expected, on state and trait anxiety, comorbid disorders (depression and anxiety), medication use (SSRIs and Benzodiazepines), CAPS and MASQ scores. Five participants were removed because of plasma levels that were far above the expected range. The only female participant was excluded because of known gender differences with regard to ECS activity[39].

Table 1. Demographic and clinical characteristics of PTSD patient and combat controls (N=80)

	Combat Control (n=26) mean (sd) or n (%)	PTSD (n=54) mean (sd) or n (%)	Test statistic	p-value
Age (in years)	36.65 (9.61)	36.33 (10.02)	F=.018	.892
Education Level (ISCED)	5.31 (1.81)	5.26 (1.43)	F=.014	.908
Number of missions	2.42 (1.45)	2.65 (2.95)	F=.141	.708
Early traumatic experiences	3.13 (3.01)	5.23 (4.73)	F=3.777	.056
State anxiety	30.70 (6.92)	54.15 (9.52)	F=110.638	<.001*
Trait anxiety	31.78 (4.77)	52.26 (7.97)	F=128.588	<.001*
Substance use[1]				
Cannabis use (ever during life)	12 (63.2%)	27 (50.9%)	X^2=.840	.359
Cigarettes (average per week)	4.56 (14.24)	4.73 (6.94)	F=.005	.946
Comorbid disorders (number)[1]				
Depression current	0 (0%)	30 (55.6%)	X^2=23.111	<.001*
Anxiety disorder current	0 (0%)	18 (33.3%)	X^2=11.183	.001*
Alcohol dependence	0 (0%)	2 (4.1%)	X^2=1.049	.306
Medication[1]				
SSRI	0 (0%)	15 (28.3%)	X^2=9.083	.003*

Table 1. Demographic and clinical characteristics of PTSD patient and combat controls (*N*=80) (continued)

	Combat Control (n=26) mean (sd) or n (%)	PTSD (n=54) mean (sd) or n (%)	Test statistic	p-value
BENZO's	0 (0%)	10 (18.9%)	X^2=5.617	.018*
PTSD symptoms				
Re-experiencing (CAPS B)	0.62 (1.20)	23.56 (5.05)	*F*=519.951	<.001*
Avoiding (CAPS C)	1.04 (2.31)	23.44 (9.55)	*F*=138.434	<.001*
Hyperarousal (CAPS D)	3.08 (3.14)	24.63 (4.65)	*F*=457.323	<.001*
Total (CAPS TOTAL)	4.73 (4.81)	71.63 (12.89)	*F*=653.021	<.001*
MASQ				
Anhedonic Depression	45.87 (8.95)	76.23 (12.17)	*F*=112.963	<.001*
Anxious Arousal	21.61 (8.46)	38.11 (12.52)	*F*=32.560	<.001*
General Distress Depression	16.65 (8.12)	29.94 (9.15)	*F*=34.950	<.001*
General Distress Anxiety	14.65 (4.91)	29.00 (7.67)	*F*=66.820	<.001*
General Distress Mixed	22.91 (8.76)	45.53 (9.17)	*F*=96.636	<.001*

ISCED = International Standard Classification of Education
MASQ = Mood & Anxiety Symptom Questionnaire
CAPS = Clinician Administered PTSD Scale
[1] Because of missing data values and percentages will not always equal the total sample size
*Significant with a p<.05

3.2. Endocannabinoid and Cortisol Levels in PTSD and Combat Controls

The mixed ANOVA for differences in AEA levels (see Table 2 and Figure 1 for data) demonstrated no effect of Group, $F(1,71) = .004$, $p = .947$, Time $F(1,71) = .025$, $p = .874$, or Time × Group interaction, $F(1,71) = 1.388$, $p = .243$. For 2-AG levels no main or interaction effects were found either (Group $F(1,71) = .085$, $p = .771$; Time $F(1,71) = .789$, $p = .377$; Time × Group $F(1,71) = 1.021$, $p = .316$). A main effect of time for cortisol was observed, $F(1,71) = 4.313$, $p = .041$, partial $\eta^2 = .06$, reflecting that cortisol levels decreased over time. Finally, there was no main effect of group $F(1,71) = 1.668$, $p = .201$ or Time × Group interaction $F(1,71) = .274$, $p = .603$ for cortisol levels. The mixed ANOVA for differences in AEA levels and 2-AG levels was then tested with the addition of several covariates tested independently of each other. Out of these age, comorbidity (depression and anxiety), medication (SSRIs and Benzodiazepines), early childhood experience, units of alcohol the day before blood sampling, and cigarettes per week were tested (see supplementary data Table S1), but these covariates did not change the tested model, so none of these were retained in further analyses.

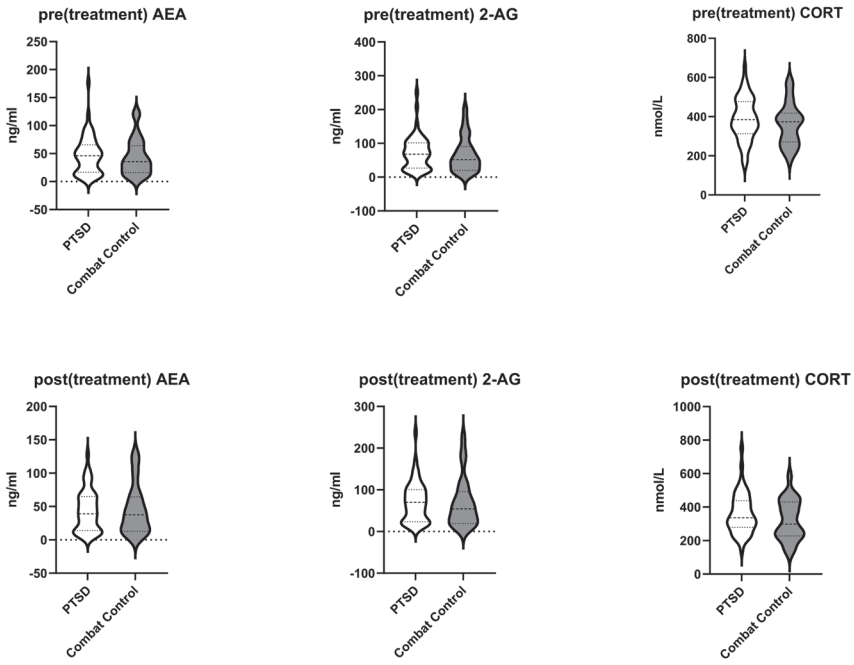

Figure 1. Pre- and post(treatment) endocannabinoid and cortisol levels for PTSD and combat controls.

Table 2. Pre and post endocannabinoid and cortisol levels for PTSD and Combat Controls

		PTSD (*n*=54)		**Combat Controls (*n*=26)**	
		M	***SD***	***M***	***SD***
AEA (ng/ml)	Pretreatment	45.81	35.10	43.27	33.08
	Posttreatment	44.08	31.01	45.53	36.76
2-AG (ng/ml)	Pretreatment	70.61	50.35	64.75	51.60
	Posttreatment	70.35	48.00	68.88	58.64
Cortisol (nmol/L)	Pretreatment	381.63	111.61	355.38	108.09
	Posttreatment	361.27	121.02	321.29	119.26

3.3. Cannabis use Associated with Post AEA, and Pre and Post 2-AG Levels

Adding Cannabis use as a covariate to the previous tested model resulted in a significant effect of Cannabis use (yes or no) on both AEA ($F(1,62) = 4.780$, $p = .033$, partial $\eta^2 = .07$) and 2-AG levels ($F(1,62) = 6.559$, $p = .013$, partial $\eta^2 = .09$). Furthermore, there was a significant Time × Cannabis use effect for both AEA ($F(1,62) = 4.649$, $p = .035$, partial $\eta^2 = .07$) and 2-AG ($F(1,62) = 4.879$, $p = .031$, partial $\eta^2 = .07$). Simple effect analysis revealed that pre(treatment) AEA did not differ between the Cannabis use groups, $F(1,71) = 3.184$, $p = .79$, contrary to post(treatment) AEA levels ($F(1,64) = 7.541$, $p = .008$, partial $\eta^2 = .11$). Pre(treatment) 2-AG levels ($F(1,71) = 5.033$, $p = .028$, partial $\eta^2 = .07$; and post(treatment) 2-AG levels $F(1,64) = 8.841$, $p = .004$, partial $\eta^2 = .12$) also differed between the Cannabis use groups, in which lifetime cannabis users demonstrated lower levels in comparison to individuals who never used cannabis during their life, see also Figure 2. This suggests that cannabis use was associated with lower post AEA, and lower pre and post 2-AG levels independent of PTSD diagnosis.

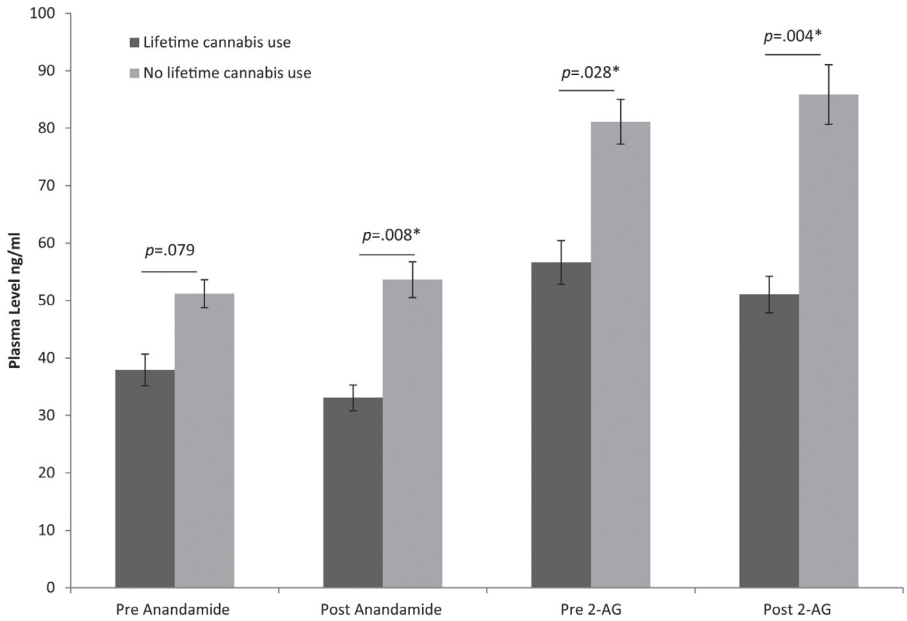

Figure 2. Post-Hoc test on mean difference and SEM on pre and post AEA and 2-AG levels between individuals who reported to have used cannabis during their lifetime and non-cannabis users, independent of PTSD diagnosis. *Significant with a $p < .05$.

3.4. No Differences in Endocannabinoid Levels Between Combat Controls, Treatment Responders and Treatment Non-Responders

The groups were divided into combat controls, treatment responders and non-responders to investigate differences in endocannabinoid levels between these three groups.

Clinical characteristics of the differentiation are displayed in the supplementary data Table S2. The mixed ANOVA analysis demonstrated the same pattern of effects as the analysis with the 2 groups differentiation (combat controls and PTSD). For mean scores and standard deviations see supplementary data Table S3 and for the covariate analysis see supplementary data Table S4.

3.5. Pretreatment Endocannabinoid Levels are Not Predictive of Symptom Reduction

AEA, 2-AG and cortisol levels were added separately into a regression model to investigate their predictive value on treatment success. Because cannabis use has an influence (of effect) on endocannabinoid levels we also added this variable into our regression model. Regression analysis demonstrated that neither pretreatment AEA ($F(2,45) = 1.222$, $p = .304$), 2-AG ($F(2,45) = .986$, $p = .381$) nor cortisol ($F(2,45) = .035$, $p = .965$) were able to predict percentage symptom reduction in PTSD symptomatology as measured with the CAPS.

3.6. Pretreatment 2-AG Levels are Associated with Anxious Arousal and Avoidance, and Posttreatment AEA and 2-AG Levels are Associated with Trait Anxiety, General Distress Depression and Anxious Arousal

Correlations between endocannabinoid levels (AEA and 2-AG), cortisol and clinical symptoms (CAPS subscales, STAI and MASQ) were only investigated in the PTSD group (see supplementary data Table S5, S6 and S7), because of predominantly low scores for the combat controls on these questionnaires. The correlations were tested for both pre- and post-treatment levels and pre- and post-treatment clinical symptoms. Only the associations that survived the Benjamini-Hochberg procedure are reported, for the other correlations see supplementary data Table S5, S6 and S7.

For the analysis on pre-treatment 2-AG and pre-treatment clinical symptoms the association between pretreatment 2-AG and pretreatment MASQ anxious arousal symptoms ($r(45) = .354$, $p = .015$. and pretreatment 2-AG and pretreatment CAPS avoidance symptoms ($r(52) = -.271$, $p = .048$) were significant, see Figure 3. This suggests that elevated 2-AG levels in PTSD are associated with higher anxious arousal and with less avoidance symptoms.

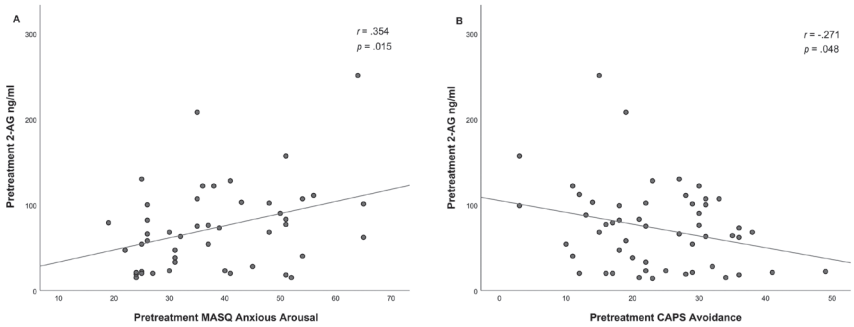

Figure 3. Correlation between pretreatment 2-AG levels and the pretreatment MASQ anxious arousal subscale (A) and correlations between pretreatment 2-AG and pretreatment CAPS avoidance symptoms (B) in PTSD patients.

Additionally posttreatment 2-AG levels demonstrated positive correlations with posttreatment trait anxiety ($r(41) = .423$, $p = .006$), general distress depression ($r(41) = .374$, $p = .016$) and anxious arousal ($r(41) = .311$, $p = .048$), see Figure 4.

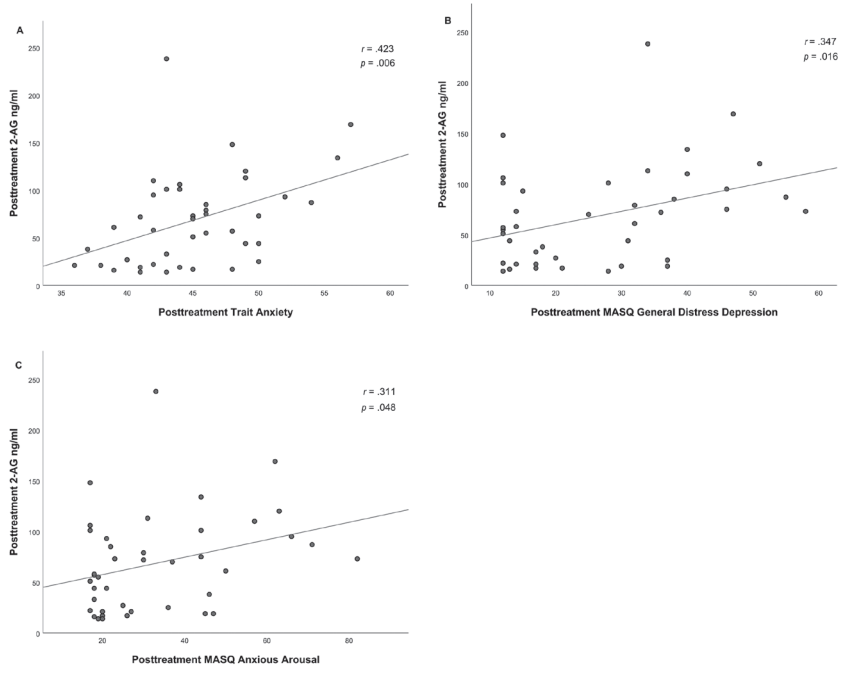

Figure 4. Correlation between posttreatment 2-AG levels and posttreatment trait anxiety (A), posttreatment MASQ general distress depression (B) and posttreatment MASQ anxious arousal (C) PTSD patients.

The same pattern was demonstrated between posttreatment AEA levels and posttreatment trait anxiety ($r(41) = .459$, $p = .003$), general distress depression ($r(41) = .414$, $p = .007$) and anxious arousal ($r(41) = .351$, $p = .024$), see Figure 5.

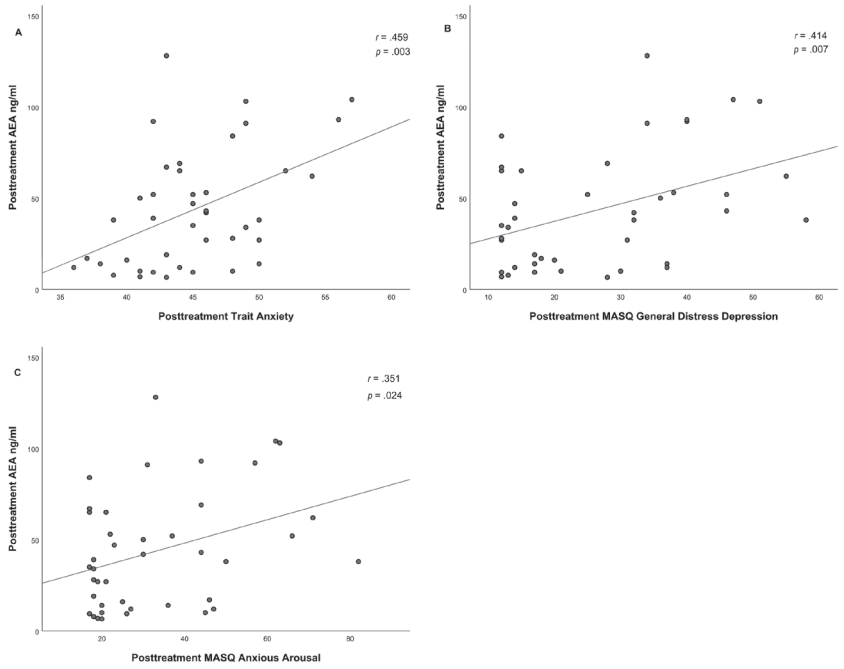

Figure 5. Correlation between posttreatment AEA levels and posttreatment trait anxiety (A), posttreatment MASQ general distress depression (B) and posttreatment MASQ anxious arousal (C) PTSD patients.

4. DISCUSSION

Our study indicated that baseline endocannabinoid levels (AEA and 2-AG) did not differ between PTSD and combat controls nor between combat controls, treatment responders, and non-responders. Pretreatment endocannabinoid levels were also not predictive of treatment induced PTSD symptom reduction. However, our findings indicated that endocannabinoid levels were reduced in individuals who reported to have used cannabis during their life, independent of PTSD diagnosis. Lastly, pretreatment 2-AG levels in PTSD were observed to be associated with pretreatment anxious arousal and avoidance symptoms. Additionally, both posttreatment AEA and 2-AG levels were associated with posttreatment trait anxiety, general distress depression and anxious arousal.

Contrary to our expectations no baseline differences in endocannabinoid levels were found between combat controls and PTSD patients. Additionally, endocannabinoids remained stable over time, eg no differences between pre- and post-endocannabinoid

levels. To date, most studies reported contradictory findings, namely either a reduction or an increase, or no differences in baseline endocannabinoid levels in PTSD patients compared to controls[26–28]. Preclinical and clinical studies suggest that endocannabinoids are recruited in the brain during stress, which supports to terminate the stress response[10,16]. Chronic elevation of these endocannabinoid levels might lead to a downregulation of endocannabinoid signaling. This could explain the reduction in endocannabinoid levels reported in some studies. On the other hand, it may not be the case that such chronic stress and corresponding endocannabinoid reactivity leads to ECS downregulation, which could explain studies that reported an increase in endocannabinoid levels in PTSD[28].

Our findings, however, correspond with the studies that reported no baseline differences in endocannabinoid levels between PTSD and controls[14,40]. In addition one of these studies reported that in contrast to healthy controls, psychosocial stress did not induce an increase in 2-AG levels is PTSD[14]. This suggests an alternative explanation in which PTSD may be associated with an unresponsiveness of the ECS in reaction to stressful situations, also supported by data from healthy individuals[15,16]. An increase in endocannabinoid levels during stress has been observed in individuals who successfully adapt to stressful situations, in contrast to those who displayed a maladaptive stress response[15,16]. A possible cause that we found no differences in endocannabinoid levels is that they were measured at baseline instead of during a stress induction. More research is needed to gain further insight into which factors play a role in the elevation or reduction in baseline endocannabinoid levels and the reactivity of the ECS under different forms of stress in PTSD patients.

Given the important role that the ECS and specifically AEA has during extinction learning[41,42], we hypothesized that lower endocannabinoid levels prior to trauma-focused therapy could possibly predict reduced treatment effect, ie, less treatment induced reduction of symptoms. However, our study did not demonstrate a relationship between endocannabinoid levels and treatment success, possibly because our study has measured endocannabinoid levels during baseline conditions. Fear reduction during extinction depends on first activating this fear, and with that potentially also the ECS. This may explain why baseline endocannabinoid levels that we compared between combat controls, treatment responders and non-responders in our study were not representative of the level of ECS activation in patients during treatment. By measuring endocannabinoid levels during both baseline conditions and during fear extinction, future studies can elucidate possible difference in reactivity of the system during fear extinction.

With post-hoc analysis we observed that reduced endocannabinoid levels were associated with people who used cannabis occasionally during their life, independent of PTSD diagnosis. It is known that chronic cannabis use is associated with CB1 receptor desensitization and down-regulation of endocannabinoid signaling[43]. Reduction of endocanna-

binoid levels in chronic cannabis users have been explained by the impact of externally administered cannabinoids that cause the ECS to adapt. In our study, we observed that also more occasional use of cannabis is associated with lower endocannabinoid levels. In our study individuals with a diagnosis of cannabis use disorder or active users at the time were excluded, which makes it less likely that adaptation of the ECS could explain the findings. An alternative explanation could be that the difference between user and non-user already existed before starting to use cannabis. Future studies need to elucidate the association between occasional use of cannabis and the ECS.

The exploratory correlation analysis between clinical symptoms and endocannabinoid levels indicated two moderate associations with pretreatment 2-AG levels. Pretreatment endocannabinoid levels in the PTSD group were positively associated with pretreatment anxious arousal subscale of the MASQ, and negatively correlated with the pretreatment avoidance subscale of the CAPS. When focusing on posttreatment levels, six moderate associations were revealed, namely three with posttreatment 2-AG levels and three with posttreatment AEA. Both 2-AG and AEA were positively associated with trait anxiety, general distress depression and anxious arousal as measured with the MASQ. Anxious arousal is characterized by somatic symptoms (eg sweating, racing heart and muscle tension) and exaggerated physiological responses to stressful events[44,45]. A study[46] indicated that in patients with panic disorder 2-AG correlated with different measures of panic and anxiety (bodily sensations and agoraphobic cognitions), which is in line with our findings. This relationship has also been confirmed by an animal study demonstrating a crucial role for 2-AG and not AEA in panic symptoms[47]. The negative association between 2-AG and avoidance behavior is contrary to a previous study that reported a positive correlation between 2-AG and avoidance behavior in PTSD patients[26]. The positive correlation with general depression symptoms is also contrary to previous studies[48,49]. Since these aforementioned findings are a result of exploratory analysis, interpretation must be with caution and future research is needed to elucidate the association between AEA, 2-AG and clinical symptomatology.

Our study has a couple of limitations that must be addressed. Firstly, endocannabinoid levels were assessed on single time points which makes the interpretation difficult because of known impact of circadian rhythm on endocannabinoid levels[50]. Although it must be noted that all blood draws were taken place between 08:00–11:00 and differences in endocannabinoid levels were previously found based on a single time point[26–28]. Furthermore, our study indicated that overall, endocannabinoid levels remained stable over time. Multiple time point and additional reactivity measures on the ECS could contribute to our understanding of the ECS. Secondly, in our study plasma levels were determined with the enzyme-linked immunosorbent assay method (ELISA). Most of the previous studies made use of chemical ionization liquid chromatography/mass spectrometry (LC-APCI-MS) to quantify AEA and 2-AG levels. Although they are both reliable and valid methods in determining AEA and 2-AG levels, ranges for these two approaches differ

which can make it difficult to compare results between these methods. Thirdly, information about frequency and duration of cannabis use from the SCID-I interview was not documented. Therefore, this study only allowed the distinction between whether or not cannabis had been used before, and no detailed information on frequency and duration of cannabis use was available. Future studies in which the frequency and duration of cannabis use is archived more precisely may shed more light on how this is related to AEA and 2-AG levels. Lastly, our study consists of a male population and findings may not be generalized. For example, women generally show a higher CB1 receptor availability and AEA levels already under basal conditions, which is also confirmed in animal research[27]. Additionally, our sample consisted only of male participants which can also explain why we did not replicate the results from earlier studies which consisted of 50–80% females[14,26–28].

5. CONCLUSION

Our study did not find indications that pretreatment endocannabinoid levels are associated with either PTSD or treatment outcome in PTSD patients. Furthermore, our findings confirm earlier findings that cannabis use is associated with reduced endocannabinoid levels. Lastly, pretreatment 2-AG in PTSD was associated with pretreatment anxious arousal and avoidance symptoms. Furthermore both posttreatment 2-AG and AEA was associated with posttreatment trait anxiety, general distress depression and anxious arousal. However, further research is needed to obtain more insights in these relations. Since endocannabinoids are mainly generated 'on demand' future work will benefit from investigating endocannabinoid circulation under both rest and stressful conditions. This will lead to a better understanding about how the ECS (dys)functions under stressful conditions and during extinction therapy sessions.

Declaration of Conflicting Interests

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SUPPLEMENTAL MATERIAL

Table S1. Additional covariates added to the mixed model on differences in endocannabinoid levels between PTSD (*n*=54) and Combat Controls (*n*=26)

	AEA			2-AG		
	df	*F*	*p*	*df*	*F*	*p*
Age	1,70	.634	.429	1,70	.194	.661
Comorbidity						
Depression	1,70	.001	.981	1,70	.458	.501
Anxiety	1,70	.008	.927	1,70	.098	.755
Medication						
SSRIs	1,70	.298	.587	1,70	.627	.431
BENZO's	1,70	1.197	.278	1,70	1.430	.236
Early traumatic experiences	1,65	.056	.814	1,65	.032	.859
Cigarettes (average per week)	1,67	1.625	.207	1,67	1.055	.308
Alcohol use (unit the day before blood measures)	1,70	.037	.848	1,70	.004	.952

3.4. No differences in endocannabinoid levels between combat controls, treatment responders and treatment non-responders (clinical characteristics)

Table S2. Demographic and clinical characteristics of combat controls, treatment responders, and treatment non-responders

	Combat Control (n=24)	Treatment responders (n=25)	Treatment non-responders (n=24)	Test statistic	p-value
	mean (sd) or n (%)	mean (sd) or n (%)	mean (sd) or n (%)		
Age (in years)	37.00 (9.85)	34.84 (9.09)	39.96 (9.84)	*F*=1.753	.181
Education Level (ISCED)	5.25 (1.87)	5.72 (1.28)	4.87 (1.46)	*F*=1.812	.171
Number of missions	2.54 (1.44)	3.24 (4.03)	2.18 (1.26)	*F*=.991	.376
Early traumatic experiences	3.13 (3.01)	5.17 (4.38)	5.64 (5.34)	*F*=2.154	.124
State anxiety[1]	30.70 (6.92)	50.55 (8.66)	57.09 (9.48)	*F*=60.561	<.001*
Trait anxiety[2]	31.78 (4.77)	50.32 (6.39)	53.82 (9.27)	*F*=64.308	<.001*
Substances[4]					
Cannabis use (ever during life)	10 (58.8%)	14 (58.3%)	12 (50%)	X^2=.447	.800
Cigarettes (average per week)	4.43 (14.76)	4.83 (7.15)	5.33 (7.23)	*F*=.045	.956
Comorbid disorders (number)[4]					
Depression current	0 (0%)	14 (56%)	13 (54.2%)	X^2=21.004	<.001*
Anxiety disorder current	0 (0%)	5 (20%)	11 (45.8%)	X^2=14.811	.001*
Alcohol dependence	0 (0%)	0 (0%)	2 (9.1%)	X^2=4.492	.106
Medication[4]					

Table S2. Demographic and clinical characteristics of combat controls, treatment responders, and treatment non-responders (continued)

	Combat Control (n=24)	Treatment responders (n=25)	Treatment non-responders (n=24)	Test statistic	p-value
	mean (sd) or n (%)	mean (sd) or n (%)	mean (sd) or n (%)		
SSRI	0 (0%)	4 (16%)	10 (41.7%)	X^2=13.689	.001*
BENZO's	0 (0%)	6 (24%)	4 (16.7%)	X^2=6.232	.044*
PTSD symptoms					
Re-experiencing (CAPS B)[2]	0.67 (1.24)	24.08 (3.73)	22.79 (6.45)	*F*=221.383	<.001*
Avoiding (CAPS C)[2]	1.13 (2.38)	23.24 (11.82)	24.75 (6.63)	*F*=65.785	<.001*
Hyperarousal (CAPS D)[2]	3.17 (3.20)	24.36 (5.13)	24.83 (4.62)	*F*=190.601	<.001*
Total (CAPS TOTAL)[2]	4.96 (4.91)	71.68 (14.57)	72.38 (11.85)	*F*=285.644	<.001*
MASQ					
Anhedonic Depression[2]	45.87 (8.95)	73.14 (11.25)	79.55 (11.66)	*F*=63.714	<.001*
Anxious Arousal[2]	21.61 (8.46)	34.59 (11.98)	41.18 (11.81)	*F*=19.079	<.001*
General Distress Depression[2]	16.65 (8.12)	26.95 (6.85)	32.45 (10.26)	*F*=20.026	<.001*
General Distress Anxiety[2]	14.65 (4.91)	26.55 (7.06)	31.27 (7.48)	*F*=38.571	<.001*
General Distress Mixed[2]	22.91 (8.78)	43.00 (9.37)	47.73 (8.79)	*F*=48.679	<.001*
At T6					
PTSD symptoms					
Re-experiencing (CAPS B)[1]	1.42 (2.28)	8.60 (7.61)	22.71 (6.30)	*F*=81.502	<.001*
Avoiding (CAPS C)[1]	0.75 (1.62)	7.44 (6.03)	22.88 (8.00)	*F*=90.050	<.001*

Table S2. Demographic and clinical characteristics of combat controls, treatment responders, and treatment non-responders (continued)

	Combat Control (n=24)	Treatment responders (n=25)	Treatment non-responders (n=24)	Test statistic	p-value
	mean (sd) or n (%)	mean (sd) or n (%)	mean (sd) or n (%)		
Hyperarousal (CAPS D)[1]	3.17 (2.63)	12.60 (6.76)	24.17 (6.45)	*F*=84.005	<.001*
Total (CAPS TOTAL)[1]	5.33 (4.19)	28.64 (16.97)	69.75 (16.19)	*F*=133.985	<.001*
MASQ					
Anhedonic Depression[3]	46.77 (10.40)	54.17 (16.80)	74.75 (21.06)	*F*=17.505	<.001*
Anxious Arousal[3]	21.00 (8.97)	24.50 (9.13)	41.70 (20.03)	*F*=15.433	<.001*
General Distress Depression[3]	14.92 (5.08)	19.46 (9.22)	34.20 (13.66)	*F*=24.156	<.001*
General Distress Anxiety[3]	14.88 (5.82)	17.88 (6.12)	28.55 (11.38)	*F*=18.079	<.001*
General Distress Mixed[3]	23.12 (9.35)	27.83 (9.23)	47.10 (14.73)	*F*=28.447	<.001*

[1] Bonferroni: All groups differ
[2] Bonferroni: Combat controls differ from responders and non-responders
[3] Bonferroni: Combat controls differ from non-responders and responders differ from non-responders
[4] Because of missing data values and percentages will not always equal the total sample size
ISCED = International Standard Classification of Education
MASQ = Mood & Anxiety Symptom Questionnaire
CAPS = Clinician Administered PTSD Scale
*Significant with a p<.05

The mixed ANOVA demonstrated no significant main effect of time for AEA $F(1,70) = .061$, $p=.805$ or group $F(1,70) = 1.058$, $p=.353$. Time x Group interaction was also not significant $F(2,70) = .685$, $p=.507$. A similar pattern was demonstrated for 2-AG, no significant main effect of time $F(1,70) = .346$, $p=.558$ or group $F(1,70) = .635$, $p=.533$ was found. Time x Group was also not significant $F(2,70) = .554$, $p=.577$. Contrary, for cortisol a main effect of time $F(1,70) = 4.078$, $p=.047$, partial $\eta^2 = .06$ was found, which suggests a decrease in cortisol levels over time. There was no main effect of group $F(1,70) = .905$, $p=.409$ or Time x Group interaction $F(2,70) = .221$, $p=.802$ for cortisol levels found. For mean scores and standard deviations see Table S3.

Table S3. Pre and posttreatment endocannabinoid and cortisol levels for Combat Controls, Treatment responders and Treatment non-responders

		Combat Controls (*n*=24)		Treatment responders (*n*=25)		Treatment non-responders (*n*=24)	
		M	*SD*	*M*	*SD*	*M*	*SD*
AEA (ng/ml)	Pretreatment	43.27	33.08	39.09	26.70	52.80	41.56
	Posttreatment	45.53	36.76	37.46	24.43	50.98	35.88
2-AG (ng/ml)	Pretreatment	64.75	51.60	63.32	39.11	78.21	59.80
	Posttreatment	68.88	58.64	62.28	40.06	78.75	54.68
Cortisol (nmol/L)	Pretreatment	355.38	108.09	384.40	103.40	378.75	121.75
	Posttreatment	321.29	119.26	370.16	103.34	352.00	138.73

When adding covariates only cannabis use demonstrated a significant effect for both AEA ($F(1,61) = 4.382$, $p=.040$, partial $\eta^2 = .07$) and 2-AG ($F(1,61) = 6.148$, $p=.016$, partial $\eta^2 = .09$). Additionally it demonstrated a significant interaction with time for both AEA ($F(1,61) = 4.669$, $p=.035$, partial $\eta^2 = .07$) and 2-AG levels ($F(1,61) = 4.744$, $p=.033$, partial $\eta^2 = .07$). This again suggests that cannabis use was associated with lower pretreatment AEA and 2-AG levels, independent group. For the other covariate analysis see Table S4.

Table S4. Additional covariates added to the mixed model on differences in endocannabinoid levels between combat controls (*n*=24), treatment responders (*n*=25), and treatment non-responders (*n*=24)

	AEA			2-AG		
	df	*F*	*p*	*df*	*F*	*p*
Age	1,69	.247	.621	1,69	.044	.834
Comorbidity						
Depression	1,69	.000	.998	1,69	.433	.513
Anxiety	1,69	.261	.611	1,69	.406	.526
Medication						
SSRIs	1,69	1.014	.317	1,69	1.332	.252
BENZO's	1,69	.946	.334	1,69	1.212	.275
Early traumatic experiences	1,69	.000	.986	1,69	.044	.834
Cigarettes (average per week)	1,66	1.722	.194	1,66	1.096	.299
Alcohol use (unit the day before blood measures)	1,69	.000	.988	1,69	.048	.828

3.6. Pretreatment 2-AG levels are associated with anxious arousal and avoidance symptoms (correlational analysis)

Table S5. Correlations between pretreatment endocannabinoid and cortisol levels and pretreatment CAPS subscales

	Pretreatment		
	AEA	2-AG	Cortisol
Pretreatment Re-experiencing (CAPS B)	-.143	-.084	-.195
Pretreatment Avoiding (CAPS C)	-.205	-.271*	-.137
Pretreatment Hyperarousal (CAPS D)	-.069	.002	.054
Pretreatment Total (CAPS TOTAL)	-.233	-.233	-.158

*significant with $p<.05$, after Benjamini-Hochberg procedure

Table S6. Correlations between posttreatment endocannabinoid and cortisol levels and posttreatment CAPS subscales

	Posttreatment		
	AEA	2-AG	Cortisol
Posttreatment Re-experiencing (CAPS B)	.168	.122	.007
Posttreatment Avoiding (CAPS C)	.112	.021	-.011
Posttreatment Hyperarousal (CAPS D)	.263	.244	-.168
Posttreatment Total (CAPS TOTAL)	.195	.135	-.058

*significant with $p<.05$, after Benjamini-Hochberg procedure

Table S7. Correlations between pretreatment endocannabinoid and cortisol levels and pretreatment State and Trait anxiety

	Pretreatment		
	AEA	2-AG	Cortisol
Pretreatment State Anxiety	.149	.166	-.112
Pretreatment Trait Anxiety	.133	.132	-.101

*significant with $p<.05$, after Benjamini-Hochberg procedure

Table S8. Correlations between posttreatment endocannabinoid and cortisol levels and posttreatment State and Trait anxiety

	Posttreatment		
	AEA	2-AG	Cortisol
Posttreatment State Anxiety	.247	.282	-.082
Posttreatment Trait Anxiety	.459*	.423*	-.022

*significant with $p<.05$, after Benjamini-Hochberg procedure

Table S9. Correlations between pretreatment endocannabinoid and cortisol levels and pretreatment MASQ subscales

	Pretreatment		
	AEA	2-AG	Cortisol
Pretreatment Anhedonic Depression	.194	.188	-.026
Pretreatment Anxious Arousal	.281	.354*	-.060
Pretreatment General Distress Depression	.139	.145	-.087
Pretreatment General Distress Anxiety	.236	.256	-.082
Pretreatment General Distress Mixed	.159	.218	.037

*significant with $p<.05$, after Benjamini-Hochberg procedure

Table S10. Correlations between posttreatment endocannabinoid and cortisol levels and postreatment MASQ subscales

	Pretreatment		
	AEA	2-AG	Cortisol
Posttreatment Anhedonic Depression	.229	.148	.486
Posttreatment Anxious Arousal	.351*	.311*	-.127
Posttreatment General Distress Depression	.414*	.373*	-.071
Posttreatment General Distress Anxiety	.300	.248	-.139
Posttreatment General Distress Mixed	.283	.218	-.202

*significant with $p<.05$, after Benjamini-Hochberg procedure

CHAPTER 6

The role of genetic variations in the FAAH rs324420 polymorphism and its interaction with CRHR1 rs110402 and CNR1 rs2180619 in anxiety and- trauma related symptoms after military deployment

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ABSTRACT

Background: During military deployment stress regulation is vital to protect against the development of anxiety and trauma related symptoms. Brain endocannabinoids play an important role in stress regulation and previous research has shown that genetic variations in the FAAH rs324420 polymorphism demonstrate protective effects during stress. In addition, this polymorphism shows interactions with the CRHR1 and CNR1 polymorphisms on anxiety. The present study examines whether genetic variations of the FAAH, CRHR1 and CNR1 polymorphisms interact with the development of anxiety and trauma related symptoms in military veterans.

Methods: Veterans (*N*=949) who went on military deployment and experienced a stressful event were genotyped for FAAH rs324420, CRHR1 rs110402 and CNR1 rs2180619. Anxiety and trauma symptoms were measured pre-deployment and 6 months after deployment. Anxiety was measured with the anxiety subscale of the Symptom Checklist-90 (SCL-90) and trauma with the Self-Rating Inventory for PTSD (SRIP).

Results: Covariance Pattern Models demonstrated no significant relation of genetic variations in FAAH rs324420 on anxiety and PTSD symptoms from pre-deployment to 6 months after military deployment. Additionally, we investigated interactions between the FAAH s324420, CRHR1 rs110402 and CNR1 rs2180619 polymorphisms. This also demonstrated no significant effects on anxiety and PTSD symptoms pre- to post deployment. However, the covariate of childhood trauma that was included in de models was significant in all these models.

Conclusion: Genetic variations in FAAH rs324420 and its interactions with CRHR1 rs110402 and CNR1 rs2180619 are not related to the development of anxiety and trauma-related symptoms. The study however indicates the importance of considering childhood trauma in the investigation of the effects of polymorphisms that are related to the endocannabinoid system on the development of anxiety and PTSD symptoms.

1. INTRODUCTION

Post-traumatic stress disorder (PTSD) is a debilitating disorder that can develop after being exposed to, or by witnessing a traumatic event[1]. Certain professions have a higher risk for the development of PTSD such as soldiers, firefighters, and first responders[2,3]. Individuals with these occupations often deal with stressful events in which adaptive stress regulation and emotional processing is vital to protect against the development of trauma and anxiety related symptoms. For example, prevalence rates demonstrated that approximately 9% of soldiers develop PTSD symptomatology six months after returning from military deployment[4,5]. Converging evidence from animal and human studies suggests that brain endocannabinoids play an important role in regulating stress and emotional processing, besides their already well-established role in the extinction of aversive memories[6–8].

The yet known elements of the endocannabinoid system (ECS) are the cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), the endogenous cannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and the catabolic enzymes for the degradation of these cannabinoids, fatty acid amide hydrolase (FAAH) for AEA and monoacylglycerol lipase (MAGL) for 2-AG[9]. The well-established role of the ECS in the extinction of fear is repeatedly demonstrated in animal and human studies. Studies showed that blocking or genetically deleting the CB1 receptor resulted in a failure to extinguish fear[6,10,11]. On the other hand, augmenting endocannabinoid signaling by CB1 agonists or the pharmacological blockade of the enzyme FAAH enhanced fear extinction (reconsolidation)[6,7,12]. However, the ECS also plays an important role in the regulation of stress. Inhibition of the FAAH enzymes prevents the reduction in AEA that is normally accompanied by stress and anxiety[13]. Therefore, FAAH inhibitors are a promising new agent in the treatment of anxiety and stress related symptoms[14,15]. Additionally, research suggest that FAAH inhibition is not anxiolytic per se but protects against anxiogenic effect of stress during high environmental aversiveness[13,16–18].

In humans, several studies have focused on a single nucleotide polymorphism in the FAAH gene because of its potential protective effects on stress and anxiety during high environmental aversiveness as was demonstrated in animal studies[13,16–18]. The polymorphism FAAH rs324420, A-allele, has an frequency of approximately 25% in populations of Caucasian ancestry and is associated with reduced FAAH activity and elevated levels of AEA[19–21]. Several studies demonstrated that A-allele carriers were associated with decreased anxiety, enhanced fear extinction learning and extinction recall, decreased threat related amygdala reactivity, increased fronto-amygdala connectivity, and protected against stress-induced decreases in AEA and negative emotional consequences of stress[22–28]. Taken together, these studies support the role of increased FAAH activity and increased AEA levels in buffering stress responses besides its enhancement of fear extinction.

In dealing with stress and anxiety corticotropin-releasing hormone receptor 1 (CRHR1) also plays a significant role. Therefore, recent studies do not focus solely on the FAAH rs324420 polymorphism, but also on its interaction(s) with different CRHR1 polymorphisms [29,30]. The ECS plays an important role in the activation and regulation of Hypothalamic–Pituitary–Adrenal (HPA) responses to stress. Namely, stress-related reduction in AEA is driven by activation of FAAH within the basolateral amygdala (BLA)[8,31]. This in turn activates the HPA axis. The association between FAAH rs324420 genotypes and amygdala habituation, which is thought to be associated with anxiety, was shown to depend on CRHR1 genotypes[29]. Blunted amygdala habituation was not directly affected by FAAH rs324420 AA/AC genotypes, but was observed in A-carriers that also had CRHR1 rs110402 AA genotype[29]. Moreover, blunted left amygdala habituation mediated between these genotypes and increased risk for anxiety disorders[29]. Another study investigated the interactions on self-reported anxiety of the FAAH rs324420 genotypes with minor alleles of several other CRHR1 polymorphisms (i.e. rs110402 AA; rs242924 TT; rs7209436 TT)[30]. This study concluded that FAAH rs324420 AA/AC genotypes and CRHR1 minor alleles were related to lower scores on the Beck Anxiety Inventory (BAI). Participants with the FAAH rs324420 CC genotype only reported lower anxiety when they also possessed a combination on three CRHR1 SNPs (rs110402 AA; rs242924 TT; rs7209436 TT).

Finally, variations in the CB1 cannabinoid receptor gene (CNR1) have been associated with anxiety and is of interest when investigating effects of the FAAH rs324420 polymorphism, considering that FAAH impacts on the neurotransmitter AEA that binds to CB1 receptor[32]. A study on the role of the CNR1 rs2180619 polymorphism in fear learning demonstrated that G-carriers were associated with better fear extinction learning and less anxiety in comparison to A-homozygotes[33]. This seems to contradict the higher trait anxiety in G-carriers that also carried the s-allele of the serotonin transporter gene[34]. Although these findings are contradictory, further investigation into CNR1 genotypes and especially how they interaction with genetic variability in FAAH is needed to establish the role of the in anxiety more precisely[35]. It is especially important to investigate this interaction because the CB1 receptors play a significant role in anxiety behavior and are mediated by the FAAH enzymes[36].

So far studies have mainly focused on populations that already have developed anxiety and trauma related symptoms or in healthy individuals on stress and anxiety in an experimental setting[22–28]. However, it remains unknown whether potential genetic variations of the FAAH, CRHR1 and CNR1 polymorphisms are protective in the development of anxiety and trauma related symptoms in real life events. Therefore, the primary aim of the current study is to examine the relationship between genetic variations in the FAAH rs324420 polymorphism (i.e. AA/AC-allele carriers) and the development of anxiety andtrauma related symptoms after the experience of stressful events in military veterans who have been deployed in Afghanistan. Our secondary aim is to investigate whether the

relation of the FAAH polymorphism on the development of anxiety and- trauma related symptoms interact with the CRHR1 rs110402 and CNR1 rs110402 polymorphisms after returning from military deployment.

2. METHODS

2.1. Participants

For this study we analyzed data that was collected for the Prospective Research in Stress-Related Military Operations (PRISMO) study[37,38]. PRISMO is a large prospective cohort study on the long-term effects of military deployment on mental health and the contribution of biological and psychological factors in the development of these mental health symptoms. Participants were Dutch military personnel who were deployed to Afghanistan between 2005-2008. From this sample participants were included if they experienced at least one stressful event. We selected this subsample because we were interested in the relation of the differences in genotypes on anxiety and trauma symptoms during high environmental aversiveness. To select participants from the dataset we selected participants from the collected data who went on military deployment and had a minimum score of 1 (which means they experienced at least one stressful/traumatic combat related stressor) on the Deployment Experience Scale (DES)[5]. The DES consist of 19 questions regarding events experienced during military deployment. Examples of combat related stressors were for example: witnessed people suffering, enemy fire, witnessed wounded, and a colleague injured or killed. The DES was assessed 1 month after military deployment. The study was approved by the Institutional Review Board of the University Medical Center Utrecht, The Netherlands. All participants provided verbal and written informed consent before participation in the study.

2.2. Questionnaires

The Symptom Checklist-90 (SCL-90) is a self-report questionnaire to measure a broad range of psychiatric symptoms [39,40]. It measures different symptom dimensions but for this research question we were primarily interested in the anxiety subscale as an anxiety-outcome measure. The Dutch Self-Rating Inventory for PTSD (SRIP) was assessed to measure PTSD symptoms[41,42]. The Early Trauma Inventory Short Form (ETI-SF) was used to assess traumatic experiences during childhood. The ETI-SF was used as a covariate in the analyses because of the known effects of childhood trauma on alternations in the ECS[43–45]. Questionnaires were assessed 1 month pre-deployment and 6 months after military deployment. Except for the ETI-SF, which was only assessed pre-deployment.

2.3. Genotyping

Blood samples were obtained via venipuncture and standard protocol was used for DNA extraction. The concentration and quality of the DNA were examined using Nanodrop (Thermo Fisher Scientific, MA, USA). Genotyping was conducted using Illumina Human

OmniExpress 24 v1.1. The genetic variations of the FAAH rs324420, CRHR1 rs110402, and CNR1 rs2180619 polymorphisms were extracted using PLINK software version 1.9[46].

2.4. Statistical Analysis

Before starting the analysis missing data from the SCL-90, SRIP, ERI-SF and DES were handled by Multivariate Imputation by Chained Equations (MICE)[47]. The missing values in the data were assumed to be missing at random and all the variables and covariate used in the analyses were included in the imputation model. Details about the missing value analyses and multiple imputation procedure are shown in the Supplemental Data (1. Multiple Imputations).

Covariance Pattern Models were then used for the remaining analysis. Covariance Pattern Models are a form of linear mixed models that specifies a unique pattern of change over time in correlation among repeated measurements on the outcome measure[48]. We first analyzed the effect of FAAH rs324420 genotypes on development of anxiety and PTSD symptoms (separate analyses per outcome measure) from pre-deployment to 6 months after military deployment. In these analyses FAAH rs324420 (CC/AC/AA) was added as fixed effect and Time (anxiety or PTSD scores pre-deployment and 6 months after deployment) the dependent variable. The total score on the ETI-SF (childhood trauma) was used as a covariate in the analysis.

We then added the CRHR1 rs110402 (GG/GA/AA) and CNR1 rs2180619 (AA/GA/GG) genes to test interactions with FAAH rs324420 (CC/AC/AA). The Covariance Pattern Model now included the three polymorphisms as fixed effects and tested their (interaction) effects on the development of anxiety symptoms, also with childhood trauma as a covariate. Subsequently, this was repeated for the PTSD symptoms as an outcome measure. In case of any significant interaction effects or main effects we adjusted the model so that it would include only the significant interactions or main effect and then test this model again to see whether the effect remained. The Satterthwaite approximation was used in all models. This is recommended for small sample sizes or when the model has a complicated covariance type (i.e. unstructured as was used in the model)[49]. This correction can result in atypical denominator degrees of freedom compared to traditional repeated measures models (e.g., denominator degrees of freedom that may actually be higher than the number of subjects)[50]. Analyses and the imputation of missing data were carried out with IBM SPSS Statistics (version 27). Pooled fixed effects from the Covariance Pattern Model analysis were calculated using the miceadds package in R (Version 4.3.1.)[51,52]. A p-value of $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Sample characteristics

From the PRISMO sample 949 participants, reported experiencing at least one stressful event. In this subsample mean age was 28.61 years (*SD* = 8.99) and male/female ratio was 866/83 (91.3/8.7%). Other characteristics of the sample, with regard to military deployment are displayed in Table 1. All three polymorphisms (FAAH rs324420, CRHR1 rs110402 and CNR1 rs2180619) were in Hardy–Weinberg equilibrium, see Table 2.

Table 1. Sample demographic characteristics.

	N	%
Rank		
Private	372	39.2
Corporal	195	20.5
Non-commissioned officer	342	36.0
Officer	34	3.6
Education*		
Low	357	37.7
Middle	430	45.3
High	101	10.6
Previous deployments		
Yes	459	48.4
No	420	44.3

Sample sizes might not add up to total participants due to missing data.
*Education (International Standard Classification of Education levels): Low = primary and lower secondary education; Moderate = upper secondary, postsecondary non-tertiary and short cycle tertiary education; High = bachelor, master and doctoral education.

Table 2. Genotype frequencies and Hardy–Weinberg equilibrium.

Polymorphism	*n*	%	*n*	%	*n*	%	X^2	*p*
	CC		AC		AA			
FAAH rs324420	575	60.6	278	29.3	30	3.2	.289	.865
	GG		GA		AA			
CRHR1 rs110402	282	29.7	440	46.4	166	17.5	1.753	.416
	AA		GA		GG			
CNR1 rs2180619	320	33.7	429	45.2	140	14.8	.030	.985

Sample sizes might not add up to total participants due to missing data.

3.2. FAAH rs324420 genotypes and anxiety and PTSD symptoms

The Covariance Pattern Model test of fixed effects on development of anxiety from pre-deployment to 6 months after military deployment for the different FAAH rs324420 (CC/AC/AA) genotypes was not significant, $F(2, 63)=2.635$, $p=.080$. Also with regard to the PTSD symptoms the Covariance Pattern Model test of fixed effects demonstrated no significant effects, $F(2, 405)=1.048$, $p=.352$, see also Table 3 for the pooled estimated marginal mean and standard errors. In both of the tested models the covariate of childhood trauma was significant, both models $p<.001$.

Table 3. Pooled estimated marginal mean and standard error for the different FAAH rs324420 genotypes per outcome (anxiety and PTSD).

	CC	*n*=575	AC	*n*=278	AA	*n*=30
	Mean	*SE*	*Mean*	*SE*	*Mean*	*SE*
SCL Anxiety	11.86	.139	11.64	.169	12.70	.429
SRIP PTSD	30.36	.298	29.73	.387	30.40	1.06

3.3. FAAH rs324420, CRHR1 rs110402 and CNR1 rs2180619 interactions

The Covariance Pattern Model was then conducted first to examine the interaction effect of FAAH rs324420 (CC/AC/AA), CRHR1 rs110402 (GG/GA/AA) and CNR1 rs2180619 (AA/GA/GG) genotypes on anxiety symptoms and secondly to examine the effects on PTSD symptoms six months after military deployment. For the Covariance Pattern Model with anxiety as an outcome measure the interaction between the three genes was also not significant, $F(7, 34)=1.455$, $p=.216$. However, this model demonstrated two significant main effects of FAAH ($F(2, 107)=5.275$, $p=.007$) and CRHR1 ($F(2, 716)=4.213$, $p=.016$).

This was followed by an adjustment of the model so that it only included the main effects. However, when testing the effects in isolation they did not remained significant (CRHR1 $F(2, 371)=.002$, $p=1$; FAAH, see above under 3.2). In addition, the tests of fixed effects demonstrated no statistically significant three-way interaction between FAAH rs324420 (CC/AC/AA), CRHR1 rs110402 (GG/GA/AA) and CNR1 rs2180619 (AA/GA/GG) genotypes on PTSD symptoms six months after military deployment, $F(7, 17278)=1.154$, $p=.326$. Also, the other interaction term and main effects were not significant, see also Supplemental Data (2. Tests of Fixed effects, Table 3). Again in all of our models the covariate childhood trauma reached a significance of $p<.001$.

3.4. Explorative analysis on childhood trauma

In all our tested models childhood trauma was significant as a covariate. For exploratory purpose we tested the same Covariance Pattern Model as in our primary analysis (under 3.2). Because the AA genotype group was small we added the AA to the AC group. In addition, childhood trauma was divided into three groups no childhood trauma (score of 0 on the ETI-SF), low childhood trauma (ETI-SF score 1-3) and high childhood trauma (ETI-SF score 4 or higher), based on the median split and previous exploratory analysis on childhood trauma and FAAH rs324420 genotypes[44]. In the explorative analysis FAAH rs324420 (CC and AC/AA) and childhood trauma (no childhood trauma, low and high childhood trauma) were added as fixed effect and Time (anxiety or PTSD scores pre-deployment and 6 months after deployment) the dependent variable.

Both models demonstrated a significant interaction effect of FAAH rs324420 and childhood trauma on anxiety ($F(5, 17)=3.555$, $p=0.02$) and PTSD symptoms ($F(5, 30)=8.899$, $p=<.001$). Table 4 and 5 shows the estimated fixed effects and standard error for the different groups in comparison to the reference group (high childhood trauma and AA/AC genotype). Both models demonstrated significant differences between the reference group and both genotypes with low childhood trauma and the CC genotype with no childhood trauma. These groups reported less anxiety and PTSD symptoms 6 months after military deployment.

Table 4. Pooled estimated fixed effects and standard error for the differences in anxiety scores in relation to the reference group per genotype and childhood trauma category

Childhood trauma	Genotype	Estimates of Fixed Effects	*SE*	*p*
No	CC	-0.78	0.33	.024*
No	AA/AC	-0.50	0.41	.226
Low	CC	-0.64	0.25	.013*
Low	AA/AC	-0.90	0.27	.001*
High	CC	.05	0.25	.853
High	AA/AC	reference		

*significant with a p=<.05

Table 5. Pooled estimated fixed effects and standard error for the differences in PTSD scores in relation to the reference group per genotype and childhood trauma category

Childhood trauma	Genotype	Estimates of Fixed Effects	*SE*	*p*
No	CC	-2.59	0.84	.002*
No	AA/AC	-2.33	1.09	.033
Low	CC	-2.36	0.66	<.001*
Low	AA/AC	-2.96	0.77	<.001*
High	CC	0.63	0.67	.350
High	AA/AC	reference		

*significant with a p=<.05

4. DISCUSSION

Our study indicated that genetic variations in the FAAH rs324420 polymorphism are not related to the development of anxiety- and trauma related symptoms after the experiences of a stressful event in military veterans who were deployed in Afghanistan. Anxiety and PTSD symptoms did not differ pre- to 6 months post-deployment between the different FAAH rs324420 genotypes. Additionally, we investigated interactions between the FAAH rs324420, CRHR1 rs110402, and CNR1 rs2180619 polymorphisms. Again, interactions between FAAH rs324420, CRHR1 rs110402, and CNR1 rs2180619 on anxiety and PTSD symptoms six months after military deployment did not reach statistical significance. Interestingly, the covariate of childhood trauma was significant in all the models that we tested.

Contrary to our expectations based on prior studies, in our sample genetic variation in the FAAH rs324420 polymorphism did not relate to differences in anxiety and PTSD symptoms from pre- to post-deployment. Prior studies demonstrated differences in anxiety between A-allele carriers (individuals with AA or AC genotypes) in comparison with individuals with the CC genotype. These studies demonstrated that A-allele carriers of this polymorphism reported lower levels of anxiety, showed enhanced fear extinction and recall and decreased threat-related amygdala activity and this group seemed to be protected against negative consequences of stress[22–28,53]. Contrary to previous studies that investigated genetic variations of the FAAH rs324420 polymorphism in experimental designs, our prospective study was the first to investigate stress and anxiety related to real life events. From each participant anxiety and trauma symptoms had been assessed before and after a stressful event (military deployment) and analyzed in relation to variations in the FAAH gene. It must be noticed, however, associations with the A-allele appeared to be more robust when assessed with experimental fear and stress tasks than on any of the subjective measures of anxiety and stress that have been used in the aforementioned studies. Furthermore, participants in our sample have been faced with high environmental stress due to being deployed, and witnessed people suffering and wounded, were target of enemy fire, or witnessed a colleague who was injured or killed. In experimental studies so far, not all have demonstrated effects of the FAAH polymorphism in the same direction. One study[53] used aversive pictures, which induces a strong emotional context relative to the emotional faces task used in the studies by Hariri and colleagues[23,29], and reported stronger fear responses in the A-carriers as indexed by startle potentiation. This was explained as potentially resulting from bidirectional effects of the ECS as a function of the intensity of the emotions experienced in a context[53]. Yet, the emotional intensity of watching negative pictures on a screen pales compared to the emotional context that the sample in this experiment experienced. On the other hand, the outcome measure was also not just intensity of emotion experienced at the time of the emotional event, but rather a farther removed consequence, i.e., trauma and anxiety reported 6 months later. In short, the studies so far differ widely in study design and outcome parameters, and conclusions based on their comparison are at most preliminary.

As our secondary aim we investigated the interactions between the genetic variations in the FAAH rs324420, CRHR1 rs110402, and CNR1 rs2180619 polymorphisms on anxiety and trauma symptoms after military deployment. Again, interactions between FAAH rs324420, CRHR1 rs110402, and CNR1 rs2180619 on both anxiety and PTSD symptoms six months after military deployment did not reach statistical significance. However, in all these models childhood trauma as a covariate was significant. Interestingly, none of the aforementioned studies included childhood trauma as a covariate[22–28,53]. Although it must be interpreted with caution because of its preliminary nature our exploratory analysis demonstrated interaction effects between FAAH rs324420 genotypes and childhood trauma on de development of anxiety and PTSD symptoms after military deployment. Traumatic experiences during childhood are related to the development of different

psychiatric disorders like anxiety depression and schizophrenia later in life[54]. In addition, childhood trauma is also related to disturbances in development of the endocannabinoid and related systems[45]. For example, corticotropin-releasing hormone, a central regulator of the hypothalamic-pituitary-adrenal (HPA) axis, for which the CRHR1 rs110402 gene codes can be permanently disturbed by childhood trauma[55–57]. Furthermore, clinical and preclinical studies demonstrated that trauma during childhood is related to an upregulation of endogenous cannabinoid and a down regulation of CB1 receptor availability[45]. One study that investigated the FAAH rs324420 polymorphism demonstrated that the chronically elevated AEA levels in A-carriers may be a risk factor in the case of chronic childhood adversity, having both is associated with higher levels of anxiety and depression[44]. These findings indicated that childhood trauma may interact in important ways with the ECS and needs to be taking into account when investigating the role of genetic variation on the development of anxiety and PTSD.

Our study has a couple of limitations that must be addressed. Firstly, our sample consisted of a predominantly male population (91.4%), although this is representative for the military. Additionally, we only examined veterans with a specific type of trauma exposure, namely related to combat. While this resulted in a homogeneous sample, future research with a larger group of women and different types of stressors is warranted, especially since it is known that females show differences in AEA levels and CB1 receptor density which is confirmed in both human and animal research[58–61]. Secondly, our group of FAAH rs324420 AA carriers (*n*=30) was small. Future studies would benefit from using a prospective genotyping strategy to create balanced genotype groups, since the FAAH rs324420 A-allele was found to be associated with gene-dose-dependent increase in basal peripheral AEA levels[25]. In this way it is possible to investigate the AA and AC genotypes separately instead of putting them together in one group as is common practice in most studies. Thirdly, our study focused on a single polymorphism (FAAH rs324420) in combination with two other polymorphisms (CRHR1 rs110402 and CNR1 rs2180619). Future studies would also benefit from additionally investigating haplotypes, a set of DNA variants along a single chromosome that tend to be inherited together[32]. For example, the CB1 receptor site polymorphisms rs806379, rs1535255, and rs2023239 may be important as well[62]. Furthermore, more work is needed to investigate other signaling systems known to interact with the ECS, such as interactions between the 5-HTTLPR serotonin transporter gene and the CNR1 rs2180619 gene[34]. Lastly, we did not determine AEA concentrations in our study, so we can only assume that AEA concentrations were higher in A-carriers. However prior preclinical and clinical research have consistently demonstrated higher AEA in A-carriers[15,19–21,25].

In conclusion, FAAH rs32442, CRHR1 rs110402, and CNR1 rs2180619 were not associated with the development of anxiety and- trauma related symptoms after military deployment in Afghanistan. There was, however, a significant effect of childhood trauma as a covariate in all our models. Future research on the endocannabinoid system could

benefit from assessing effects of genetic variations in the FAAH rs324420 polymorphism together with genotypes in the cannabinoid receptors, such as CNR1 rs2180619. This could lead to a better understanding of complexity and heterogeneity in endocannabinoid signaling. Finally, these results underline the importance of considering childhood trauma in the investigation of the effects of polymorphisms that are related to the endocannabinoid system on the development of anxiety and PTSD symptoms.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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SUPPLEMENTAL DATA

1. Multiple Imputations

1.1. Missing data analyses

The missing values on the SCL-90, SRIP, ETI-SF and DES were investigated to assume the most plausible missing data mechanism. The original dataset consisted of 1032 participants. Before looking at missing data patterns we selected the following participants: 1) have been on military deployment, 2) have completed at least 1 questionnaire of the SCL-90 or SRIP. This resulted in a sample size of 949 participants. The missing data pattern are presented in Table 1 and Table 2. Little's MCAR test was significant with a $p<.001$. Little's MCAR test to assess the differences of the observed mean and the estimated mean in each missing data pattern. Based on the results of the missing data analysis we assume a missing at random mechanism for the missing values of SCL-90, SRIP, ETI-SF and DES.

Table 1. Missing data patterns for the SCL-90 (Anxiety subscale), SRIP, ETI-SF and DES.

Variable	*N*	Missing – Count	Missing – Percent
SRIP pre-deployment	701	248	26.1
SRIP post-deployment	745	204	21.5
SCL-90 pre-deployment	846	103	10.9
SCL-90 post-deployment	750	199	21
ETI-SF	922	27	2.8
DES	758	191	20.1

Table 2. Missing data patterns for the SCL-90 (Anxiety subscale), SRIP, ETI-SF and DES.

SRIP pre-deployment	SRIP post-deployment	SCL-90 pre-deployment	SCL-90 post-deployment	ETI-SF	DES	*n*
						488
X						69
	X		X			81
X	X		X			24
X	X		X		X	27
	X		X		X	64
					X	62
X					X	23

Table 2. Missing data patterns for the SCL-90 (Anxiety subscale), SRIP, ETI-SF and DES. (continued)

SRIP pre-deployment	SRIP post-deployment	SCL-90 pre-deployment	SCL-90 post-deployment	ETI-SF	DES	*n*
X		X				69
X		X		X		19

Note: X indicates a missing value; *n* indicates the number of participants that had the corresponding missing data pattern.

1.2. Multiple imputation procedure

Multivariate imputation by chained equations (MICE) was performed in SPSS. A total of 5 imputed datasets were generated and 50 iterations per dataset was used. The imputation model included the SCL-90, SRIP, ETI-SF and DES as predictor and to be imputed. Other variables that were used only as a predictor were baseline demographic variables (age, gender, deployment year, function during deployment, rank, number of previous deployments) and other questionnaires that were completed pre- and post-deployment (CIS20R – Checklist Individual Strength; CMHS – Cook-Medley Hostility Scale; DS-14 – Type D personality; UBOS – Utrecht burnout scale; VTCI – Temperament and Character Inventory). The missing item variables that were describe above were imputed at the item level. Total scores and subscale were calculated after imputation. The performance of the imputation procedure was checked by inspecting the iteration plots for each imputed variable.

2. Tests of Fixed effects

Table 3. Interaction term, main effects and tests of fixed effects for the different genotypes (FAAHxCNR1x CRHR1) on PTSD.

Interaction/Main effect	***df***	***F*-value**	***p*-value**
FAAHxCNR1x CRHR1	7,17278	1.154	.326
FAAH x CNR1	4, 579	.505	.732
FAAH x CRHR1	4, 1385	1.271	.279
CNR1 x CRHR1	4, 4432	2.221	.064
FAAH	2, 503	1.936	.145
CNR1	2, 2227	.858	.424
CRHR1	2, 290	1.854	.158

Table 4. Interaction term, main effects and tests of fixed effects for the different genotypes (FAAHxCNR1x CRHR1) on Anxiety.

Interaction/Main effect	***df***	***F*-value**	***p*-value**
FAAHxCNR1x CRHR1	7, 34	1.455	.216
FAAH x CNR1	4, 482	1.818	.124
FAAH x CRHR1	4, 742	2.032	.088
CNR1 x CRHR1	4, 525	1.901	.109
FAAH	2, 107	5.275	.007*
CNR1	2, 279	2.772	.064
CRHR1	2, 716	4.213	.016*

*Significant with a $p < .05$.

CHAPTER 7

General discussion

As stated in the introduction of this thesis anxiety, trauma- and stressor related disorders (collective referred to as anxiety related disorders hereafter) are the most prevalent mental health disorders worldwide[1]. Because current psychological and pharmacological treatments are unfortunately insufficient for approximately 40% of patients more insight into the development, maintenance, and treatment of these disorders is very much needed[2,3]. This is especially the case for individuals who are prone to develop these disorders because they are at higher risk to be exposed to stressful and sometimes even traumatic situations, as is the case in the military[4]. In the first part of this thesis, *fear conditioning and extinction learning,* we made use of a fear conditioning task and analyzed behavioral responses with the use of latent class growth analysis (LCGA) to focus on individual differences in task performance. We investigated whether different fear learning classes could be distinguished based on behavior in a fear conditioning and extinction experiment and whether these behavioral classes were associated with treatment outcome. In the second part, *the endocannabinoid system*, we focused on the role of the endocannabinoid system in the treatment of anxiety related disorders. Our systematic review and meta-analysis investigated different endocannabinoid enhancing compounds and its anxiolytic effect in preclinical and clinical studies. In addition, we investigated endogenous cannabinoid levels and genetic variations in the endocannabinoid system in cohorts of military veterans in relation to the development and treatment of anxiety related symptoms.

Fear conditioning and extinction learning

As was demonstrated in previous studies, individuals differ in how they learn (fear acquisition) and unlearn fear associations (fear extinction)[5–7]. Individuals that display a failure to extinguish fear are more prevalent among patients with post-traumatic stress disorder (PTSD) or anxiety disorders than healthy control subjects[8–10]. Since extinction is a central and very important part of exposure therapy, a first-choice anxiety treatment, a failure in these learning processes might account for part of the 40% non-response of this type of treatment. Additionally, generalization has been suggested as an additional mechanism that poses a risk factor for the onset of anxiety disorders because generalization is an indicator for impaired safety learning[11]. To gain more insight into these two possible maladaptive fear learning classes, their characteristics, and the possible association to treatment outcome, we developed a fear conditioning and extinction experiment suitable for use in clinical settings. **Chapter 2** describes this 15 minute task, which used subjective measures of fear and US expectancy ratings as its outcome measure. In a group of healthy subjects (*N*=300) we tested this task and demonstrated that it was able to distinguish between maladaptive fear learning classes of poor extinguishers and generalizers. In addition, these maladaptive fear learning classes were associated with higher state and trait anxiety, and more intrusions and fear one week after completion of the experiment. These maladaptive classes are hypothesized to be associated with a poorer treatment response. In **Chapter 3** we therefore implemented the same task in several clinics to assess a group of patients with various anxiety-related

disorders (*N*=122). Again, maladaptive fear learning classes of poor extinguishers and generalizers were demonstrated. However, fear learning classes were not associated with symptom reduction measured twelve weeks after starting the initial anxiety related treatment. Because these were preliminary results (the study is still ongoing) and because the group of poor extinguishers was relatively small (*n*=8 for the rating of fear to the CS+ and *n*=22 for the ratings of US expectancy to the CS+) this must be tested in a larger sample to draw definitive conclusions.

Critical Evaluation

Although our research regarding the different fear learning classes yielded some interesting results there are some critical notes that can be made. In both studies we used latent class growth analyses (LCGA), a data-driven approach to investigate latent homogenous classes within a larger heterogeneous sample[12,13]. The advantage of LCGA is its usefulness in cases with smaller sample sizes (from *N*=150 onwards) and that it requires fewer parameters than other growth models[14,15]. However, one of the main disadvantages is that LCGA does not take into account between-subject variability within a class[12,16]. LCGA is therefore often used as an initial modelling step before specifying a growth mixture model (GMM)[12]. Increasing sample size allows the application of GMM models and could therefore, as a next step, give more insight into the individual differences with regard to fear learning classes. In comparison to an LCGA model, in GMM between-subject variability in slopes and intercept can by determined by adding predictors of change and of baseline values to the model[17]. In relation to our second study, where large variability in e.g. the perceived aversiveness of the scream was observed (partly caused by the way the samples were collected caused), such a variable such could be added to the model to control for these differences when using GMM. Since our two studies with LCGA has been one of the first applications of these kind of growth models on fear conditioning data, these findings can be regarded as pioneering step in investigating individual differences in fear learning classes. Future studies could benefit from bigger dataset and making the step to analyzing the data with GMM.

Another important aspect of our research is the choices and assumptions we made in experimental design of the fear conditioning and extinction task that potentially could have affected the classes. For example, the choice for the stimuli (faces, abstract forms, spiders) that are used and the US (scream, shock). Previous research has demonstrated that a strong relationship between CS and US (for example angry face/scream are more strongly associated than landscape/scream) show stronger acquisition and is more resistant to extinction[18]. Especially with a task as short as ours (15 minutes) acquiring fear acquisition in such a short time is really important. More importantly future research needs to investigate a possible titration of the scream to make it equal aversive to all participants. Furthermore, subjective measures and physiological measures (e.g. fear potentiated startle reflex, skin conductance response) can yield different responses to a fear conditioning task[19,20]. For future research it would be interesting measuring all

these outcome measure simultaneously since different classes were found both on physiological and subjective measures[5–7,21]. Additionally more research is needed with regard to the context and timing of the experiment in relation to the anxiety treatment. In our current patient study participants performed the experiment in a fearful environment right before starting their anxiety related treatment, and therefore were already in their context of fear.

Lastly, although LCGA is a promising new way to analyze fear conditioning and extinction data it also has an arbitrary component in choosing the best fitting model because of multiple model criteria and taking into account clinical relevance when interpreting the analysis. Therefore it is important to transparently report all of de model criteria and arguments for choosing your model. Unfortunately, because this way of analyzing data is quite new not every study tends to report these choices transparently[22]. This transparency is important because there are different criteria on which you decide the choice for the best fitting model, and none of the established criteria is superior. Although some statisticians and researchers suggest a recommendable order of criteria, this still makes it more subjective than other analysis methods[23].

Future directions

Future research could benefit from implementing LCGA in fear conditioning and extinction learning research. More knowledge about individual response differences is necessary to see how this is related to patients' characteristics, and the development and eventually treatment of anxiety related disorders. It is therefore important to conduct this research in large groups of patients with various anxiety related disorders in order to explore the prognostic potential of these classes. Additionally, larger sample sizes would make it possible to conduct GMM and, perhaps even going a step further by implementing the fear learning classes as a variable in machine learning models to predict treatment reponse[24]. To use the different fear learning classes as a good predictor the first step is to study different aspects in the timing of the assessment of the task and design of the fear conditioning and extinction task. In addition, the replication of the task within different groups of anxiety patients before the implementation of this task into clinical practice. Lastly, our two studies also demonstrated that quite a large group of patients show normal patterns of fear extinction learning. One interesting aspect to add to the experiment is adding a follow-up experiment to measure the retention of fear a couple of days or weeks later [25]. This could give more insight into the maintenance of an emotional memory and the resistance against extinction training. Lastly, it would be of interest to study if these fear learning classes are stable over time or can change, for example after successful anxiety treatment.

Clinical implications

Currently research is more and more focused on heterogeneity across individuals and across disorders. Some see this as a first step in the implementation of precision psy-

chiatry in which a specific treatment is chosen per individual, although not everyone is convinced that this will be beneficial[26,27]. We investigated whether different fear learning classes were associated with treatment outcome. We recognize that the individual differences in fear behaviors can be attributed to both genetic and environmental factors. On the other hand, the identification of behavioral and physiological predictors, regardless of their initial cause, could provide actual key insights into anxiety related disorders, and promote developments in treatment. It is the identification of fear learning classes that can be one of the factors that could contribute to a better selection of treatment and the prediction of treatment response in individuals suffering from anxiety related disorders.

The endocannabinoid system

In the second part of this thesis, we examined the role of the endocannabinoid system in anxiety related symptoms. The endocannabinoid system plays an important role in stress regulation and the extinction of aversive memories and is therefore seen as a promising new target in the understanding and treatment of anxiety related disorders[28–32]. However, the translation from animal research to healthy individuals and anxiety patients is still largely lacking[33]. In **Chapter 4** we therefore started with a systematic review and meta-analysis to investigate different endocannabinoid enhancing compounds for its anxiolytic effect in pre-clinical (*N*=114) and clinical studies (*N*=6). Our systematic review and meta-analysis demonstrated beneficial effects of FAAH inhibitors and inhibitors of AEA transport in preclinical tests of anxiety. Furthermore, in animals it was found that a pre-existing condition of anxiety predicted larger effects of CBD as treatment compound. However, the quality of the evidence was low and unfortunately human studies are still scarce. In **Chapter 5** we investigated differences in endogenous endocannabinoid levels (AEA and 2-AG) in war veterans with a PTSD diagnosis (*n*=54) and combat controls (*n*=26) before and after 6–8 months of trauma-focused therapy. In addition, we investigated whether pretreatment endogenous endocannabinoid levels in individuals with PTSD were associated with treatment outcome. Although pretreatment endocannabinoid levels were not associated with treatment outcome, our study showed indications of a possible association of lower endocannabinoid levels with lifetime cannabis use. Surprisingly we also found some positive associations between endocannabinoid levels with depression and anxiety symptoms in individuals with PTSD. In our last study, **Chapter 6,** we investigated genetic variability in the FAAH rs324420 polymorphism and if these genetic variations explain variances in the development of anxiety and trauma related symptoms. This was investigated in veterans (*N*=949) who went on military deployment and experienced a stressful and possibly traumatic event during deployment. Although genetic variations in FAAH rs323320 were not related to the development of anxiety and trauma related symptoms, childhood trauma that was included as a covariate in our analysis was highly significant, both as a main effect and in interaction with the FAAH rs324420 genotype. These results advocate for more research into interactions between genetic variations in FAAH rs323320 and childhood trauma on the development of anxiety and PTSD symptoms.

Critical Evaluation

Our research contributes to the growing body of literature on the endocannabinoid system over the last 20 years since the discovery of the system[34]. However, as our systematic review and meta-analysis demonstrated, there is a great lack of translational research from animals to humans and the absence of high quality randomized double-blind placebo controlled (RDBPC) studies, which are considered the "gold standard" in clinical practice[33,35,36]. With regard to anxiety related disorders only *n*=17 clinical studies on anxiety and *n*=1 study on PTSD were completed[33]. Most of these studies were characterized by small sample sizes and were often not placebo controlled. In addition, these studies showed a high variation in application of cannabinoids, such as the type of cannabinoid administered, duration of study medication and dosage that was used. Because of the lack of RDBPC studies, in September 2019, we started our own RDBPC aimed at boosting the endocannabinoid system before the treatment of anxiety symptoms (BOOSTCAMP). The primary aim of this study is to investigate if two weeks of oral Cannabidiol (CBD; 3 times a day, 200 mg) is effective in alleviating anxiety symptoms. In addition, the study has three secondary aims. First, to experimentally study the effect of CBD on both fear extinction and extinction consolidation with the use of a laboratory fear conditioning and extinction task. Second, to experimentally study the effects of CBD on stress regulation with the use of the Maastricht Acute Stress Task (MAST)[37]. And thirdly, to investigate if CBD administration improves sleep quality and reduces nightmares. With this study we aim to contribute to the gap in knowledge that exists on translating fundamental research into applicable solutions for clinical practice. As we experienced ourselves, setting up a RDBPC with cannabinoids is accompanied with a lot of challenges which may be one of the reasons these studies are still lacking in the field[38].

One of the first challenges in setting up a RDBPC study is the choice of cannabinoid compound, composition (for example dissolved in oil of powder in capsules), dosage, and route of administration. Most cannabis products are not standardized, do not satisfy strict quality and safety criteria necessary for production of medicinal (research) compounds (the Good Manufacturing Practice or GMP guidelines), and pharmacokinetics have not been established yet for most of these products[39]. In addition, much of the existing pharmacokinetic data focuses primarily on THC[40]. Unfortunately, pharmacokinetic data of CBD is lacking, even though CBD has a more favorable safety profile[41,42]. Also, a great discrepancy exists between the route of administration used in clinical research and what individuals normally use. Most clinical studies in anxiety make use of cannabinoids in capsule form[43–51], while in the Netherlands cannabis for medicinal use is primarily distributed as CBD oil (49.4%), inhalation of THC/CBD combinations (37.5%) and THC oil (18.7%)[52]. It is therefore difficult to generalize findings from clinical research with CBD capsules to the majority of the clinical population that uses oil or inhalation as a route of administration. Lastly, related to this topic the product quality varies greatly and lacks standardization; it is not only dependent on the cannabinoid used, but also on the country in which it is distributed[53].

After choosing the cannabinoid compound, dosage, and route of administration the method of establishing measuring endocannabinoids or exogenous cannabinoids is crucial. Therefore, it is of importance to monitor activation of the endocannabinoid system. This can be achieved in human subjects by measuring circulating endocannabinoid levels (e.g. AEA and 2-AG) from blood samples[49]. Recently, endocannabinoid levels can also be reliably determined in saliva and hair[54–57]. However, the question remains what determination of endocannabinoids in human blood, hair or saliva actually tells us about endocannabinoid levels within the whole brain and the different brain regions. Circulating endogenous cannabinoids originate from multiple organs and tissues, including brain, muscle, adipose tissue and circulating cells[58]. In rodents endocannabinoid circulation can be determined accurately in particular brain regions. In humans however, measurements in blood does not say anything about more fine-grained distribution, such as particular increases or decrease in endocannabinoid levels at a certain location, making it very difficult to determine what is going on in the brain. This is reflected by contradictory results of endocannabinoid levels in relation to anxiety, stress and depression symptoms[59–62]. Unfortunately this makes it difficult to use endocannabinoid levels of AEA and 2-AG as a potential biomarker.

In addition, when measuring cannabinoid levels, to draw conclusions about the functional state of the endocannabinoid system it is also important to have more information about the state of endocannabinoid receptors in the brain. With chronic high levels, they can be downregulated which would mean that functionally, the high levels have negligible effect. Also, the state of different aspects of a neurotransmitter systems depend on many factors. For example, regularly smoking cannabis is associated with down regulation of the CB1 receptor. The experience of childhood trauma can also result in upregulation of endogenous cannabinoid and a down regulation of CB1 receptor availability[63]. In addition, there are significant differences between the sexes in CB1 receptor density[61,64]. Sex differences could also have played a role in our two studies conducted in military veterans which consisted of predominantly male participants. Future studies should focus on sex differences in the endocannabinoid system and the effects of these differences in augmenting the system with cannabinoids. In relation to chronic stress animal research shows an upregulation in FAAH levels in both sexes, however, CB1 receptors are downregulated in males and upregulated in females[65–68]. In females it is thought that this is associated with impaired endocannabinoid signaling[69]. In addition sex hormones, muscle mass and fat tissue account for sex differences in the endocannabinoid system[70]. Unfortunately this is not yet studied in human subjects that experienced chronic stress. Knowledge about these receptors is important since it could have an impact on vulnerability to develop anxiety but also for the effectivity of cannabinoid compounds when intervening with the endocannabinoid system. One way to establish endocannabinoid activation in humans in vivo is by brain imaging, using Positron Emission Tomography (PET). PET is a technique that allows in vivo quantification of biochemical and pharmacological processes under healthy and diseased conditions[71].

Until now only one study focused on PTSD and the endocannabinoid system, demonstrating elevated CB1 receptor availability in PTSD compared to controls[61]. Future studies that make use of PET could increase our understanding of circulating cannabinoid levels. Several PET tracers for CB1 and CB2 receptor and the degradation enzymes MAGL and FAAH are being developed that would allow to study these processes in more detail and eventually provide more insight into the complexity of the system[72,73].

Finally, we must conclude that we currently know only a small part of how the endocannabinoid systems works. Research is still discovering new receptors that play a more important role in the endocannabinoid system than one previously was aware of. For example, the possibility of GPR55 as the third endocannabinoid receptor besides the already established CB1 and CB2 receptor is under debate[74]. In addition, molecules that act on the endocannabinoid system and that might be a therapeutical compound are being developed rapidly and new interesting phytocannabinoids are isolated from the cannabis plant. For example, the phytocannabinoid Cannabigerol (CBG)[75] that inhibits the FAAH enzyme[76], and molecules that inhibit MAGL activation, are potential therapeutics in the treatment of anxiety and stress and trauma related disorders[77,78].

Future directions

As discussed above further research is necessary to gain more insight into the endocannabinoid system. More RDBPC studies are needed to translate findings from pre-clinical studies to the clinical context. Although RDBPC are the golden standard for evidence based treatment I also recommend to conduct more non-inferiority trials and three-arm trials. In BOOSTCAMP I noticed that some patients did not wanted to participant because of the 50% chance to get a placebo. Non-inferiority trials and three-arm trials have the advantage of including more patients[79]. In addition, it will included more people with severe symptoms since it is know that RDBPC often include patients with mild symptoms because of the possibility to be allocated to the placebo group[80]. I would also advocate for a whole system approach in future studies into this fascinating and complex lipid signaling system. This approach is not limited to solely one receptor or single endocannabinoid but aims to integrate as much of the system as possible. Also taking into account genetic variation of the system between humans, which can influence metabolism and efficiency of endocannabinoids and exogeneous cannabinoid compounds. With regard to measuring endocannabinoid levels in blood, saliva or hair, I would consider time of sampling, and preferably, determine endocannabinoid levels at multiple time points or after a stress challenge. Subsequently, the translation from pre-clinical research to clinical research contributes to obtaining more knowledge about the kinetics, dosage, and route of administration of different medicinal cannabis products. Off course, taking into account gender differences, previous cannabis use, genetics and other factors (e.g. childhood trauma, smoking) that can influence the endocannabinoid system. Lastly, it is also of importance to investigate the effects of the long-term effect of the use of

medicinal cannabis on clinical symptoms, side effect and the possible development of tolerance for these different compounds.

Clinical implications

Nowadays a lot of people use medicinal cannabis, and half of these individuals name anxiety as one the main reasons for their use[81]. Trimbos Institute in the Netherlands did research in cannabis users and concluded that 3.5% of adult used cannabis for medicinal purposes. Of this group 92.7% did this without a prescription from a general practitioner or specialist[52]. This demonstrates the importance of more research on medical cannabis, because research shows many people are self-medicating without professional guidance[52]. It is necessary to conduct high quality research about the use of medicinal cannabis for anxiety and other psychological symptoms. Besides investigating acute effects of medical cannabis on stress and anxiety, studies should also research its potential to enhance fear extinction learning. Especially in combination with treatments that are based on fear extinction mechanisms, like exposure treatment. Investigation of these effects in isolation and in combination with psychological treatments is therefore important before considering implementation in clinical practice. Pre-clinical research already demonstrated the importance of the endocannabinoid system in the enhancement of fear extinction learning. Since a subset of people demonstrate a failure to extinguish fear, enhancement of the ECS is seen as a promising target in the treatment of anxiety related disorders. Using cannabinoids in the treatment of anxiety related disorders might be a promising way to improve the success rate of exposure therapy, but more clinical research in patient population are needed.

Conclusion

The studies in this thesis aimed to contribute to a better understanding of individual differences in fear acquisition and extinction learning and the role of the endocannabinoid system in anxiety related disorders. We demonstrated in two studies that a fear conditioning and extinction task can reveal different fear learning classes. In both healthy individuals and patients with various anxiety-related disorders maladaptive fear learning classes of poor extinction and generalization were demonstrated. How these maladaptive fear learning classes are related to treatment outcome must be elucidated in future studies. The endocannabinoid system might be a possible candidate to investigate in the treatment and understanding of anxiety related disorders. Our review demonstrated reduction in anxiety across different endocannabinoid enhancing compounds on anxiety which was more profound in pre-existing anxiety. However, future research must investigate this finding in both healthy individuals and anxiety patients. In our other two studies on endogenous cannabinoids and genetics we found that endogenous cannabinoids were associated with levels of depression, anxiety and lifetime cannabis use. Genetics on the others hand showed no association between different FAAH enzyme genotypes and anxiety and PTSD symptoms. However, they advocate more research into interactions between genetic variations in FAAH rs323320 and childhood trauma

on the development of anxiety and PTSD symptoms. We still have a lot to learn about the pharmacokinetics, the precise working mechanism of the endocannabinoid system and should implement this knowledge into randomized, placebo controlled clinical trials. The studies in this thesis on the endocannabinoid system contribute to the growing body of literature on the endocannabinoid system. Although these are some small steps to the understanding and treatment of anxiety related disorders all these small steps will eventually have impact on the way we understand and treat anxiety related disorders. This will contribute to relieving the burden of many individuals and especially military personnel and veterans who are suffering from anxiety related disorders in their everyday lives.

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APPENDICES

NEDERLANDSE SAMENVATTING

Angst, trauma en stressor gerelateerde stoornissen (hierna collectief aangeduid als angst gerelateerde stoornissen) komen veelvuldig voor in Nederland en de rest van de wereld. In sommige beroepen is de kans op het ontwikkelen van deze stoornissen groter doordat individuen meer kans hebben om te worden blootgesteld aan stressvolle en traumatische ervaringen, zoals bijvoorbeeld in het leger Het is daarom belangrijk om meer inzicht te krijgen in de ontwikkeling en behandeling van deze stoornissen om zo het ontstaan van deze stoornissen te voorkomen en nieuwe manieren te vinden om patiënten te behandelen. Dit is belangrijk omdat 40% van de patiënten niet reageert op de huidige behandelingen. Een belangrijke vraag die we ons hierbij kunnen stellen is welke mechanismes liggen ten grondslag aan het ontwikkelen van deze stoornissen en kunnen we deze kennis gebruiken om iets te zeggen over de slagingskans van een bepaalde behandeling. Daarnaast is het belangrijk om te kijken naar alternatieve behandelingen die gebruikt kunnen worden om deze stoornissen te behandelen. Een van de mechanismes die een belangrijke rol speelt bij het uitdoven van angst is het endocannabinoïde systeem. Onderzoek naar het endocannabinoïde systeem is de laatste 20 jaar exponentieel gestegen en het versterken van dit systeem wordt gezien als een belangrijke vooruitgang in het behandelen van angst gerelateerde stoornissen.

Angstconditionering en extinctie leren

Om meer inzicht te krijgen in het ontstaan en de behandeling van angst gerelateerde stoornissen wordt gebruik gemaakt van een angstconditionering en uitdoving (extinctie) paradigma. In dit paradigma wordt een neutrale stimulus (CS), bijvoorbeeld een plaatje van een gezicht, gekoppeld aan een ongeconditioneerde stimulus (US), bijvoorbeeld een hard geluid. Op deze manier ontstaat er een associatie tussen de CS en de US (CS+). Angstuitdoving (extinctie leren) vindt plaats door de CS te laten zien zonder de US. Daarnaast wordt er gebruik gemaakt van een 'veilige' stimulus (CS-), deze is niet gekoppeld aan een US. Behandelingen van angst gerelateerde stoornissen zijn gebaseerd op dit paradigma. Tijdens exposure therapie wordt een patiënt herhaaldelijk blootgesteld aan de angststimulus om zo de angst reactie te verminderen of uit te doven. Als we echter kijken naar verschillen in reacties van gezonde mensen en angstpatiënten op dit soort paradigma's vallen er twee dingen op:

1) Angstpatiënten laten een hogere angstreactie zien op de CS+ tijdens het aan- en afleren van angst.
2) Angstpatiënten laten een hogere angstreactie zien op de CS- tijdens het aanleren van angst.

Deze bevindingen wijzen op twee mogelijke mechanismes die ten grondslag liggen aan de ontwikkeling van angst:

1) Moeite met het uitdoven van de angstreactie, omdat een angst die is aangeleerd niet wordt afgeleerd op het moment dat deze niet meer voorspellend is voor een aversieve uitkomst.
2) Het generaliseren van angst naar stimuli die aanwezig zijn tijdens het aanleren van angst maar nooit gepaard zijn gegaan met een aversieve uitkomst.

De vergelijking tussen patiëntengroepen en gezonden controles wordt nog vaak gedaan in onderzoek. Echter is er recentelijk steeds meer aandacht voor het kijken naar individuele verschillen binnen patiëntengroepen. Hierbij is aangetoond dat angstpatiënten in verschillende klassen kunnen worden opgedeeld aan de hand van leerpatronen in het aan en afleren van angst. We kunnen ons hierbij afvragen in hoeverre deze klassen die gekenmerkt worden door moeite met de uitdoving van angst en angstgeneralisatie mogelijk klassen zijn die verschillen in behandelsucces van groepen die een meer 'normaal' patroon laten zien.

Het endocannabinoïde systeem

Het endocannabinoïde systeem is een biologisch systeem dat een belangrijke rol speelt in het centrale zenuwstelsel, het uitdoven van angst, en het reguleren van stress. Het endocannabinoïde systeem bestaat uit de cannabinoïde receptor 1 (CB1) en cannabinoïde receptor 2 (CB2), de lichaamseigen cannabinoïden Anandamide (AEA) en 2-arachidonoylglycerol (2-AG), evenals de enzymen die deze lichaamseigen cannabinoïden afbreken: vetzuur-amide-hydrolase (FAAH) voor AEA en monoacylglycerol-lipase (MAGL) voor 2-AG. De lichaamseigen cannabinoïden (AEA en 2-AG) worden aangemaakt als er veranderingen optreden die de homeostase verstoren, bijvoorbeeld als reactie op een stressvolle gebeurtenis. Het werkt op een retrograde manier om de afgifte van neurotransmitters te reguleren, voornamelijk door remming van GABAergische en glutamaterge neurotransmissie.

Het endocannabinoïde systeem kan ook worden gestimuleerd door exogene stoffen die lijken op AEA en 2-AG. De meest bekende stoffen komen van de cannabisplant, namelijk delta-9-Tetrahydrocannabinol (THC) en Cannabidiol (CBD). THC werkt als een directe agonist op de CB1-receptor, en CBD werkt door het FAAH-enzym te inhiberen dat normaal AEA afbreekt. In vergelijking met THC laat het gebruik van CBD veel van de bijwerkingen die we zien bij THC niet zien. Deze voordelen dragen bij aan de populariteit van CBD, waardoor het veel wordt gebruikt bij angst, depressie en slaapproblemen. Wetenschappelijk bewijs voor deze effecten ontbreekt echter nog echt

Zoals we eerder hebben besproken, kunnen lichaamseigen endocannabinoïden het endocannabinoïde systeem ook activeren. Verschillende studies tonen aan dat mensen die PTSS hebben ontwikkeld in de meeste gevallen lagere lichaamseigen endocannabinoïden (AEA en 2-AG) hebben dan gezonde controles. Dit zou kunnen wijzen op een kwetsbaarheid voor het ontwikkelen van PTSS of op een verandering in het endo-

cannabinoïde systeem als gevolg van het ontwikkelen van PTSS. Echter worden er ook tegenovergestelde effecten en nul effecten gevonden. De vraag blijft of controles en mensen met PTSS verschillen in endocannabinoïde levels, en in hoeverre dit gerelateerd is aan behandelsucces. Met name omdat hogere endocannabinoïde levels geassocieerd zijn met snellere angstuitdoving.

Naast lichaamseigen cannabinoïden spelen genetische variaties in het endocannabinoïde systeem mogelijk ook een rol in de ontwikkeling en behandeling van angst, stress en trauma. Met name genetische variaties in het FAAH-gen krijgen veel belangstelling. Mensen met de A-allel laten een verlaagde FAAH enzymactiviteit zien wat geassocieerd is met verhoogde AEA levels. Normaal gesproken vermindert de remming van FAAH de anxiogene effecten van stress, omdat het verlagingen van AEA voorkomt die normaal gesproken gepaard gaan met stress en angst. Mensen die drager zijn van het A-allel laten dan ook verlaagde angst, verhoogde angstuitdoving en betere bescherming zien tegen de negatieve gevolgen van stress. Daarnaast wordt gesuggereerd dat FAAH-remming op zich niet alleen anxiolytisch is, maar ook beschermt tegen het anxiogene effect van stress tijdens hoge omgevingsaversie. Echter is dit nog nooit onderzocht in een real-life situatie.

Studies en bevindingen in dit proefschrift

In het eerste deel van dit proefschrift onderzochten we individuele verschillen in het aan- en afleren van angst en hoe deze verschillen gerelateerd waren aan behandelsucces. In hoofdstuk 2 deden we onderzoek om te bepalen of we verschillende klassen konden onderscheiden in hoe mensen angst aan- en afleren. Dit werd onderzocht bij gezonde studenten (N=300). Dit onderzoek toonde aan dat verschillende maladaptieve klassen konden worden onderscheiden, met name de maladaptieve klassen van ‘verminderde angstuitdoving’ en ‘generaliseerders’. In hoofdstuk 3 gebruikten we het voorgaande onderzoek van een gezonde populatie om te kijken naar een populatie patiënten met verschillende angststoornissen. Hier onderzochten we of de eerder gevonden klassen konden worden gerepliceerd en of deze klassen geassocieerd waren met behandel succes. We pasten de eerder gebruikte taak toe bij patiënten met verschillende angststoornissen (N=122) en vonden opnieuw de maladaptieve klassen van ‘verminderde angstuitdoving’ en ‘generaliseerders’, hoewel deze klassen niet geassocieerd waren met behandeluitkomsten.

In het tweede deel onderzochten we de rol van het endocannabinoïde systeem in angst gerelateerde stoornissen. In hoofdstuk 4 startten we met een systematische review en meta-analyse om verschillende endocannabinoïde versterkende verbindingen te onderzoeken op hun anxiolytische werking in preklinische (N=114) en klinische onderzoeken (N=6). Onze systematische review en meta-analyse toonden gunstige effecten aan van FAAH-remmers en remmers van AEA-transport in preklinische angsttests. Bovendien werd bij dieren gevonden dat een reeds bestaande angsttoestand grotere effect-

en van CBD als behandelingsmiddel voorspelde. De kwaliteit van het bewijsmateriaal was echter laag, en helaas zijn studies bij mensen nog steeds schaars. In hoofdstuk 5 onderzochten we verschillen in endogene endocannabinoïdeniveaus (AEA en 2-AG) bij oorlogsveteranen met een PTSS-diagnose (n=54) en gevechtscontroles (n=26) voor en na een traumagerichte therapie van 6-8 maanden. We onderzochten ook of de endogene endocannabinoïdenniveaus vóór de behandeling bij personen met PTSS geassocieerd waren met het behandelresultaat. Hoewel de endocannabinoïdenniveaus vóór de behandeling niet geassocieerd waren met de uitkomst van de behandeling, toonde ons onderzoek aanwijzingen voor een mogelijk verband van lagere endocannabinoïdeniveaus en cannabisgebruik. Verrassend genoeg vonden we ook enkele positieve associaties tussen endocannabinoïdeniveaus en depressie- en angstsymptomen bij personen met PTSS. In onze laatste studie, Hoofdstuk 6, onderzochten we de genetische variabiliteit in het FAAH rs324420-polymorfisme en of deze genetische variaties beschermend zijn bij de ontwikkeling van angst- en trauma gerelateerde symptomen. Dit is onderzocht bij veteranen (N=949) die op militaire uitzending gingen en tijdens de uitzending een stressvolle gebeurtenis meemaakten. Hoewel genetische variaties in FAAH rs324420 niet beschermend waren bij de ontwikkeling van angst- en trauma gerelateerde symptomen, was de toevoeging van kindertrauma als covariaat in onze analyse significant. Dit suggereert een belangrijke rol voor het corrigeren van trauma uit de kindertijd bij het onderzoeken van de effecten van polymorfismen die verband houden met het endocannabinoïdensysteem op de ontwikkeling van angst- en PTSS-symptomen.

Kritiek en vervolgonderzoek

In het eerste deel van dit proefschrift hebben we gebruikgemaakt van latent class growth analyses (LCGA) om verschillende klassen te analyseren die te onderscheiden zijn aan de hand van gedrag op een angstconditionering- en extinctietaak. Het uitbreiden naar een nog geavanceerder model, zoals een growth mixture model (GMM), zou nog meer inzicht kunnen geven in individuele verschillen. Daarnaast dient er ook aandacht te worden besteed aan het ontwerp van de angstconditioneringstaak en de context waarin deze is afgenomen. Deze aspecten kunnen van invloed zijn geweest op de uiteindelijke klassen die we hebben gevonden. Tegenwoordig richt onderzoek zich steeds meer op de heterogeniteit tussen individuen en tussen stoornissen. Het identificeren van angstleerklassen kan bijdragen aan een betere selectie van behandelingen en het voorspellen van de behandelrespons bij individuen die lijden aan angst gerelateerde stoornissen.

In het tweede deel van het proefschrift hebben we meer inzicht gekregen in de rol van het endocannabinoïdensysteem bij angst gerelateerde stoornissen. Doordat er nog steeds een groot tekort is aan de vertaalslag van preklinisch naar klinisch onderzoek, is het opzetten van gerandomiseerde, dubbelblinde, placebo-gecontroleerde studies essentieel. Gerelateerd aan het opzetten van deze studies komt het maken van keuzes en het doen van onderzoek naar type cannabinoïde, keuze van de samenstelling (bijvoorbeeld opgelost in olie of poeder in capsules), dosering en toedieningsweg. Hiervan ontbreekt

het voor het grootste gedeelte nog aan goede kinetiekstudies. De volgende uitdaging is het meten van deze kinetiek in bijvoorbeeld bloed, haar of speeksel. Hoewel dit niet heel specifiek is, kan het gezien worden als de minst invasieve manier om inzicht te krijgen in lichaamseigen en endogene cannabinoïde niveaus. Daarnaast is het ook van belang om de beschikbaarheid en werkzaamheid van de CB1-receptor mee te nemen. Toekomstige studies kunnen baat hebben bij de combinatie van bepalingen in bloed en PET om zo ook meer inzicht te krijgen in de CB1-receptor en de complexiteit van het systeem. Als laatste weten we nog maar slecht voor een klein gedeelte hoe het endocannabinoïdensysteem werkt. Onderzoek leidt nog steeds tot de ontdekking van betrokkenheid van specifieke receptoren en nieuwe manieren en stoffen om het systeem te stimuleren. Het gebruik van cannabinoïde bij de behandeling van angst gerelateerde stoornissen zou een veelbelovende nieuwe behandeling kunnen zijn bij het verbeteren van het succespercentage van exposuretherapie.

Conclusie

De onderzoeken in dit proefschrift dragen bij aan een beter begrip van individuele verschillen in het aan- en afleren van angst en de rol van het endocannabinoïdensysteem bij angst gerelateerde stoornissen. In twee onderzoeken hebben we aangetoond dat een angstconditionerings- en extinctietaak verschillende angstleerklassen aan het licht kan brengen, zowel bij gezonde individuen als patiënten met verschillende angst gerelateerde stoornissen. Maladaptieve angstleerklassen met slechte uitdoving en generalisatie werden aangetoond, en hoe deze klassen verband houden met de uitkomst van de behandeling zal in toekomstige onderzoeken moeten worden opgehelderd.

Het endocannabinoïdensysteem zou een mogelijke kandidaat kunnen zijn om te onderzoeken bij de behandeling en het begrip van angst gerelateerde stoornissen. Onze review toonde een vermindering van angst aan bij verschillende endocannabinoïde-versterkende stoffen op angst, die sterker was in reeds bestaande angst. Toekomstig onderzoek moet deze bevinding niettemin onderzoeken bij gezonde individuen en angstpatiënten. In onze andere twee onderzoeken naar endogene cannabinoïden en genetica ontdekten we dat endogene cannabinoïden geassocieerd waren met niveaus van depressie, angst en cannabisgebruik. Genetica daarentegen liet geen verband zien tussen verschillende FAAH-enzym genotypen en angst- en PTSS-symptomen. De covariaat kindertrauma was wel significant in al de getesten modellen. Dit benadrukt de noodzaak van meer onderzoek naar de effecten van kindertrauma op de mogelijke ontwikkeling en veranderingen in het endocannabinoïdensysteem en de effecten die dit kan hebben op de ontwikkeling en behandeling van angst.

Hoewel we nog veel moeten leren over de farmacokinetiek, het precieze werkingsmechanisme van het endocannabinoïdensysteem en de implementatie van deze kennis in gerandomiseerde, placebo-gecontroleerde klinische onderzoeken, kan het endocannabinoïdensysteem worden gezien als een veelbelovende kandidaat in het begrijpen en

behandelen van angst, stress en trauma. Hopelijk zal dit bijdragen aan de last van veel individuen, met name militairen en veteranen die in hun dagelijks leven lijden aan angst gerelateerde stoornissen."

LEKENSAMENVATTING

Angst, trauma en stressor gerelateerde stoornissen zijn de meest voorkomende psychische stoornissen ter wereld. De kans op het ontwikkelen van deze stoornissen is groter in beroepen waar er een verhoogde kans is op blootstelling aan stressvolle en traumatische situaties, zoals in het leger. Meer inzicht in de ontwikkeling en behandeling van deze stoornissen is van groot belang omdat huidige psychologische en farmacologische behandelingen onvoldoende werken voor 40% van de patiënten.

Dit proefschrift onderzoekt twee belangrijke systemen die meer inzicht kunnen geven in de ontwikkeling en behandeling van angst, trauma en stressor gerelateerde stoornissen. Ten eerste, individuele verschillen in het aan en afleren van angst en het gebruik van deze verschillen om meer inzicht te krijgen in behandeluitkomsten. Ten tweede, de rol van het endocannabinoïde systeem in deze stoornissen.

De studies in dit proefschrift laten zien dat met het gebruik van een angst conditioneringstaak verschillende klassen in het aan- en afleren van angst onderscheiden kunnen worden. De klassen van ‘generaliseren’ en ‘slechte angstuitdoving’ werden gevonden maar waren niet geassocieerd met behandeluitkomst. In het tweede deel werd gevonden dat stoffen die het endocannabinoïde systeem versterken mogelijk kunnen helpen bij de behandeling van angst. Ook waren er aanwijzingen voor associaties tussen endogene cannabinoïde en angst symptomen.

Het onderzoek draagt bij aan het maken van een vertaalslag van meer fundamenteel onderzoek naar toepasbaarheid in de klinische praktijk in het ontstaan en de behandeling van angst, trauma en stressor gerelateerde stoornissen. Dit kan mogelijk bijdrage aan het voorkomen en het beter behandelen van deze stoornissen.

LAYMAN'S SUMMARY

Anxiety, trauma and stressor-related disorders are the most common mental disorders in the world. The likelihood of developing these disorders is higher in occupations where there is an increased likelihood of exposure to stressful and traumatic situations, such as in the military. More insight into the development and treatment of these disorders is of great importance because current psychological and pharmacological treatments are insufficient for 40% of patients.

This thesis investigates two important systems that can provide more insight into the development and treatment of anxiety, trauma and stressor-related disorders. First, individual differences in fear learning and the use of these differences in association with treatment outcome. Second, the role of the endocannabinoid system in these disorders.

The studies in this thesis show that different fear learning classes can be distinguished using a fear conditioning task. The classes of 'generalizing' and 'poor fear extinction' were found but were not associated with treatment outcome. In the second part, it was found that compounds that enhances the endocannabinoid system may alleviate anxiety symptoms. There was also evidence of associations between endogenous cannabinoid and anxiety symptoms.

This research contributes to translating more fundamental research into applicability in clinical practice in the development and treatment of anxiety, trauma and stressor-related disorders. This may contribute to the prevention and better treatment of these disorders.

OVER DE AUTEUR

Nadia Leen werd geboren op 12 mei 1991 te Haarlem. Nadia zat op de Nicolaasschool in Zandvoort en heeft haar HAVO afgerond aan het Eerste Christelijke Lyceum te Haarlem. In 2008 startte zij met de opleiding Media en Entertainment Management aan Hogeschool InHolland Haarlem. Dit combineerde zij met het behalen van haar propedeuse Theaterwetenschappen aan de Universiteit van Amsterdam. Tijdens en na haar opleiding was zij betrokken bij de productie van verschillende theatervoorstellingen. Zo liep zij stage bij Theater M-Lab en de voorstelling Mighty Society 9 van Toneelgroep Amsterdam. Aansluitend werkte zij bij Bos Theaterproducties. In 2013 besloot Nadia om opnieuw de schoolbanken op te zoeken en startte met de Bachelor Psychologie aan de Universiteit van Utrecht gevold door de Research Master Neuroscience & Cognition. Nadia haar interesse ging uit naar angst, trauma- en stress gerelateerde stoornissen en onderzoek naar nieuwe psychofarmaca in de behandeling van deze stoornissen. Tijdens haar Master deed zij onderzoek naar individuele verschillen in angstconditionering, en de associatie tussen de persoonlijkheidstrek psychopathie en amygdala resting-state functionele connectiviteit in veteranen. Tijdens haar opleiding werd zij tevens geselecteerd om een beursaanvraag te schrijven voor NWO met als onderwerp het versterken van het endocannabinoïdensysteem in de behandeling van PTSD. Hoewel de aanvraag niet werd gehonoreerd ging zijn na haar stage bij het expertisecentrum aan de slag als onderzoeksassistent. Een jaar later mocht zij alsnog bij het expertisecentrum haar onderzoeksvoorstel als promotietraject gaan uitvoeren. In 2022 werkte Nadia naast haar promotie tevens als monitor van laag risico onderzoek voor het UMC Utrecht Julius Centrum. Vanaf 1 september 2023 werkt Nadia als Clinical Research Associate bij Bedrocan. Bedrocan is wereldwijd het oudste bedrijf gespecialiseerd in de productie van medicinale cannabis. Zij werkt op de Clinical Research Unit te Utrecht waar zij verantwoordelijk is voor de coördinatie, uitvoering en gegevensverzameling van het klinische onderzoek.

LIST OF PUBLICATIONS

Peer reviewed Publications:

Kwee, C. M., **Leen, N. A.,** Van der Kamp, R. C., Van Lissa, C. J., Cath, D. C., Groenink, L., & Baas, J. M. (2023). Anxiolytic effects of endocannabinoid enhancing compounds: A systematic review and meta-analysis. European Neuropsychopharmacology, 72, 79-94.

van der Zwaag, N., **Leen, N.A.,** Baas, J.M.P., & van Houtem, C.M.H. H. (2022). Behandelangst en alexithymie, onderzoek naar angstleerprocessen. Nederlands tijdschrift voor tandheelkunde, 129(11), 519–524.

Leen, N.A., de Weijer, A.D., van Rooij., S.J.H., Kennis, M, Baas, J.M.P., & Geuze, E. (2022). The Role of the Endocannabinoids 2-AG and Anandamide in Clinical Symptoms and Treatment Outcome in Veterans with PTSD. Chronic Stress, 6, 1-12.

Leen, N.A., Duits, P., & Baas, J.M.P. (2021). Trajectories of fear learning in healthy participants are able to distinguish groups that differ in individual characteristics, chronicity of fear and intrusions. Journal of Behavior Therapy and Experimental Psychiatry, 72, 101653.

Non Peer-Reviewed Publications:

Klein, M.G., Kouwer, K., **Leen, N.A.,** op ten Noort, C.B., Geuze, S.G., & de Weijer, A.D. Stimuleren van fysieke activiteit bij militair personeel en veteranen die deelnemen aan een oplossingsgerichte groepstherapie: effect op therapieresultaten. Nederlands Militair Geneeskundig Tijdschrift, 76(4), 123-136.

DANKWOORD

Het grote gemis in mijn proefschrift is natuurlijk de BOOSTCAMP studie, een dubbelblind, gerandomiseerd, placebo-gecontroleerd onderzoek naar de effecten van Cannabidiol bij angst, stress en slaapproblemen bij militairen en veteranen met angst-, stress- en traumaklachten. Een studie waar heel veel mensen keihard aan hebben gewerkt maar wat helaas geen plek heeft gekregen in dit proefschrift. Ik ben dankbaar dat de studie wordt voortgezet door het Expertisecentrum. *Remco,* ik weet dat het onderzoek bij jouw in goede handen is en ben heel benieuwd naar de resultaten van de studie. Daarnaast wil ik met dit dankwoord de vele mensen bedanken die voor mij van onschatbare waarde zijn geweest voor mijn onderzoeken en de mensen die altijd aan mijn zijde zijn blijven staan tijdens deze bijzondere reis.

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Nadia

'I said to myself, again: What moves me so deeply, about this little prince who is sleeping here, is his loyalty to a flower – the image of a rose that shines through his whole being like the flame of a lamp, even when he is asleep…'

'Now my sorrow is comforted a little. That is to say – not entirely. But I know that he did go back to his planet…'

Antoine de Saint-Exupéry, The Little Prince

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