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Nicotine and endocannabinoid plasma levels as risk factors for maladaptive fear learning

Dissertation

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Content

Summary

Anxiety disorders (AD) are a widespread mental illness that cause great suffering among those affected. Pathological anxiety can develop from maladaptive fear learning processes, which include impaired discrimination between safety and danger. There are various factors that can impair fear learning processes and in the long run may lead to pathological anxiety. In three studies, this thesis investigates nicotine and endocannabinoid plasma levels as risk factors for maladaptive fear learning processes in healthy humans.

Previous studies found that smokers show altered fear learning, as opposed to non smokers. Furthermore, acute nicotine in animal models enhances fear learning, when compared to controls. The first study therefore tested the effect of acute nicotine exposure (i.e. smoking) in smokers on fear acquisition (ACQ), memory retrieval and generalisation in 202 healthy participants in an online paradigm. Smokers were pseudo-randomly sorted into three groups that either smoked before fear acquisition, directly after fear acquisition or were restricted from smoking for 6 h after fear acquisition. A fourth group consisted of non-smokers only. All groups underwent a two-day paradigm with a fear acquisition on day 1 and a generalisation test 24 h later. Acute smoking had no effect on either fear acquisition or generalisation. However, smokers showed increased self-reported fear towards the safety stimulus after generalisation and overgeneralised their expectancy of the aversive, unconditioned stimulus (US expectancy) onto novel stimuli, when compared to non-smokers. This indicates that smoking disrupts safety learning and might therefore be a risk factor for maladaptive fear learning.

Acute nicotine influences fear learning in rodents, but no study has yet translated these findings in humans. Thus, the second study investigated the pharmacological effect of acute nicotine on fear acquisition and extinction training (EXT) in healthy non-smokers. In this functional imaging study, participants were pseudo-randomly and double-blindly sorted into three groups that either received 1 mg orally administered nicotine before fear acquisition, before extinction training or they received a placebo. Acute nicotine administration before fear acquisition resulted in decreased discrimination between danger and safety stimuli in reported fear and hippocampal activity, when compared to placebo controls. This effect was driven by decreased fear towards the danger stimulus (CS+) in the group that received nicotine. This shows an impaired fear learning process that lead to maladaptive learning in the group that was exposed to nicotine.

The role of endocannabinoids (eCBs) in stress buffering has yet mainly been studied in the extinction of learned fear. Hence, the third study examined the relationship between the plasma levels of the eCBs N-arachidonoylethanolamine (AEA), its metabolite arachidonic acid (AA), 2-arachidonoylglycerol (2-AG) and fear acquisition in healthy males. A stronger stimulus discrimination measured as neuronal activity in the amygdala (AMY), self reported fear and

US expectancy was associated with elevated eCB plasma levels. This indicates that changes in eCB plasma levels play a role in discriminatory fear learning and are flexible responses to learned threats.

Both, nicotine and eCBs are part of the dopaminergic system, which plays a crucial role in fear learning. This thesis discusses new links between maladaptive fear learning in healthy humans and the influences of these risk factors. The results suggest an implementation of nicotine restrictions, especially in patients suffering from pathological anxiety.

Zusammenfassung

Angststörungen (AD) sind eine weit verbreitete psychische Erkrankung, die bei den Betroffenen großes Leid verursacht. Pathologische Angst kann sich aus maladaptiven Angstlernprozessen entwickeln, zu denen eine verringerte Unterscheidung zwischen Sicherheit und Gefahr gehört. Es gibt verschiedene Faktoren, die Angstlernprozesse beeinträchtigen und langfristig zu pathologischer Angst führen können. In dieser Arbeit werden in drei Studien Nikotin und die Plasmaspiegel von Endocannabinoiden als Risikofaktoren für maladaptive Angstlernprozesse in gesunden Menschen untersucht.

Frühere Studien haben gezeigt, dass Raucher im Vergleich zu Nichtrauchern ein verändertes Furchtlernen aufweisen. Darüber hinaus verstärkt akutes Nikotin in Tiermodellen das Furchtlernen im Vergleich zu Kontrollen. In der ersten Studie wurde daher die Wirkung einer akuten Nikotinexposition (d. h. Rauchen) bei Rauchern auf das Furchtlernen (Akquisition: ACQ), den Gedächtnisabruf und die Generalisierung bei 202 gesunden Teilnehmern in einem Online-Paradigma untersucht. Die Raucher wurden zufällig in drei Gruppen eingeteilt, die entweder vor der Furchtakquisition, direkt nach der Furchtakquisition oder 6 Stunden nach der Furchtakquisition nicht rauchen durften. Eine vierte Gruppe bestand nur aus Nichtrauchern. Alle Gruppen durchliefen ein zweitägiges Paradigma mit einer Furchtakquisition an Tag 1 und einem Generalisierungstest 24 Stunden später. Akutes Rauchen hatte keinen Einfluss auf die Furchtakquisition oder die Generalisierung. Allerdings berichteten Raucher im Vergleich zu Nichtrauchern nach der Generalisierung eine erhöhte Furcht vor dem Sicherheitsreiz und übergeneralisierten ihre Erwartung des aversiven Stimulus (US-Erwartung) auf neue Reize. Dies deutet darauf hin, dass Rauchen das Sicherheitslernen stört und daher ein Risikofaktor für maladaptives Angstlernen sein könnte.

Akutes Nikotin beeinflusst das Furchtlernen bei Nagetieren, aber keine Studie hat diese Erkenntnisse bisher auf den Menschen übertragen. Daher wurde in der zweiten Studie die pharmakologische Wirkung von akutem Nikotin auf den Furchterwerb und das Extinktionstraining (EXT) bei gesunden Nichtrauchern untersucht. In dieser Studie zur funktionellen Bildgebung wurden die Teilnehmer zufällig und doppelt verblindet in drei Gruppen eingeteilt, die entweder 1 mg oral verabreichtes Nikotin vor der Furchtakquisition, vor dem Extinktionstraining oder ein Placebo erhielten. Die akute Verabreichung von Nikotin vor der Furchtakquisition führte im Vergleich zu den Placebo-Kontrollen zu einer verringerten Unterscheidung zwischen gefährlichen und sicheren Reizen bei der berichteten Furcht und der Aktivität des Hippocampus. Dieser Effekt wurde durch eine verringerte Furcht vor dem Gefahrenreiz (CS+) in der Gruppe, die Nikotin erhielt, verursacht. Dies zeigt einen gestörten Furchtlernprozess, der in der nikotinexponierten Gruppe zu maladaptivem Lernen führte.

Die Rolle der Endocannabinoide (eCBs) bei der Stressregulierung wurde bisher hauptsächlich im Zusammenhang mit der Extinktion erlernter Angst untersucht. Daher wurde in der dritten

Studie die Beziehung zwischen den Plasmaspiegeln der eCBs N-Arachidonoylethanolamin (AEA), seinem Metaboliten Arachidonsäure (AA) und 2-Arachidonoylglycerol (2-AG) und dem Furchtlernen untersucht. Eine stärkere Stimulusdiskriminierung, gemessen als neuronale Aktivität in der Amygdala (AMY), selbstberichtete Furcht und US-Erwartung, war mit erhöhten eCB-Plasmaspiegeln verbunden. Dies deutet darauf hin, dass Veränderungen des eCB-Plasmaspiegels eine Rolle beim Erlernen diskriminierender Angst spielen und flexible Reaktionen auf erlernte Bedrohungen sind.

Sowohl Nikotin als auch eCBs sind Teil des dopaminergen Systems, das eine entscheidende Rolle beim Angstlernen spielt. Diese Arbeit zeigt neue Zusammenhänge zwischen maladaptivem Furchtlernen bei gesunden Menschen und den Einflüssen dieser Risikofaktoren. Die Ergebnisse legen eine Einschränkung des Nikotinkonsums nahe, insbesondere bei Patienten, die unter pathologischer Angst leiden.

Abbreviations

2-AG	2-arachidonoylglycerol
AA	Arachidonic acid
ACQ	fear acquisition
AD	Anxiety Disorder
AEA	N-arachidonoylethanolamine
Ag/AgCl	silver / silver chloride
AMY	amygdala
BOLD	blood-oxygenation-level-dependent
CB_1-receptors	cannabinoid receptor type 1
CR	conditioned response
CS	conditioned stimulus
CS-	conditioned stimulus paired without unconditioned stimulus/with neutral unconditioned stimulus
CS+	conditioned stimulus paired with unconditioned stimulus
dACC	dorsal anterior cingulate cortex
DEBRA	German Study on Tobacco Use (Deutsche Befragung zum Rauchverhalten)
DRKS	German Clinical Trials Register (Deutsches Register Klinischer Studien)
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th Edition
eCB	endocannabinoid
e-cigarettes	electronic cigarettes
EXT	extinction training
FAAH	fatty acid amide hydrolase
fMRI	functional magnetic resonance imaging
GAD	generalised anxiety disorder
GS	generalised stimulus
HC	hippocampus
IAPS	International affective picture system
INS	insula
LTP	long term potentiation
mPFC	medial prefrontal cortex
NAcc	Nucleus Accumbens
nAChR	nicotinic Acetylcholine receptor

Nic1	group that received nicotine before fear acquisition in Study II
Nic2	group that received nicotine before extinction training in Study II
nUS	neutral unconditioned stimulus
Pla	placebo group in Study II
PTSD	post-traumatic stress disorder
RoF	return-of-fear manipulation
ROI	region of interest
SCR	skin conductance response
T1	blood sample pre fear acquisition in Study III
T2	blood sample post fear acquisition in Study III
TR	repetition time
TE	echo time
US	unconditioned stimulus
vmPFC	ventromedial prefrontal cortex
VTA	ventral tegmental area

1. Introduction

The trait emotions “fear” and “anxiety” are conserved over a long lineage of mammalian history (LeDoux, 2021). Fear and anxiety do overlap partly, but also have some distinct features. The neuroscientific literature defines anxiety as pre-encounter hyper-vigilance in anticipation of threat. Fear, on the other hand, is described a post-encounter reaction towards acute threat that entails a fight, flight or freeze behaviour in mammals (Sylvers *et al.*, 2011; Perusini & Fanselow, 2015). To survive in a changing environment, the identification and recollection of potential new threats is essential and needs to be frequently updated and translated in defensive behaviour. One mechanism for this is aversive learning.

To study aversive learning under experimental conditions, fear conditioning protocols have been widely established and used to investigate a wide spectrum of animals, including humans. They have proven effective to study “threat detection and defense responses” (LeDoux, 2014; Lonsdorf *et al.*, 2017). During fear acquisition (ACQ) a conditioned stimulus (CS+) is predictive for an aversive unconditioned stimulus (US, e.g. an electrotactile stimulation). Over time, this learning procedure leads to a (defensive) conditioned response (CR) towards the CS+. Another conditioned stimulus (CS-) is predictive for the omission of the US. The CS- is therefore a safety signal. A stronger discrimination between the two CSs after fear acquisition can be interpreted as elevated differential learning success. If the CS+ is presented repeatedly without the US, the CR decreases over time. This procedure is called extinction training (EXT). A third possible procedure of fear conditioning protocols is the “return-of-fear” manipulation (RoF). Here, different approaches are possible, e.g. the reinstatement, where the US is presented again, but without any CS information beforehand (Lonsdorf *et al.*, 2017).

The conditioned response as indicator of conditioned fear can be translated and quantified by several outcome measurements. For example, in non-human animals an observation of defensive behaviour, such as freezing, indicates conditioned fear. Human subjects can report their subjective experience in affective or cognitive ratings. Physiological reactions such as a change in skin conductance level or fear potentiated startle response additionally quantify the conditioned response. Moreover, neurobiological changes such as discriminatory activation within brain regions of the fear network can be interpreted as a result of conditioned fear (Lonsdorf *et al.*, 2017).

The adaptive identification and discrimination of threat and safety is important for survival. Uncontrolled and maladaptive fear towards harmless situations and safe environments can manifest and the experience of fear can be overwhelming and change a life drastically. Such maladaptive and extensive defensive behaviour can be observed in patients with anxiety disorders (AD) (Penninx *et al.*, 2021). Although being called “anxiety” disorders, the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) summarizes mental disorders that share

excessive fear and anxiety under that term, such as specific phobias, social anxiety, general anxiety disorder or panic disorder (American Psychiatric Association, 2013). Many people suffer from AD with increasing tendency in the last years (Śniadach *et al.*, 2021). Apart from medication, established tools for treating AD are cognitive and/or behavioural therapy, such as exposure therapy (Kaczkurkin & Foa, 2015). In exposure therapy patients are confronted with situations or objects that they normally avoid because they overestimate the associated danger. After constant and safe exposure, patients acquire this new safety-association, resulting in decreased fear/anxiety. This procedure can be explained by reinforcement learning.

1.1. The dopaminergic system

Reinforcement learning depends on the dopaminergic reward system. If a behaviour leads to reward then dopamine is released, which reinforces the neural pathways that resulted in this reward-inducing behaviour (Bromberg-Martin *et al.*, 2010). Similarly, a non-rewarding result would lead to the inhibition aforementioned neuronal circuit. This mechanism enables individuals to learn to discriminate between rewarding and not rewarding - even harmful - behaviour. Therefore, the dopaminergic system is crucial for fear acquisition and extinction training. The dopamine release in the amygdala during ACQ links to learning strength and fear memory formation, as stimulus discrimination and dopamine release are positively correlated (Frick *et al.*, 2022). A recent study has shown that dopaminergic neurons in the ventral tegmental area (VTA) of rodents activate during safety learning (US omission during CS-presentation) (Yau & McNally, 2022). Furthermore, dopaminergic VTA neurons are also activated especially during the beginning of extinction learning (Salinas-Hernández *et al.*, 2018). These activations during safety and extinction learning are possibly due to the rewarding feeling of relief that can be experienced when the US is omitted (Kalisch *et al.*, 2019). The dopaminergic system is mostly associated with the reward system and its motivational value processing. However, it is assumed that dopamine is also important for motivational salience processing (Bromberg-Martin *et al.*, 2010). The difference is that dopaminergic neurons coding motivational value are excited by reward and inhibited by aversive events, whereas neurons coding motivational salience are excited by both. In a changing environment, both types of dopaminergic neurons are necessary, as salient stimuli independent of value are often important to detect e.g. to gain an estimation of motivational value. Importantly, dopamine sythesis and transmission can be influenced by exogene substances, e.g. nicotine (Rademacher *et al.*, 2016).

1.2. Fear and nicotine

A recent survey conducted by the German Study on Tobacco Use (DEBRA) has found that the tobacco consumption especially in German teenagers is increasing. While the prevalence of current tobacco smokers in individuals from age 14-17 has been 8.7% in 2021, it already increased to 15.9% in 2022 (Kotz *et al.*, 2022). In the age group between 18-24 years even 40.8% of individuals stated tobacco consumption in 2022. These are drastic numbers demonstrating a need for further investigation of nicotinic effects and education on smoking behaviour.

Nicotine is a psychoactive drug that is an agonist to the nicotinic acetylcholine receptor (nAChR). These ligand gated ion channels consist of a combination of subunits, resulting in a great variety of nAChR subtypes with different characteristics (Hogg *et al.*, 2003). The subtypes α4β2 and α7 are prominently expressed in the central nervous system and seem to be most sensitive to upregulation and desensitization by chronic nicotine exposure. Especially the subtype α4β2 shows very high affinity among nAChRs (Paterson & Nordberg, 2000). Interestingly, the activation of nAChRs can directly induce long term potentiation (LTP), which is crucial for building long-term (fear) memory (Matsuyama *et al.*, 2000). Raybuck and Gould conducted a study in mice where either nicotine, an antagonist for α4β2 nAChRs or an antagonist for α7 nAChRs were directly infused into the dorsal or ventral hippocampus, or in the medial prefrontal cortex (mPFC) before trace or context conditioning (Raybuck & Gould, 2010). They found that the high affinity α4β2 nAChR in the dorsal hippocampus is involved in the acquisition of trace fear conditioning and that nicotine infusion enhanced fear conditioning. In contrast, they found that nicotine infusion into the ventral hippocampus disrupted fear conditioning (likely due to connectivity to the amygdala). In addition, nicotine infusion into the mPFC before fear conditioning enhanced learning. These results show that specific subtypes of nAChRs are critically involved in fear conditioning and that infusion of nicotine can have differential effects on fear learning. When activated regularly, like in chronic nicotine users, the density of nAChRs is increasing (Wüllner *et al.*, 2008). Similarly, the density of β2-nAChRs in non-smoking patients suffering from post-traumatic stress disorder (PTSD) is higher, compared to healthy non-smoking controls (Czermak *et al.*, 2008). This suggests that patients suffering from pathological anxiety show an upregulation of nAChRs, similar to healthy smokers.

Inversely, anxiety disorders are closely linked to nicotine dependence. Patients suffering from pathological anxiety are more likely to smoke than healthy individuals (Ziedonis *et al.*, 2008). Smoking can have a short-term anxiolytic effect and might therefore be a possibility for anxiety-relief, e.g. in social phobia (Sonntag *et al.*, 2000). However, the connection between nicotine and anxiety seems to be bi-directional. Studies found that the degree of nicotine dependence and symptom severity in PTSD patients are positively correlated (Thorndike *et al.*, 2006;

Baschnagel *et al.*, 2008). Furthermore a prospective longitudinal study investigating the effect of nicotine dependence on the development of panic disorders found an increased risk of new onsets of panic attacks (Isensee *et al.*, 2003). Hence, smoking does not only lead to a more serious course of anxiety disorders in patients, it also seems to be a risk factor for the development of pathological anxiety.

The influence of smoking on pathological anxiety can be observed in experimental environments by nicotine interventions incorporated into fear acquisition protocols. To determine differences between adaptive aversive learning and maladaptive aversive learning, which can be observed in patients with AD, fear conditioning protocols are widely used. A sign of adaptive aversive learning is the successful discrimination between threat and safety signals after fear acquisition and the behavioural flexibility to reduce conditioned fear during extinction training (Lonsdorf *et al.*, 2017). Maladaptive aversive learning can for example be observed in patients suffering from AD (Duits *et al.*, 2015).

Chronic nicotine interventions incorporated into the aversive learning process during fear acquisition have been shown to impair discrimination between threat and safety in rodents (Kutlu *et al.*, 2018). Nicotine administration specifically seems to disrupt safety learning. That means that the safety stimulus elicits increased fear reactions in rodents exposed to nicotine, when compared to placebo controls. Furthermore, early nicotine administration during pre-adolescence or adolescence can lead to long lasting impairments in contextual fear learning (Portugal *et al.*, 2012).

1.2.1. Nicotine effects fear acquisition and extinction training

Animal studies in rodents found that acute nicotine administration dose-dependently results in impaired discrimination between threat and safety, when compared to saline treated controls (Kutlu *et al.*, 2014). Specifically, safety learning was disrupted by acute nicotine infusion in the dorsal hippocampus of rodents (Connor *et al.*, 2017).

Nevertheless, not only fear acquisition, also extinction training seems to be impaired by acute nicotine administration. During extinction training, the acquired fear memory is suppressed by an inhibitory learning process (Myers & Davis, 2002). Studies in mice found delayed extinction learning after nicotine administration, compared to saline controls (Kutlu & Gould, 2014). Interestingly, this delay indicated by increased freezing behaviour in the nicotine treated group was only the case for contextual extinction, rather than extinction of cued fear. Although there was no effect of acute nicotine administration on retrieval of unextinguished fear memories in mice, spontaneous recovery, which is a form of return-of-fear, was enhanced by nicotine (Kutlu *et al.*, 2016). Whilst widely investigated in animal models, translational approaches examining these effects of acute nicotine on fear learning in non-smoking humans are still lacking.

1.2.2. Memory consolidation and nicotine

Memory consolidation is a process in which short-term memory is transformed into long-term memory (Dudai *et al.*, 2015). Sleep studies have shown that the time up to 6 h after the learning phase is crucial for memory consolidation (Stickgold & Walker, 2005). Previous studies found that nicotine treatment in rodents enhances freezing (as measure of conditioned fear response) during memory retrieval after reactivation or extinction (Tian *et al.*, 2011; Kutlu *et al.*, 2017). Hence, nicotine seems to disrupt threat learning, as well as memory consolidation processes.

1.3. Fear and endocannabinoids

The circulating endocannabinoids (eCB) 2-arachidonoylglycerol (2-AG) and N-arachidonoylethanolamine (AEA) are endogenous ligands that are postsynaptically released to the presynaptic cannabinoid receptors and consequently reduce the presynaptic neurotransmitter release (Kano *et al.*, 2009). Arachidonic acid (AA) is a metabolite of AEA. Cannabinoid receptors type 1 (CB_1-receptors) in the human central nervous system show a high density in areas such as the amygdala, the hippocampus or the cerebral cortex (Petrie *et al.*, 2021). These are areas that are also typically involved in fear and anxiety processing (Robinson *et al.*, 2019). This way endocannabinoids (eCBs) can influence behavioural change, such as regulating stress and anxiety symptoms. The stress buffering effect of eCBs is a crucial mechanism for flexibly adjusting behaviour in a changing environment, to the extent that a dysregulation of eCBs can lead to psychiatric disorders (Lutz *et al.*, 2015).

Previous studies focussed their investigations on the effect of eCBs on extinction training. They examined rodents that show generally elevated levels of AEA (rodents were carriers of the low-expressing fatty acid amine hydrolase (FAAH) allele, which is degrading AEA). These animals show decreased anxiety-like behaviour, enhanced extinction learning, quicker habituation towards threats mirrored in amygdala activity as well as reduced trait stress-reactivity (Gunduz-Cinar *et al.*, 2013; Dincheva *et al.*, 2015). Translational approaches are essential, as patients with pathological anxiety resulting from traumatic events show altered plasma levels of eCBs (Hauer *et al.*, 2013; Hill *et al.*, 2013). Hence, a pharmacological intervention into eCB plasma levels after a traumatic event holds potential for therapeutic improvement.

2. Aims

The overall frame of this dissertation is the investigation of risk factors that have a potential harmful effect on aversive learning and therefore may lead to the development or manifestation of an anxiety disorder. The experimental key for the investigation are fear conditioning protocols, which can sensitively examine the different effects of risk factors that might manifest in maladaptive learning.

Hence, I tested the effects of acute smoking and nicotine on fear learning and memory retrieval, as well as the influence of plasma levels of endocannabinoids on fear acquisition. In detail, my aim is to investigate:

I) the effect of acute smoking on fear learning in smokers, when compared to non-smokers. Here, a generalisation task tests the disruption of safety learning and its transfer to novel stimuli in detail.

II) the effect of acute nicotine administration in healthy non-smokers on the discrimination of danger and safety in fear acquisition, extinction training and return-of-fear.

III) circulating plasma levels of endocannabinoids N-arachidonoylethanolamine (AEA), 2-arachidonoylglycerol (2AG) and its metabolite AA before and after fear acquisition and determine their relationship with neural activity during fear acquisition.

To reach these aims, three studies were conducted. Study I especially focussed on the effect of (acute) smoking on fear acquisition, memory consolidation and generalisation. The importance of investigating the effect of smoking on fear learning is evident, as smoking-induced maladaptive learning is suspected as potential origin for the development of pathological anxiety. To examine how participants apply the learned CS-US associations on novel stimuli that resemble the CS+ and CS-, Study I employed a generalisation test on day 2. A healthy, adaptive learning mechanism would be to use the information on a known threat and apply it to similar contexts or stimuli, in order to flexibly act in case of confrontation (Dunsmoor & Paz, 2015). This is especially interesting, as overgeneralisation is a characteristic of patients suffering from pathological anxiety (Dymond *et al.*, 2015). Situations, objects or contexts, even though they are harmless, are overgeneralised as dangerous and elicit increased fear in anxiety patients. Study I therefore investigated what effect smoking has on fear generalisation.

Study II focussed on the pharmacological effect of acute nicotine administration before fear acquisition and extinction training with a return-of-fear manipulation in non-smokers. Importantly, Study II tested the acute nicotine effect on non-smoking individuals, as chronic nicotine administration is upregulating nACh receptor density (Wüllner *et al.*, 2008). Since Study I found no effect of acute nicotine on fear acquisition in smokers, Study II concentrated on nicotine naïve participants.

Study III focussed on the plasma levels of eCBs during fear acquisition, as opposed to extinction training, to close this gap of research in humans. Studies in rodents found that endocannabinoids are involved in fear learning and long-term potentiation (LTP). Dose-dependent blocking of CB_1-receptors along the basolateral amygdala – medial prefrontal cortex pathway prevented the acquisition of conditioned fear (Tan *et al.*, 2010). More precisely, AEA and 2-AG seem to be involved in different regulations of fear acquisition. Blocking the degrading enzymes of AEA before aversive learning leads to enhanced fear acquisition and strong fear memory, whereas blocking the degrading enzymes of 2-AG dampens this effect (Balogh *et al.*, 2019). A closer investigation of eCB plasma level and fear acquisition in humans was necessary and hereby realised in Study III.

3. Methods

3.1. Participants

In all three studies, the general requirements for participating were similar. Participants needed to be physically and mentally healthy adults, who did not consume illicit drugs. In Study II and Study III participants additionally needed to be MRI compatible. All participant gave written informed consent and the local ethics committee in Hamburg (Ärztekammer Hamburg) approved each study.

The studies differed in smoking requirements, such as for Study I, both non-smokers and smokers were recruited, but in different groups, while in Study II only non-smokers were recruited and in Study III the smoking status was not relevant for participation. Furthermore, in Study I and Study II both female and male participants were recruited, whereas in Study III only male subjects participated.

3.2. Experimental stimuli and paradigm

Fear conditioning was chosen in all three studies as protocol to investigate risk factors for maladaptive learning. Importantly, all three studies with subsequent manuscripts are in line with the procedural and terminology framework that was recommended by a collective of European scientists from human fear conditioning labs (Lonsdorf *et al.*, 2017).

3.2.1. Study I

During the fear acquisition phase on day 1, participants were confronted with two black rings, one with a smaller diameter, and one with a larger diameter (5 cm; 11.75 cm) (Struyf *et al.*, 2017). These rings served in a counterbalanced fashion between participants as CS+ and CS-. The CS+ was predictive for an unpleasant US picture and the CS- was predictive for a neutral US (nUS). US and nUS pictures were selected from the validated International affective picture system (IAPS) database. During the generalisation test 24 hours later, participants were again confronted with CS+ and CS-, but now eight additional generalised stimuli (GS) with diameters between CS+ and CS- were presented (GS2-GS9). Study I was an online study that used PsychoPy3 (Peirce *et al.*, 2022) for the behavioural experiments.

Participants in Study I were selected into four experimental groups. Group 1 consisted of only non-smoking individuals. Groups 2-4 consisted of smoking individuals with different interventions into their smoking habit. Group 2 was asked to stop smoking for 6 hours after completing the fear acquisition. Group 3 was asked to smoke directly after the fear acquisition and Group 4 was asked to smoke directly before the fear acquisition. Outcome measures of

Study I were fear ratings before and after each experimental phase and trial-wise US expectancy ratings.

3.2.2. Study II

Study II consisted of a two-day context-dependent cue-conditioning paradigm with a fear acquisition on day 1 and an extinction training with a return-of-fear manipulation 24 hours later. The context was a virtual room on a screen which was from time to time illuminated by cues of coloured lights (yellow or blue) which served as CSs. The CS+ was predictive for an aversive electrotactile stimulus (US), whereas the CS- was predictive for the omission of a US. The extinction training was performed on the next day. In this experimental phase no US were presented with either CS. The return-of-fear was implemented in form of a reinstatement, where subjects received 4 US without any context or cue information. Both experimental days were took place in a functional magnetic resonance imaging (fMRI) scanner to find neuronal correlates of the physiological and self-reported results.

Participants were pseudo-randomly and double-blindly selected into three experimental groups. Group 1 received 1 mg of nicotine before the fear acquisition and Group 2 received 1 mg of nicotine before extinction training. On the other corresponding days, participants from Group 1 and 2 received 1 mg placebo. Group 3 received a placebo on both days and was therefore the control group. Nicotine and placebo were administered as oral spray. Outcome measures of Study II were neural activity, skin conductance responses as well as subjective fear ratings and US expectancy ratings. Study II was preregistered at the German Clinical Trials Register (Deutsches Register Klinischer Studien (DRKS); DRKS-ID: DRKS00025233).

3.2.3. Study III

Study III focused solely on fear acquisition. Here, the same paradigm as described in Study II was used. Before and after fear acquisition blood samples of the participants were taken. These were analysed for the plasma levels of the endocannabinoids 2-arachidonylglycerol (2-AG), N-arachidonoylethanolamine (AEA) and its metabolite arachidonic acid (AA). Outcome measures of Study III were similar to Study II neural activity, skin conductance responses, fear ratings and US expectancy ratings.

3.3. Subjective ratings

All three studies included subjective ratings as outcome measurements. The participants were asked for their fear or stress towards a specific stimulus (fear ratings), as well as for their US expectancy towards the same stimuli (US expectancy rating). The benefit of asking participants

directly how they experienced the experiment is to determine their conscious fear and to assure that they understand their task.

Fear ratings were employed as an affective rating of the participants experience and the US expectancy as a cognitive rating to test the participants contingency awareness. The trial-wise US expectancy ratings furthermore enables to determine a clear timeline of the participants learning progress, so that it could be used in fMRI analyses as parametric factor for threat anticipation.

3.4. Skin conductance responses

Sweating, among other functions, is controlled by the sympathetic nervous system (Drummond & Lance, 1987). If the sympathetic nervous system is highly aroused, then sweat gland activity also increases, which increases electrodermal activity or skin conductance responses. Hence, skin conductance can be a measure of psychological or physiological arousal (Dawson *et al.*, 2017). As psychophysiological measurement, skin conductance responses (SCR) were analysed in Study II and Study III. For data acquisition, self-adhesive silver/silverchloride (Ag/AgCl) electrodes were used on the hypothenar of the left hand of the participant and recorded the SCR with a BIOPAC MP-100 amplifier (BIOPAC® Systems Inc, Goleta, California, USA). Phasic responses in skin conductance to CS onset were scored manually and later normalized for each day and participant.

3.5. fMRI: Methodological considerations

Functional Magnetic Resonance Imaging (fMRI) is a method that is based on the brains' metabolism, which typically differs due to a specific task in a region over time. Active regions need to be supplied with oxygen, which is transported by haemoglobin in red blood cells. When haemoglobin is fully oxygenated, it has different magnetic properties, when compared to fully deoxygenated haemoglobin (Glover, 2011). This can be captured as Blood-Oxygenation-Level-Dependent (BOLD) contrast and is a sign of the current neuronal activity in a specific brain region. MRI data from Study II and Study III were obtained on a 3 T Magnetom-PRISMA System (Siemens, Erlangen, Germany) using a 64-channel head coil. Echo planar multiband imaging (resolution: 1.5 mm, gap: 0.5 mm) was used in a T2*-sensitive sequence (TR = 1493 ms, TE = 30 ms). Additionally, high-resolution T1-weighted structural brain images (MP-RAGE sequence, 1 mm isotropic voxel size, 240 slices) were acquired. Preprocessing and statistical analysis were performed in SPM12 (Statistical Parametric Mapping, http://www.fil.ion.ucl.ac.uk/spm). Preprocessing included realignment and unwarping, co-registration to T1-weighted structural brain images, segmentation and normalisation. On the single-subject level, a general linear model with experimental conditions (CS+, CS-, (omitted)

US, introductions, ratings and button presses) as individual regressors was calculated and betas were smoothed with a Gaussian kernel of 4 mm^3. The second level, i.e. group analysis, differed between studies. In Study II a full factorial analysis was performed for the different groups, whereas in Study III regression models were calculated.

Previous fMRI studies have identified closely connected brain regions to be involved in fear acquisition in humans, such as the bilateral insula (INS), the dorsal anterior cingulate cortex (dACC), the amygdala (AMY) and the hippocampus (HC) (Fullana *et al.*, 2016; Greco & Liberzon, 2016). During extinction training the ventromedial prefrontal cortex (vmPFC) has been observed to play a crucial role, as well as the AMY (Greco & Liberzon, 2016). These areas are the regions of interest (ROI) in both fMRI studies (Study II and Study III). Dopaminergic innervated key structures, such as the Ncl. Accumbens (NAcc) and the ventral tegmental area (VTA) show increased neural activity when nicotine is induced and are part of the reward system (Stein *et al.*, 1998; Kauer & Malenka, 2007). Furthermore a dopaminergic feedback-loop between HC, NAcc and VTA is crucial for long term memory processing and integrating novel stimuli (Lisman & Grace, 2005; Lima *et al.*, 2013). The NAcc and the VTA are therefore additional ROIs in Study II.

4. Results

4.1. Study I: Smokers show increased fear responses towards safety signals during fear generalisation, independent from acute smoking

4.1.1. Study I: Background and rationale

A study showed that smoking leads to decreased stimuli discrimination in a fear learning protocol in humans (Kutlu *et al.*, 2018). But the influence of acute smoking and smoking restrictions on fear learning had not been investigated yet. Study I examined these interventions of the normal smoking behaviour of healthy smokers and compared them with non-smoking individuals. Furthermore, it was yet unclear if acute smoking or smoking restrictions after fear acquisition influence fear memory consolidation in humans, so Study I closed this gap of knowledge. The fear memory consolidation test in Study I was combined with a generalisation protocol. Hence, in Study I acute and general smoking effects on the generalisation of threats onto novel stimuli were investigated.

The two-day study design with fear acquisition on day 1 and generalisation on day 2 was chosen, because studies have shown that a 24 h memory consolidation phase after learning is preferable for transferring short term memory in long term memory (Myers *et al.*, 2006). A critical time for memory consolidation is up to six hours after learning (Graves *et al.*, 2003; Stickgold & Walker, 2005). Nicotine administration directly after fear acquisition led to impaired extinction training in rodents (Kutlu *et al.*, 2017). This effect was not found if the rodents received nicotine 6 h after fear learning. This timeline was essential for defining different smoking manipulations between groups (smoking restriction 6h after ACQ/smoking directly after ACQ).

Four experimental groups were formed, including one group of non-smokers and three groups of smokers. One smoker group was asked to smoke directly before fear acquisition (Group 4), whereas the other two smoker groups (Group 2 & Group 3) were restricted to smoke one hour before the begin of the experiment. One expectation of Study I was to find a decreased discrimination between threat and safety stimuli during fear acquisition and generalisation as a result of acute smoking before fear acquisition, when compared to smokers restricted from smoking. One smoker group (Group 3) was asked to smoke directly after the fear acquisition, whereas another smoker group (Group 2) was restricted from smoking for 6 hours after fear acquisition. As a result, a difference in memory retrieval during generalisation between groups was expected. Finally, Study I examined the hypothesis that smokers in general (Group 2, 3 and 4) show a deficit of safety learning, when compared to non-smoking individuals (Group 1). Participants that were initially restricted from smoking for 6 h after ACQ, but smoked anyway were then assigned to Group 3 post-hoc.

Study I also investigated the transfer of learned fear towards novel stimuli by employing a generalisation task on day 2. When smoking leads to altered stimulus discrimination by a deficit of safety learning, this experimental phase was designed to investigate the changed pattern of conditioned responses in detail.

4.1.2. Study I: Results and conclusion

The results of Study I (Mueller *et al.*, 2022) indicated that smoking in general has an effect on reported fear and US expectancy during fear acquisition and generalisation, whereas interventions in smoking behaviour of smokers had no effect. Both groups showed a discrimination between CS+ and CS- after fear acquisition (Figure 1). During fear acquisition smokers showed generally increased reported fear, when compared to non-smokers. We found no differences between smoker manipulation groups.

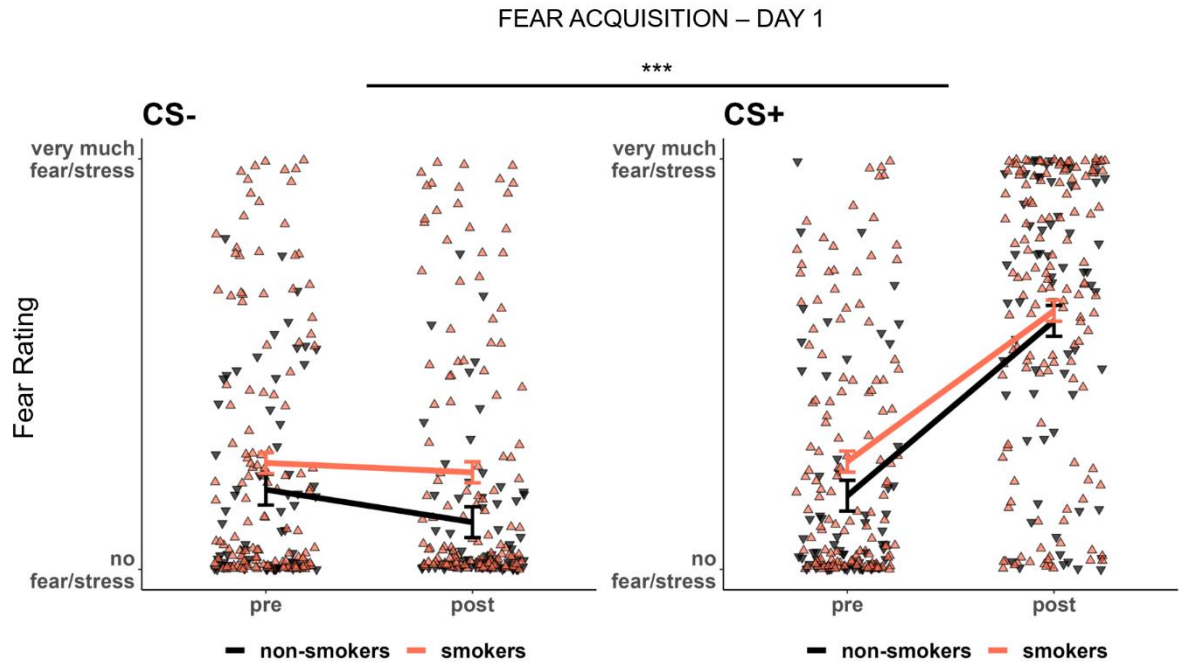

Figure 1: Study I – Fear acquisition, fear rating.
Both groups discriminated between CS+ and CS- post fear acquisition. Smokers showed increased reported fear during fear acquisition on day 1. Single subject ratings are depicted as upwards triangles for smokers and downwards triangles for non-smokers. [***] indicates p < 0.001.

After generalisation smokers showed increased reported fear specifically towards the safety stimulus (CS-), when compared to non-smokers (Figure 2). Interventions in smoking habit had no effect on generalisation over outcome measures.

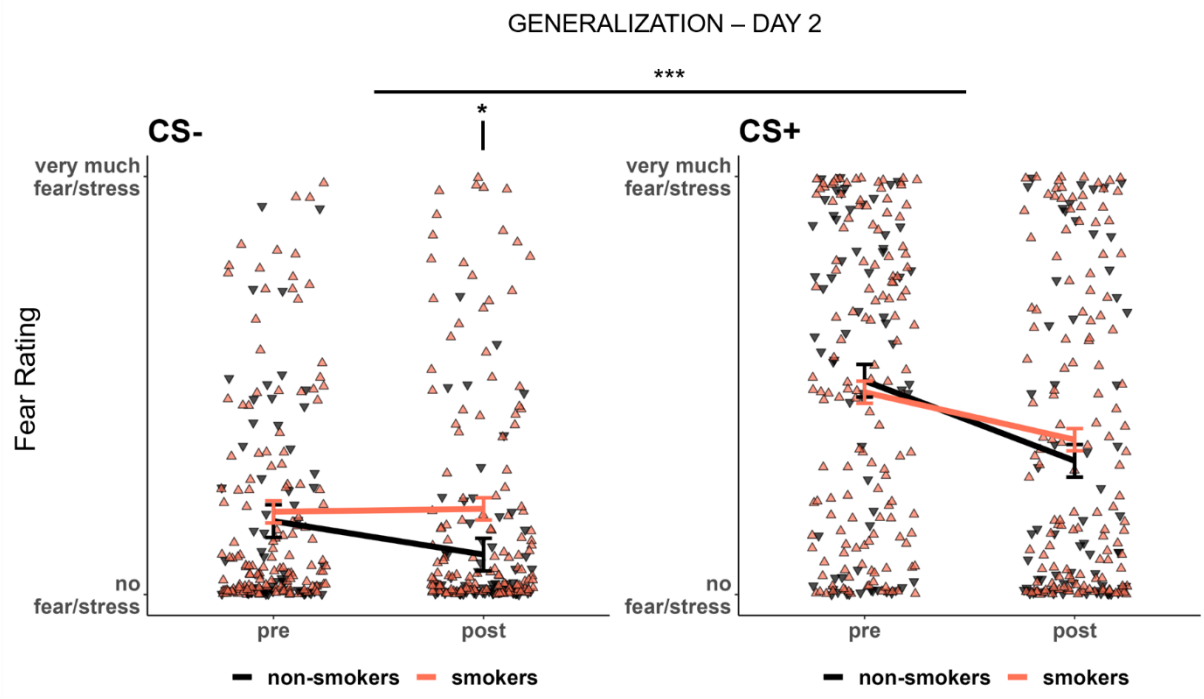

Figure 2: Study I – Generalisation, fear rating.

Smokers showed increased reported fear towards the CS- after generalisation. Single subject ratings are depicted as upwards triangles for smokers and downwards triangles for non-smokers. [***] indicates p < 0.001. [*] indicates p < 0.05.

Study I found that participants showed increased US expectancy towards generalised stimuli (GS) resembling the CS+ and decreased US expectancy towards GS that resembled the CS-. Furthermore, there was a third group of GS that was neither generalised to the CS+ nor the CS- and therefore ambiguous. Analysing these stimuli groups, Study I found a trend of increased US expectancy towards the CS- like stimuli in smokers, when compared to non-smokers (Figure 3).

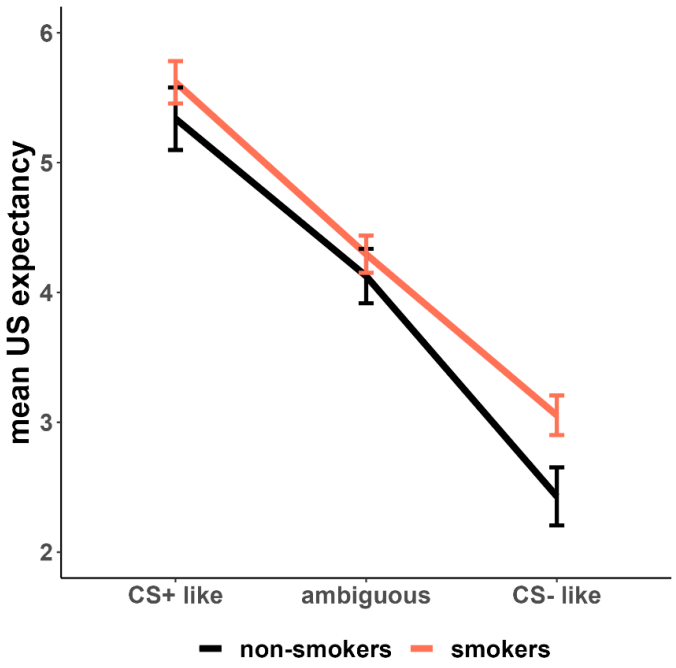

Figure 3: Study I – Generalisation, US expectancy.
Smokers show a trend-wise increased US expectancy towards CS- like stimuli during generalisation, when compared to non-smokers.

In summary, the results of Study I suggest impaired safety learning in smokers, as after generalisation (in which no US was presented) smokers still rated higher fear towards the safety stimulus. Importantly, smokers even expanded this impaired safety learning to novel stimuli. Study I did not find this specific effect during fear acquisition, although smokers showed generally increased fear when confronted with both threat and safety. Interestingly, interventions in smoking habits had no effect on fear learning and generalisation in smokers. These findings in healthy smokers can be linked to results of increased fear towards the safety stimulus in patients suffering from anxiety disorders during fear acquisition (Lissek *et al.*, 2005; Duits *et al.*, 2015). Similarly impaired safety learning processes in smokers and anxiety patients is a strong indicator for smoking as risk factor for the development or manifestation of pathological anxiety.

4.2. Study II: Nicotine reduces discrimination between threat and safety by reduction of hippocampal activations

4.2.1. Study II: Background and rationale

Previous studies associated acute nicotine with impaired fear learning and extinction in rodents (Kutlu *et al.*, 2014; Kutlu & Gould, 2014). But until now, the pharmacological effect of acute nicotine on fear acquisition and extinction in humans has not been investigated and the neural

mechanisms are unclear. Therefore, Study II analysed this relationship of nicotine and fear learning in healthy non-smokers in depth.

Study II examined multiple outcome measurements that reflect different representations and processing mechanisms of fear. Two distinctive subjective ratings were employed. Fear ratings were used to assess affective perceptions towards stimuli. Furthermore, US expectancy was acquired for each trial to receive a detailed representation of each subjects learning progress. Skin conductance responses were recorded as physiological measurement of conditioned response towards stimuli. Importantly, the whole experiment took place in the fMRI-scanner, to record neural activity and connectivity. In Study II, a decrease of stimulus discrimination in the groups that received nicotine before ACQ or EXT over all outcome measures was expected. This study creates a translational effort, as these experiments have yet been only conducted in animal models (Gould & Wehner, 1999; Davis *et al.*, 2005; Elias *et al.*, 2010).

A two-day study with a 24 h break between fear acquisition and extinction training was conducted. This “delayed extinction” between 24-72 h after fear acquisition is required for inhibition learning through memory consolidation (Myers *et al.*, 2006). The dose of 1 mg nicotine that was administered orally had been proven to elicit few side effects in participants, but also resulted in group differences in our pilot study. An increased dose of 2 mg nicotine in the non-smoking participants resulted in stronger side effects and also early termination of the experiment. In the final study all participants successfully finished the experiment and only reported light side effects. In Study II only non-smoking individuals were recruited for participation, because chronic smoking leads to an upregulation of nACh receptor density (Mukhin *et al.*, 2008) and therefore sensitivity towards the study medication would be depending on smoking habits. Furthermore, with the chosen participant criteria, Study II could show that only a single dose of nicotine affects fear learning.

4.2.2. Study II: Results and conclusion

The main finding of Study II is that nicotine administration impairs discrimination between threat and safety in healthy non-smokers. Participants that received nicotine before ACQ showed decreased stimulus discrimination in self-reported fear after fear acquisition, when compared to controls (Figure 4b). This was due to decreased fear towards the CS+ in the nicotine group (Figure 4a).

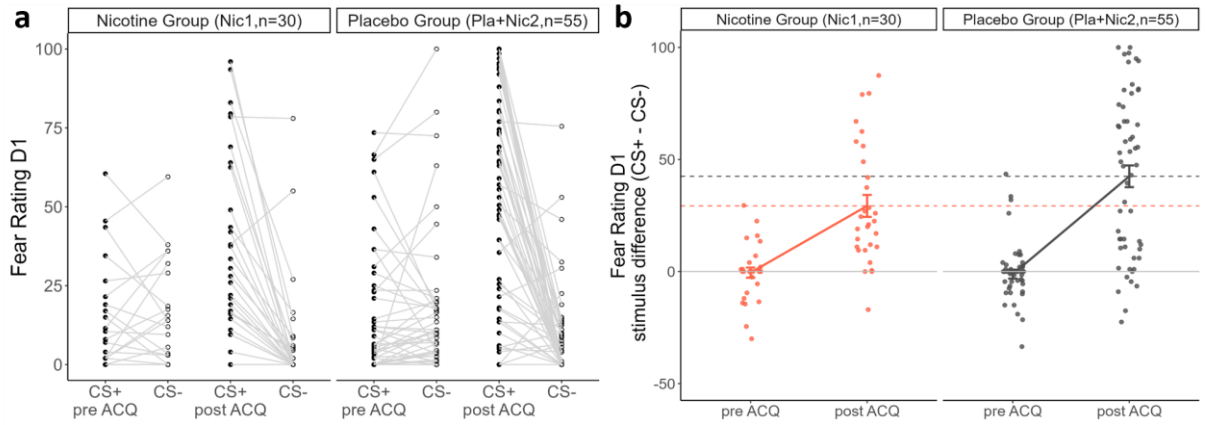

Figure 4: Study II – Fear acquisition, fear rating.

Stimulus discrimination in self-reported fear is decreased after ACQ in the group that received nicotine, when compared to placebo controls. a) Fear ratings per stimulus before and after ACQ. Connected lines indicate individual participants. b) Stimulus difference of each subject. Discrimination after ACQ is weaker in the nicotine group, indicated by dashed lines.

The effect of decreased stimulus discrimination was mirrored by neural activity in the hippocampus, especially during the last block of ACQ (Figure 5b). Similarly to the fear ratings, Study II found the main factor for decreased stimulus discrimination to be decreased neural hippocampal activity towards the CS+ in the nicotine group, when compared to placebo controls (Figure 5a). As the hippocampus plays a crucial role in learning CS-US associations (Sehlmeyer *et al.*, 2009), a weaker hippocampal activation in the nicotine group towards the CS+ is linked to impaired associative learning.

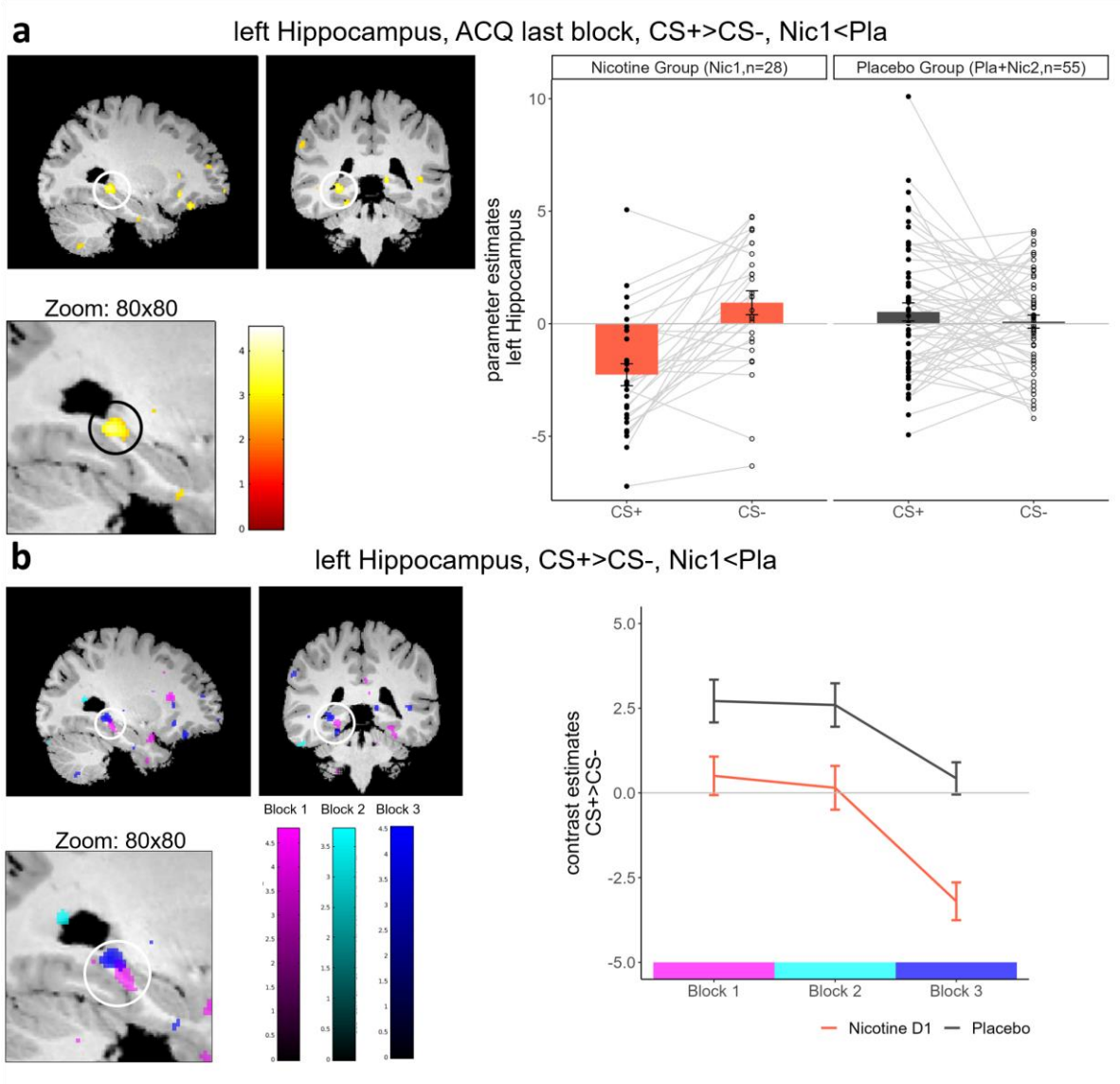

Figure 5: Study II – Fear acquisition, hippocampal activity.
Nicotine administration before ACQ leads to decreased differential activity in the left Hippocampus. a) Individual activations per stimulus reveals decreased hippocampal activation towards the CS+ in the nicotine group, when compared to placebo controls. b) Decreased differential hippocampal activation in the nicotine group over three blocks shows a robust effect.

To answer the question how exactly associative learning processes are impaired by nicotine, Study II further investigated the neural activity towards the US. Indeed, Study II found a decreased activation towards the US in multiple ROIs after nicotine administration, when compared to placebo controls (Figure 6). However, this effect is not a result of a generally decreased perception of US aversiveness, as the US valence rating showed no difference between groups. Strikingly, Study II found this decrease mainly in dopaminergic regions, such as the bilateral hippocampus, the VTA and also the bilateral Ncl. Accumbens. These regions

form a network, which is assumed to process novel and salient information and stores them in long-term memory (Lisman & Grace, 2005). Study II showed that nicotine administration decreases the activity and the connectivity of this hippocampal-accumbens-VTA-loop. That has a strong impact on fear acquisition. One can assumes that nicotine is impairing processing of novel CS-US associations and their storage in long-term memory.

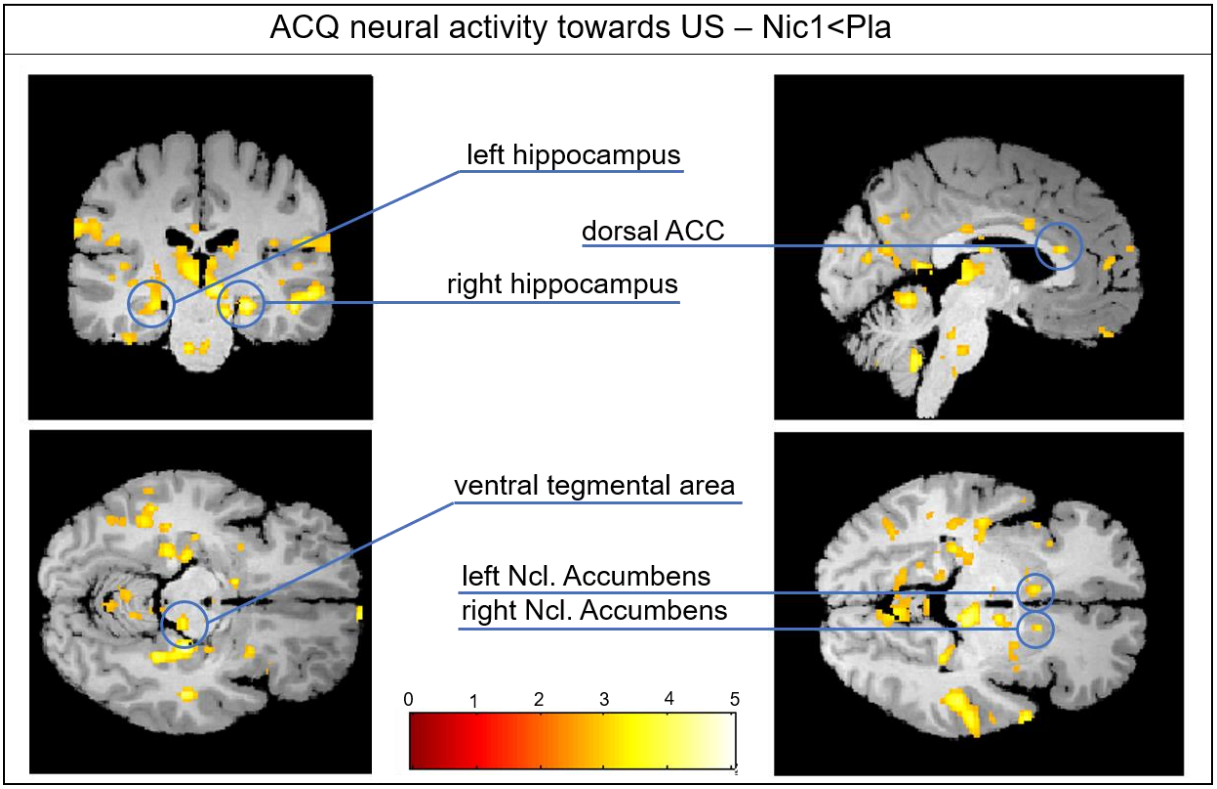

Figure 6: Study II – Fear acquisition, neural activity towards US.
Nicotine administration before ACQ leads to decreased activation towards the US in multiple ROIs.

The maladaptive associative learning processes during the fear acquisition resulting from nicotine administration are also impairing performance in extinction training. Furthermore, Study II found that acute nicotine administration before EXT has a similar effect. Both nicotine groups showed a decrease in differential memory retrieval in self-reported fear before extinction training, when compared to placebo controls (Figure 7). Similar to ACQ, Study II found a decreased stimulus discrimination in the hippocampus in the group that received nicotine before EXT, when compared to the placebo group. The return-of-fear manipulation was robust against influences of nicotine administration.

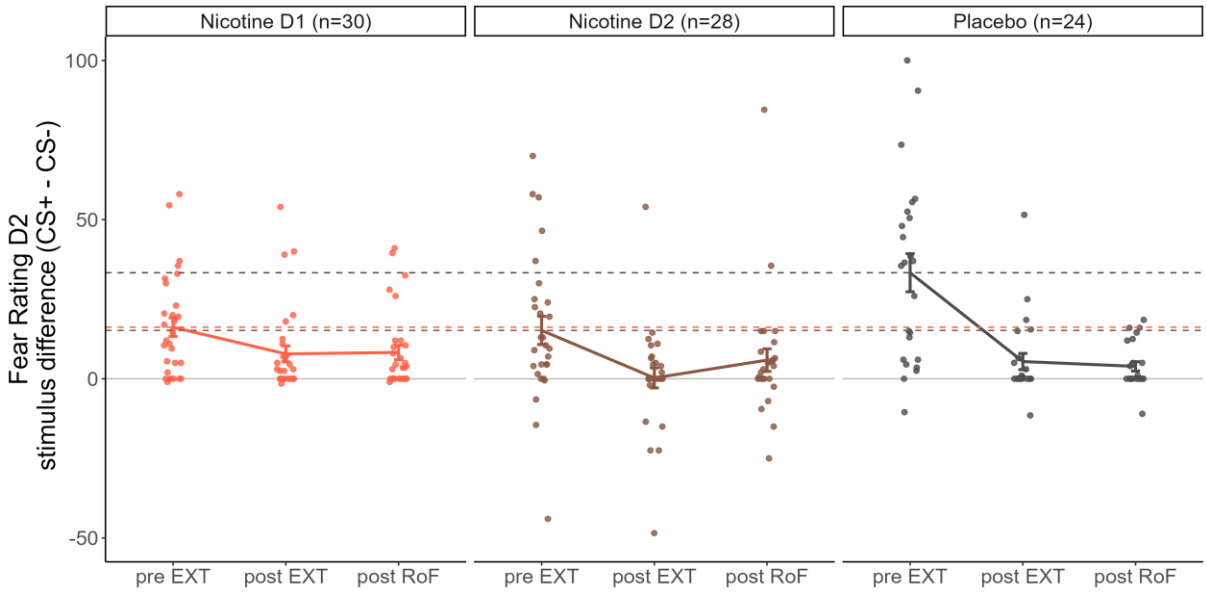

Figure 7: Study II – Extinction training, fear rating.
Both, nicotine administration before ACQ and EXT, lead to a decreased differential memory retrieval in self-reported fear before extinction training, when compared to placebo controls.

Contrary to the initial hypothesis, Study II found no effect of nicotine administration on US expectancy, indicating that cognitive contingency awareness was not disrupted by nicotine. Furthermore, Study II found an opposite than hypothesized effect in the skin conductance responses. Here, nicotine administration led to a stronger stimulus discrimination during both fear acquisition and extinction training. This effect can only be found in the last block, which suggests a difference in habituation, rather than a generally increased SCR due to nicotine administration. To ensure that the nicotine-induced disruption of fear learning processes is not a result of a generally impaired attention, participants underwent a d2-test of attention. Importantly, there were no differences between groups.

4.3. Study III: Acquisition of threat responses are associated with elevated plasma concentration of endocannabinoids in male humans

4.3.1. Study III: Background and rationale

Study III investigated changes in endocannabinoid (eCB) plasma levels (2-arachidonoylglycerol (2-AG), N-arachidonoylethanolamine (AEA) and its metabolite arachidonic acid (AA)) during fear acquisition in healthy male humans. Blood samples were collected before (T1) and after (T2) fear acquisition and the changes of AEA, 2-AG and AA (T2-T1) were examined.

Before conducting this study, research had mainly focussed on the buffering of stress and threat responses due to eCB plasma level changes as a result of extinction training. Study III established new associations between aversive learning and elevated eCB and AA plasma levels that are linked to subjective ratings, as well as physiological and neural responses.

Changing plasma levels of circulating eCBs and AA because of fear acquisition were expected in Study III, hypothesizing these changes to be related to reported fear, US expectancy, skin conductance responses and neural activity.

4.3.2. Study III: Results and conclusion

Study III (Weisser *et al.*, 2022) found that plasma levels of AEA and AA increase after ACQ, when compared to before ACQ (Figure 8a-b). However there was no general change in 2-AG plasma levels during ACQ (Figure 8c). Interestingly, Study III found a positive correlation between 2-AG plasma levels during ACQ (T2-T1) and both fear ratings and US expectancy during ACQ (T2-T1) regarding the stimulus discrimination (CS+ - CS-) (Figure 8d-e). Furthermore Study III found that a higher baseline of AEA plasma levels is associated with lower discrimination during fear acquisition.

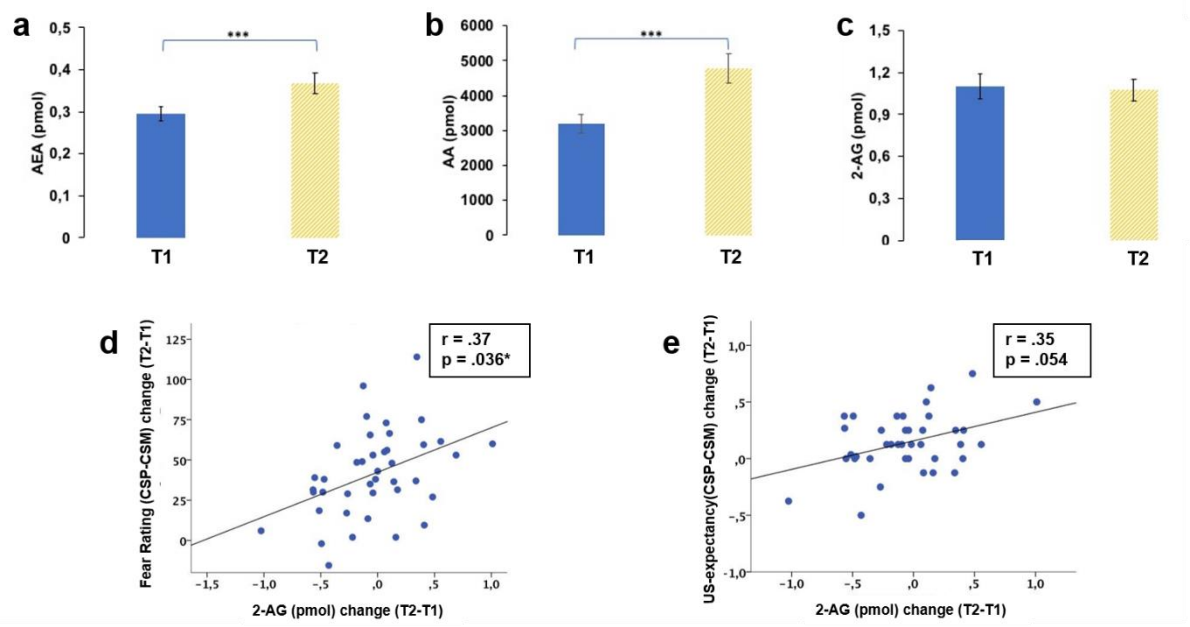

Figure 8: Study III – Fear acquisition, eCB levels and ratings.

Plasma levels of (a) AEA and (b) AA increase during fear acquisition, whereas (c) no general changes of 2-AG plasma levels were observed during fear acquisition. There is a positive correlation between (d) 2-AG plasma levels during fear acquisition (T2-T1) and discriminatory fear ratings, as well as (e) US expectancy (CS+ - CS- & T2-T1).

FMRI analyses revealed that a stronger stimulus discrimination in the right amygdala (CS+>CS-) is associated with increased AEA plasma levels during fear acquisition (T2-T1) (Figure 9a). Regarding the neural activity towards the US, Study III found that an increase in hippocampal activity is positively correlated to an increase of 2-AG plasma levels. Additionally, Study III found that an increase in neural activation in the dorsal ACC is associated with increased AEA plasma levels (Figure 9b)

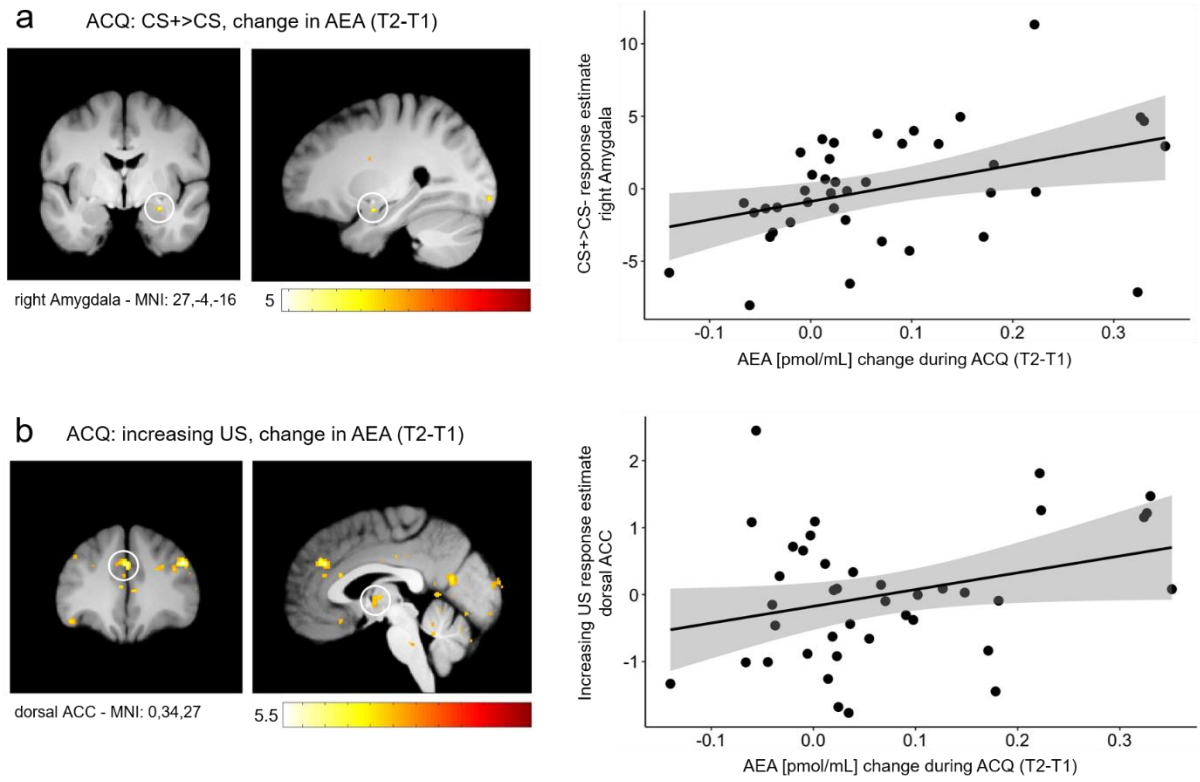

Figure 9: Study III – Fear acquisition, neural activity.
AEA plasma levels during fear acquisition (T2-T1) are positively correlated with a) differential response estimates in the right AMY (CS+ > CS-) and b) increasing response estimates towards the US in the dorsal ACC.

Altogether, Study III demonstrated that fear learning is linked to increased eCB plasma levels in AEA and AA. As eCBs are associated with buffering stress responses, this seems to be a reaction towards stress related aversive learning. Furthermore, plasma levels of 2-AG are positively correlated with discrimination of threat and safety stimuli during ACQ, meaning that eCBs specifically play a role in associative learning. A weaker discrimination between CS+ and CS- is often found in patients suffering from pathological anxiety (Lissek *et al.*, 2005; Duits *et al.*, 2015). This is mirrored by increased stimulus discrimination in the AMY, which is correlated to AEA plasma levels during ACQ. Hence, individual differences in circulating eCB

plasma levels of healthy individuals can possibly lead to changes in discriminatory fear learning and may therefore even indicate increased risk for pathological anxiety.

5. Discussion

This thesis examined different possible risk factors for the development of maladaptive fear learning, which can be observed as a decreased discrimination between threat and safety stimuli. All three investigated factors (chronic nicotine, acute nicotine and endocannabinoid plasma levels) play a potential role in maladaptive fear processing.

5.1. Nicotine-induced effects on fear learning

Both, Study I and Study II, revealed that nicotine, whether administered acutely in non-smokers or chronically in smokers results in a decreased discrimination between threat and safety. Study I found disrupted safety learning in smokers, when compared to non-smokers. Study II found decreased reported fear and hippocampal activity towards the danger stimulus during fear learning in the group that received nicotine, as opposed to placebo controls.

Importantly, the driving factor for the decreased discrimination differed between studies. Study I showed that chronic nicotine exposure leads to a decreased stimulus discrimination that was driven by increased fear towards the safety stimulus (CS-) after generalisation. This effect was independent from acute smoking. Interestingly, Study I did not find a difference between smokers and non-smokers during fear acquisition, but during memory retrieval. In line with that, a translational study in which rodent and human subjects both underwent a fear conditioning protocol found that chronic nicotine administration leads to an impaired stimulus discrimination in both species (Kutlu *et al.*, 2018). Furthermore, the analysis of the US expectancy in Study I revealed that this impaired safety memory retrieval is even overgeneralised onto CS- like novel stimuli. This mirrors the overgeneralisation of fear on safe stimuli that was found in patients with generalised anxiety disorder (GAD) (Lissek *et al.*, 2014). That implicates that smokers may develop similar characteristics known from patients suffering from pathological anxiety. Thus Study I emphasises the role that smoking plays as risk factor for the development of anxiety disorders.

As preregistered, Study II determined that already a single dose of nicotine in non-smokers impairs discrimination between safety and danger, when compared to placebo controls. This finding is in line with previous studies in rodents that also showed decreased discrimination after acute nicotine administration (Kutlu *et al.*, 2014). Interestingly, the decrease of discrimination during the fear acquisition of Study II was driven by decreased fear ratings and neural activity towards the CS+ in the group that received nicotine. As the CS+ is predictive for the aversive US, Study II also examined the processing of US presentations between groups. Study II found that the hippocampal-accumbens-VTA-loop, which is processing the storage of aversive stimuli in the long-term memory, is affected by nicotine administration, likely resulting in maladaptive learning. The VTA is a dopaminergic enervated brain structure

that plays an important role in reward, but also in processing aversive signals, such as the US (Matsumoto & Hikosaka, 2009; Bromberg-Martin *et al.*, 2010). The nicotine-induced disruption of processing the aversive CS-US association in the VTA may explain the deficit in CS discrimination. This means that the processing of the aversive US presentation that takes place in the VTA and connected regions such as the hippocampus, was disrupted by nicotine administration. The CS+ being predictive for the US might therefore be less distinct from the CS-, resulting in decreased discrimination. To exclude the possibility that participants who received nicotine simply have a reduced pain perception, compared to placebo controls, Study II analysed US valence ratings and found no group differences.

Comparing the results from Study I and Study II it is striking that chronic nicotine administration resulted in increased fear towards the safety signal during memory retrieval, whereas acute nicotine administration resulted in decreased fear towards the threat signal during fear learning. In both studies the same system is activated, which poses the question how can there be such a difference in the resulting learning and memory process? Earlier, this thesis discussed the dopaminergic VTA loop that seems to be impaired in activation and connectivity as a consequence of acute nicotine exposure. Importantly, the dopaminergic system has a variety of functions and is widely known for regulating rewarding experiences (Bromberg-Martin *et al.*, 2010). The omission of the US seems to trigger the dopaminergic reward system, because of the relief to be (unexpectedly) safe (Kalisch *et al.*, 2019). It is known that nicotine exposure differentially alters dopamine transmission in regions that are also part of the fear network, such as AMY, NAcc or VTA (Cadoni & Di Chiara, 2000; Ferrari *et al.*, 2002; Nguyen *et al.*, 2021). Hence, by influencing dopaminergic reward pathways, smoking might interfere with safety learning, consistent with Study I. One could assume that the effect of chronic nicotine stems from influencing dopaminergic neurons that process motivational value (i.e. reward). That would explain why smokers, when compared to non-smokers, show disrupted safety learning, because the processing of the rewarding US omission is impaired. Acute nicotine surely also activates the reward system, but seems to have a strong impact on dopaminergic neurons coding motivational salience (i.e. processing aversive events). Interestingly acute smoking before fear acquisition in Study I seemed to have no effect on motivational salience in smokers. This suggests that the dominant factor in smoking individuals influencing fear learning is chronic nicotine, as opposed to additional acute nicotine. This is likely due to desensitizing by upregulation of nAChR density as a result of chronic nicotine exposure.

In conclusion, Study I and Study II suggest that both chronic nicotine and acute nicotine in non-smokers impair dopaminergic pathways of learning and memory and nicotine is therefore enhancing maladaptive fear learning.

5.2. Endocannabinoid plasma levels and fear learning

Study III revealed that changes in circulating plasma levels of the endocannabinoids 2-AG, AEA and its metabolite AA are linked to fear acquisition. Regulatory changes in eCB levels have been found to be a dynamic response to buffering stress (Morena *et al.*, 2016). Study III found that ACQ elicits a general increase of AEA and AA plasma levels, but also that increased stimulus discrimination was associated with elevated plasma levels of AEA and 2-AG. The latter effect was identified across different outcome measurements. Therefore, learning success measured as affective and cognitive ratings as well as neural activity are linked to increased eCB plasma levels. Although Study III investigated a possible connection between eCB plasma levels and fear acquisition, it was not designed to examine causal effects.

A previous study in rodents receiving a 2-AG synthesis inhibitor investigated the effect of decreased 2-AG on fear conditioning (Cavener *et al.*, 2018). In line with results from Study III, they found that a decrease of 2-AG plasma levels led to an impaired fear acquisition. On the other hand, increasing 2-AG plasma levels in rodents by inhibiting 2-AG hydrolysis enhanced fear acquisition (Xu *et al.*, 2014). These results suggest a causal influence of 2-AG on fear learning and if translated to the findings from Study III might state the potential of enhancing adaptive fear learning in humans by elevating 2-AG plasma levels. Rodent studies that investigated the effect of a FAAH inhibitor (inhibition of the AEA degrading enzyme leads to an increase of AEA plasma levels) on fear conditioning and extinction found that an increase of AEA levels decreased anxiety-like behaviour, reduces stress responses and improved extinction learning (Gunduz-Cinar *et al.*, 2013; Dincheva *et al.*, 2015; Mayo *et al.*, 2020). In contrast, a recent study in male humans found no effect of FAAH inhibitors on fear conditioning (Paulus *et al.*, 2021). Nevertheless, the extensive literature on the enhancing effect of increased 2-AG and AEA levels on adaptive fear acquisition and extinction training implicates promising approaches in the future (but see (Mallet *et al.*, 2016)).

In summary, a decreased plasma level of endocannabinoids might facilitate maladaptive fear learning, such as impaired discrimination between threat and safety.

5.3. Dopaminergic pathways and fear learning

So far all three studies have individually highlighted potential factors that may lead to maladaptive learning. Chronic nicotine exposure seems to disrupt safety learning, independent from acute smoking. Acute nicotine in non-smokers seems to result in an impaired CS-US processing, leading to a decreased discrimination between threat and safety. Endocannabionid plasma levels respond to threat exposure and discriminatory fear learning processes in healthy men.

However, these factors do not only have to be discussed separately. Might there even be an increased, combined risk when naturally lower endocannabinoid plasma levels coincide with smoking? Interestingly the nicotinic cholinergic system and the endocannabinoid system have shown to influence each other (Scherma *et al.*, 2016). For example, manipulations of the endocannabinoid system are examined in research as possible target for treating nicotine dependence, as the CB_1 receptor antagonist rimonabant reduces nicotine self-administration in rats (Cohen *et al.*, 2002; Saravia *et al.*, 2021). The mutual factor between these systems is that they both belong to the dopaminergic pathways, which play an important role in fear learning (Nisell *et al.*, 1994; Fadok *et al.*, 2009; Covey *et al.*, 2017).

A rodent study found that chronic nicotine exposure altered eCB levels in different brain regions (González *et al.*, 2002). More specifically, they determined an AEA increase in the limbic forebrain (e.g. Ncl. Accumbens and amygdala), as well as an increase in both AEA and 2-AG in the brainstem (e.g. Locus coeruleus). However, they found a decrease of AEA and/or 2-AG levels after chronic nicotine exposure in the hippocampus, the striatum and the cerebral cortex. These are regions that are important for fear learning which were also affected by nicotine administration, as demonstrated in Study II. Additionally, Study I indicated an influence of chronic nicotine on fear memory. We can therefore assume that there is a relationship of nicotine administration and endocannabinoid levels in the dopaminergic system that is influencing fear learning and memory. For a definitive answer to the question how nicotine-induced changes on fear learning and memory influence eCB plasma levels, further investigations are needed. A first step would be to conduct an fMRI study that assesses eCB plasma levels before and after ACQ and EXT and then compares the results between a smoking and a non-smoking group of participants. This way, chronic nicotine-induced differences on fear learning and memory and their relation with eCB plasma levels could be determined. Regions of interest would be the already described dopaminergic fear network, especially the amygdala, hippocampus, VTA and Ncl. Accumbens.

5.4. Methodology

The line between a “side effect” and an “effect” is very thin, as a side effect is also part of the effect that a drug has. Rodent models have been widely used to investigate the effect of nicotine in fear learning. A typical behavioural indicator of fear in conditioning experiments in rodents is freezing, i.e. to stop all movement to avoid being detected by predators (VanElzakker *et al.*, 2014). As a side effect, nicotine administration can change dopaminergic pathways that are also important for locomotion, which can be challenging for the interpretation of freezing behaviour in rodent experiments (Abraham *et al.*, 2014). Typical unwanted side effects after nicotine administration in humans are vertigo, nausea or headache. Regarding the nicotine dose in Study II, a pilot study was conducted that determined 1 mg orally

administered nicotine in non-smokers to be the optimal balance between not causing too strong side effects and still affecting fear learning. If side effects like nausea would have been very strong after nicotine administration, then participants could not have concentrated on the learning task, which would also have resulted in decreased stimulus discrimination of the nicotine groups, compared to the control group. To rule this out, Study II assessed typical side effects after nicotine administration in all groups, but could not find any differences. Furthermore, the test of attention found no group differences, indicating no influence of nicotine on general attention in participants. Hence, the described group differences are not the results of side effects, indisposition or differences in attention of participants, but direct consequences of nicotine administration on systemic fear learning processes.

An important limitation of Study III is the only male sample that was included. More women than men are diagnosed with anxiety disorders and therefore they must be represented in future studies (Jalnapurkar *et al.*, 2018).

5.5. Future implications

This thesis examined the effect of different risk factors on fear learning. If adaptive, fear protects us from dangerous situations, places or things and avoiding danger is important for survival. However, increased avoidance of safe environments can have negative consequences. Through avoidance, anxiety can be reduced in the short term, but patients cannot learn that the situation is not dangerous in the long term (Thwaites & Freeston, 2005). Understanding the (neuropharmacological) mechanism of avoidance, might provide an avenue to augment key components of the therapeutic (behavioural) treatment. This thesis showed that nicotine and endocannabinoid levels alter passive fear learning, but it has not yet been investigated what influence these factors have on active avoidance behaviour. This would be the next step for extensively investigating how strong these risk factors actually impact the human fear system.

During the last years electronic cigarettes (e-cigarettes) became more popular, especially among teenagers and young adults (Walley *et al.*, 2019). E-cigarette companies are advertising their product with lower levels of carcinogens compared to classical cigarettes (Glantz & Bareham, 2018). Meanwhile most e-cigarettes still contain nicotine. Studies have shown that nicotine consumption particularly during development can lead to onsets of panic attacks in young adults (Isensee *et al.*, 2003). Therefore, not only patients suffering from pathological anxiety should get encouraged to stop smoking, but increased education on the effects of smoking and nicotine consumption is also needed in young adults.

6. Conclusion

Fear is supposed to keep individuals safe by motivating them to protect themselves from danger. To do so, it is crucial to know what is dangerous and what is safe. One of the main criteria of anxiety disorders is overestimating danger, which leads to excessive fear or anxiety with serious consequences on emotional, physical and social well-being of an individual. To understand the development or the maintenance of anxiety disorders, it is important to obtain insights into human fear learning processes. Therefore, this thesis investigated possible risk factors such as nicotine and endcannabinoid plasma levels in healthy participants. Although in different ways, discrimination between threat and safety is impaired by nicotine, whether one is exposed chronically or acutely. Furthermore, unadaptive eCB plasma levels during fear learning are also associated with impaired discrimination. It is assumed that these indicators for maladaptive fear learning seem to influence each other, as they are both part of the dopaminergic system.

A straightforward recommendation from this thesis is not to smoke, as nicotine itself affects fear learning and subsequently might affect the endocannabinoid system, too. The resulting maladaptive fear might manifest into the development of an anxiety disorder. Especially, with regard to patients already suffering from pathological anxiety, the findings of this thesis are strongly relevant. Smoking restrictions are a simple method to implement into therapy, but may lead to significant improvements in therapy sessions in the long run.

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List of Figures

Erklärung des Eigenanteils an den Publikationen

Studie I

MM, SW, JR und JH konzipierten und gestalteten das Studienparadigma. MM und SW sammelten die Daten. MM und JH analysierten und interpretierten die Daten. MM und JH verfassten das Manuskript, und SW und JR lieferten kritische Überarbeitungen. MM erstellte die Abbildungen. Alle Autoren diskutierten die Ergebnisse, kommentierten den Artikel und genehmigten das endgültige Manuskript zur Einreichung.

Studie II

MM, JR und JH konzipierten und gestalteten das Studienparadigma. MM sammelte die Daten. MM und JH analysierten und interpretierten die Daten. MM und JH verfassten das Manuskript, und TF und JR lieferten kritische Überarbeitungen. MM erstellte die Abbildungen. Alle Autoren diskutierten die Ergebnisse, kommentierten den Artikel und genehmigten das endgültige Manuskript zur Einreichung.

Studie III

JR, MM und SW analysierten die fMRI-Daten. SW und JH verfassten das ursprüngliche Manuskript, und BL, JF und MM lieferten kritische Überarbeitungen. Alle Autoren diskutierten die Ergebnisse, kommentierten und überarbeiteten den Artikel und genehmigten das endgültige Manuskript zur Einreichung.

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Studies I – III with supplementary material

STUDY I

Smokers show increased fear responses towards safety signals during fear generalization, independent from acute smoking, *Sci Rep* **12**, 8692 (2022)

Madeleine Mueller, Smilla Weisser, Jonas Rauh & Jan Haaker

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Smokers show increased fear responses towards safety signals during fear generalization, independent from acute smoking

Madeleine Mueller[1]✉, Smilla Weisser[1], Jonas Rauh[1,2] & Jan Haaker[1]

Smoking is highly prevalent among patients with anxiety disorders. Previous studies suggest that smokers show altered fear learning as compared to non-smokers. To test the effect of acute smoking on fear learning and generalization, we conducted a fear learning experiment online. 202 healthy subjects learned to differentiate a danger and a safe cue on day 1 and were tested for generalization of threat responses 24 h later. To see if the timing of smoking impacts fear learning, we formed three smoker groups with manipulations of acute smoking and withdrawal at different time-points (each group: n = 46) and one non-smoker control group (n = 64). Smoking manipulations contained a 6 h withdrawal after fear learning, smoking directly before or after fear learning. We found no group differences between smoker manipulation groups for fear learning or generalization. However, we found differences in fear generalization between smokers and non-smokers. Smokers showed increased fear ratings towards the stimulus that has been learned as safe and higher US expectancy to stimuli similar to the safe stimulus, when compared to non-smokers. Smoking might constitute a risk factor for impaired discrimination between danger and safety and smoking restrictions could be an effective way to reduce the risks of development or maintenance of anxiety disorders.

Anxiety disorders (AD) are among the most frequent mental disorders[1]. Patients suffering from AD, similar to other patients with psychiatric disorders, are more likely to smoke compared to healthy individuals (45.3% vs. 22.5% in healthy individuals)[2,3]. Furthermore, symptom severity in patients with posttraumatic stress disorder (PTSD) was positively correlated with the extent of nicotine dependence[4,5]. But not only patients with AD are affected by the influence of smoking on maladaptive responses to threats. Isensee et al. conducted a prospective longitudinal study that found a higher risk for the onsets of panic attacks in healthy individuals that smoked when compared to non-smoking individuals[6].

While being a smoker seems to increase the risks of maladaptive aversive learning, it is an open question how acute smoking affects associative aversive learning mechanisms and might drive maladaptive responses to threats.

It is assumed that one central mechanisms for the development of AD is (maladaptive) aversive associative learning, when confronted with threats[7]. Associative learning of threat responses in the laboratory is commonly examined using classical fear conditioning protocols. When employing differential fear conditioning, a conditioned stimulus (CS+) is predictive for an aversive unconditioned stimulus (US), whereas another conditioned stimulus (CS−) is not. Subjects learn the differential prediction of the two CSs for the US and express conditioned threat responses to the CS+ in comparison to the CS−. The CS− is learned as a safety stimulus and it is therefore adaptive to inhibit conditioned threat responses to the CS−[8,9].

Transfer of learned threat responses to novel objects can be very useful for coping with changing environments. Such transfer can be observed as generalization and therefore includes expression of threat responses to stimuli that resemble the CS+ and inhibition of responses to stimuli that are similar to the CS−. Such generalization can be examined across a gradient of stimuli between the CS+ and the CS−. A shallow generalization gradient indicates a stronger generalization between stimuli, whereas a steeper generalization gradient indicates a stronger discrimination between stimuli.

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Disproportionate threat responses to stimuli that resemble the CS+ (overgeneralization[10]) have been reported in patients diagnosed with Generalized anxiety disorder (GAD)[11].

A key question is if acute smoking affects associative aversive learning mechanisms by distorting the balance between expression of conditioned threat responses and their inhibition. Such effect has been found for acute nicotine (the active ingredient of cigarette smoke) on conditioned threat responses in mice. In particular, acute nicotine administration increased responses to conditioned cues and contexts, relative to saline injections[12] and nicotine impaired the inhibition of conditioned threat responses, when situations are safe. Specifically, the discrimination between dangerous and safe contexts seems to be dose-dependently disrupted when acute nicotine was acutely administered before threat learning[13].

There is further translational evidence that animal studies of chronic nicotine administration might resemble effects of smoking on threat learning in human individuals. Kutlu et al. found that mice show a reduced discrimination between CS+ and CS− during threat learning (nicotine in a chronic schedule), which was mirrored by lower CS discrimination in humans that were smokers[14]. Another study showed that smokers, as compared to non-smokers, had an impaired differentiation between danger and safety context when retrieving of conditioned threat memories[15]. While these studies underline that smokers show a deficit in safety learning, they cannot, however, delineate how acute smoking before or after learning might drive later deficits in retrieving safety information.

To this end, we employed an online experiment entailing a differential threat conditioning protocol and a generalization task (24 h later)[16,17]. With this study, we want to provide a deeper understanding of the effects of acute smoking, when comparing fear acquisition between smokers and non-smokers. Further, a generalization task should clarify if a disruption of safety learning transfers to novel stimuli. We expected that acute smoking before or after fear acquisition would lead to an impaired safety learning in fear acquisition and generalization, when compared to individuals restricted from smoking. Furthermore, we expected a deficit of safety learning in smokers, when compared to non-smokers.

Results

Fear acquisition. In order to establish that subjects learn to discriminate between CS+ and CS− during acquisition training, we examined US expectancy and fear ratings towards the CS+ , when compared to the CS−. We expected this pattern to be disrupted in regard to safety learning in groups that were acutely smoking before acquisition training and in general, when comparing smokers to non-smokers.

US expectancy results. Four groups. Participants learned to predict the US by the presence of the CS+ , indicated by a main effect of stimulus ($F(1,4376.7) = 46.857$, $p < 0.001$) with higher US expectancy for the CS+ as compared to the CS− (CS+–CS−: estimate = 3.8, SE = 0.078, z-ratio = 48.75, $p_{corr} < 0.001$) (Fig. 1a). Furthermore, we found a main effect of block ($F(2,4380.9) = 12.894$, $p < 0.001$) with an overall increasing US expectancy from block 1 to block 2 (block1–block2: estimate = − 0.5981, SE = 0.0956, z-ratio = − 6.256, $p_{corr} < 0.001$; block1–block3: estimate = − 0.6888, SE = 0.0959, z-ratio = − 7.184, $p_{corr} < 0.001$) and a stimulus by block interaction ($F(2,4378.1) = 76.523$, $p < 0.001$). The interaction consisted of higher differentiation between the CS+ and the CS− in block 2 when compared to block 1 ($t(1989) = 5.86$, $p_{corr} < 0.001$) as well as in block 3 when compared to block 2 ($t(1990) = 4.543$, $p_{corr} < 0.001$). Additionally, we found a block by group interaction ($F(6,4381) = 2.394$, $p < 0.026$), but follow-up post-hoc tests revealed no group differences.

Smokers versus non-smokers. When we compared smokers against non-smoking individuals, we found a trend towards a stimulus by block by group interaction ($F(2,4388.6) = 2.75$, $p = 0.064$), but follow-up post-hoc tests revealed no differences. We found a main effect of stimulus ($F(1,4386.7) = 46.657$, $p < 0.001$) with higher US expectancy for the CS+ as compared to the CS− (CS+–CS−: estimate = 3.85, SE = 0.083, z-ratio = 46.419, $p_{corr} < 0.001$; Fig. 1b). Furthermore, we found a main effect of block ($F(2,4390.9) = 12.829$, $p < 0.001$) with an increasing US expectancy from block 1 to block 2 (block1–block2: estimate = − 0.6365, SE = 0.102, z-ratio = − 6.271, $p_{corr} < 0.001$; block1–block3: estimate = − 0.7311, SE = 0.102, z-ratio = − 7.166, $p_{corr} < 0.001$) and a stimulus by block interaction ($F(2,4388.2) = 76.194$, $p < 0.001$). The interaction consisted of higher differentiation between the CS+ and the CS− in block 2 when compared to block 1 ($t(1992) = 15.919$, $p_{corr} < 0.001$) as well as in block 3 when compared to block 2 ($t(1995) = 4.992$, $p_{corr} < 0.001$).

Fear rating. Four groups. Participants rated higher fear for the CS+ as compared to the CS− (stimulus main effect ($F(1,197) = 75.554$, $p < 0.001$) and CS+ as compared to the CS− ($t(804) = 9.005$, $p_{corr} < 0.001$) (Fig. 1c). Furthermore, we found a main effect of time ($F(1,197) = 89.335$, $p < 0.001$) with an overall increase in fear ratings from pre ACQ to post ACQ (pre-post: $t(804) = -7.253$, $p_{corr} < 0.001$) and a stimulus by time interaction ($F(1,197) = 132.4$, $p < 0.001$). The interaction consisted of higher differentiation between the CS+ and the CS− post ACQ when compared to pre ACQ ($t(402) = 10.168$, $p_{corr} < 0.001$). Additionally we found a main effect of group ($F(3,197) = 2.701$, $p = 0.047$), but follow-up post-hoc tests revealed no differences.

Smokers versus non-smokers. When comparing smokers to non-smokers, we found a main effect of group ($F(1,199) = 7.754$, $p < 0.006$) that indicated increased fear ratings in the smoker group, when compared to the non-smoker group ($t(804) = 2.79$, $p_{corr} = 0.005$; see Fig. S1). We found a stimulus main effect ($F(1,199) = 74.254$, $p < 0.001$) with higher fear ratings for the CS+ as compared to the CS− ($t(804) = 9.005$, $p_{corr} < 0.001$). Furthermore, we found a main effect of time ($F(1,199) = 78.511$, $p < 0.001$) with an increase in fear ratings from pre ACQ to post ACQ (pre–post: $t(804) = -7.253$, $p_{corr} < 0.001$) and a stimulus by time interaction ($F(1,199) = 134.803$,

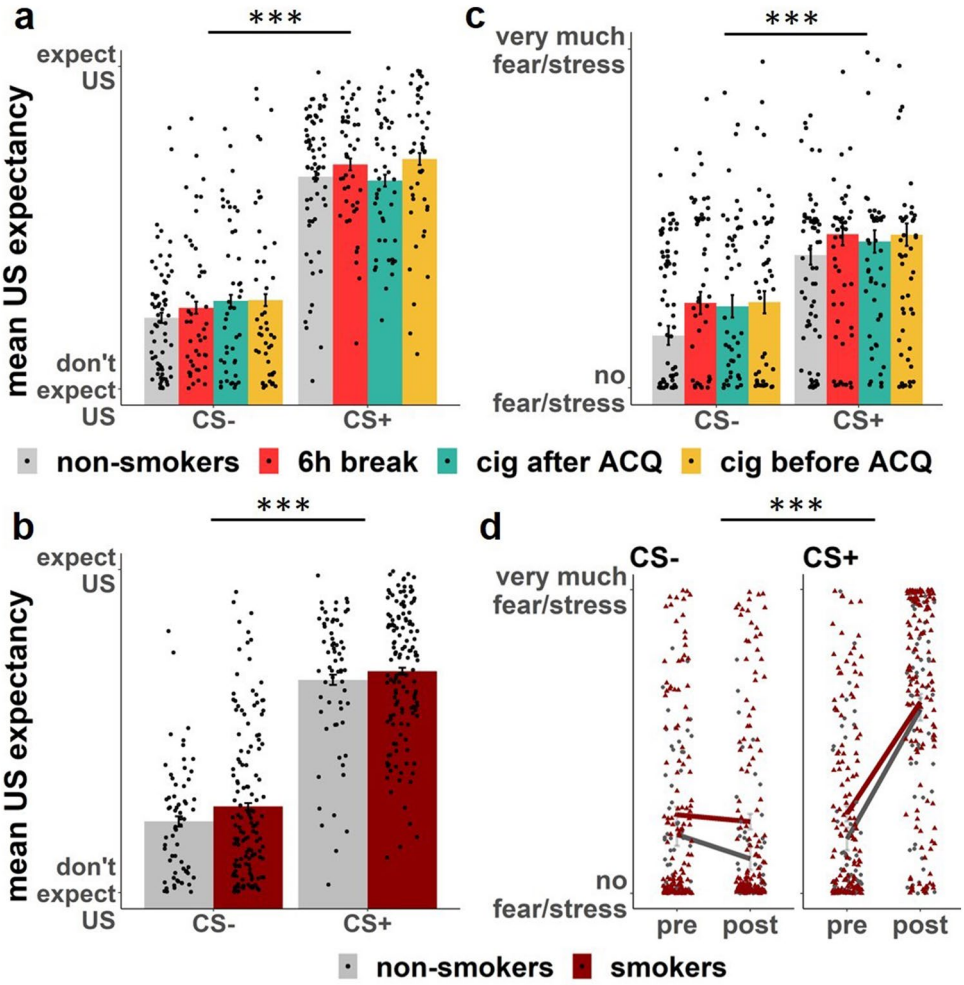

Figure 1. Rating Results Fear acquisition Day 1. Participants rated their US expectancy for every trial and their fear before and after fear acquisition. Individual representations indicate the mean for each subject. (**a**) Day 1 mean US expectancy of each group per stimulus. No group differences were found. (**b**) Day 1 mean US expectancy smokers versus non-smokers. No group differences were found. (**c**) Day 1 mean fear rating of each group. No group differences were found. (**d**) Day 1 fear rating smokers versus non-smokers. Following the colour, grey circles represent non-smokers and red triangles represent smokers. Both smokers and non-smokers showed an increased fear rating post acquisition towards the CS+, when compared to pre acquisition. Smokers showed generally increased fear ratings when compared to non-smokers. [***] indicates $p < 0.001$.

$p < 0.001$) (Fig. 1d). The interaction consisted of higher differentiation between the CS+ and the CS− post ACQ when compared to pre ACQ ($t(402) = 10.168$, $p_{corr} < 0.001$).

We found that smokers show a generally increased fear rating during fear acquisition, when compared to non-smokers. Against our hypotheses, we did not find any group differences between smoking manipulation groups.

Generalization test. To test for transfer of threat responses to the novel generalized stimuli, we employed a generalization in which we presented a gradient of new stimuli between the CS+ and the CS−. Now no stimulus was predictive for a US. We expected that retrieval of learned safety information (i.e., CS−) is altered in groups that were acutely smoking before and after acquisition training and in general, when compared to non-smokers.

US expectancy results. Four groups. Participants rated higher US expectancy for the CS+ as compared to each generalization-stimuli from GS3 to the CS− (stimulus main effect ($F(9,3576.6) = 20.601$, $p < 0.001$). Furthermore, participants rated lower US expectancy for the CS- as compared to each generalization-stimuli from GS7 to CS+ (estimates < −2.92, for details see Table S1, Fig. 2a). Furthermore, we found a main effect of block ($F(1,3576.6) = 4.959$, $p = 0.026$) with a decrease of US expectancy from block 1 to block 2 (block1–block2: estimate = 0.927, SE = 0.0756, z-ratio = 12.264, $p_{corr} < 0.001$), which is likely an effect of no US presentation during this phase (resembling extinction training).

During the generalization test, we found that participants generalized their US expectancy to stimuli that resembled the CS+ and the CS-. Hence, we could identify two generalization groups of stimuli: Stimuli that were

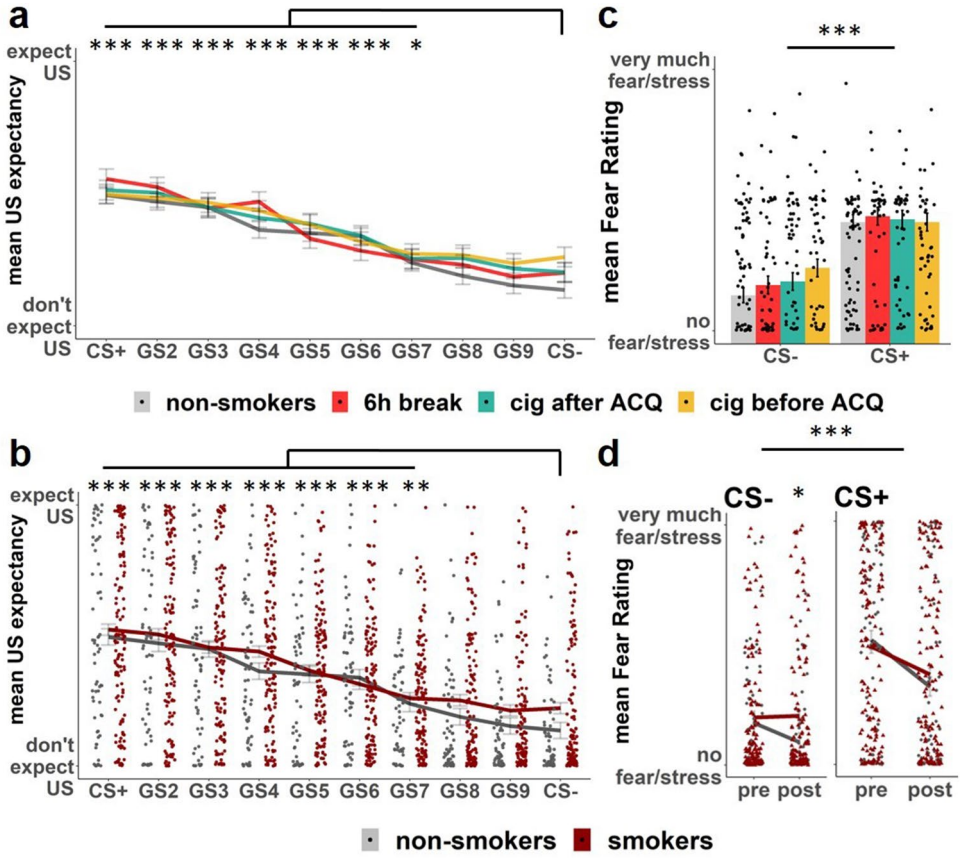

Figure 2. Rating results generalization test Day 2. Stimuli were presented in random order. Participants rated their US expectancy for every trial and their fear before and after the generalization test. Individual representations indicate the mean for each subject. (**a**) Day 2 mean US expectancy for each group per stimulus. Participants differentiated between the CS- and all stimuli between CS + and GS7 on Day 2. There were no group differences. (**b**) Day 2 mean US expectancy for all stimuli, smokers versus non-smokers. There were no group differences. (**c**) Day 2 mean fear rating for each group. No group differences were found. (**d**) Day 2 fear ratings smokers versus non-smokers. Following the colour, grey circles represent non-smokers and red triangles represent smokers. Non-smokers rated their fear/stress towards the CS- lower after the experiment when compared to smokers. [***] indicates $p < 0.001$. [**] indicates $p < 0.01$. [*] indicates $p < 0.05$.

"CS+ like" (i.e., different from the CS− and not from the CS+ (CS+ , GS2, GS3)) and "CS− like" (i.e., different from the CS+ and not from the CS− (GS8, GS9, CS−)). Additionally, we identified a third group of stimuli that was different from both, the CS+ and the CS− and hence ambiguous stimuli (GS4–GS7). For grouping these stimuli we used solely non-smoker US expectancy ratings (for details see supplementary methods). Thereby, we defined how stimuli would be grouped in a "control" population. When using these generalization groups of stimuli in the mixed-model, we found a main effect of stimulus ($F(2,3632.3) = 77.01$, $p < 0.001$) with higher US expectancy to the CS + like in comparison to novel stimuli (estimate = 1.3, SE = 0.10, z-ratio = − 12.81, $p_{corr} < 0.001$) and higher US expectancy when comparing novel stimuli to CS− like stimuli (estimate = 1.36, SE = 0.09, z-ratio = 15.32, $p_{corr} < 0.001$). We found a main effect of block ($F(1,3632.9) = 21.18$, $p < 0.001$) with a decreasing US expectancy from block 1 to block 2 (estimate = 0.97, SE = 0.08, z-ratio = 11.76, $p_{corr} < 0.001$), as in the previous model. Additionally we found a stimulus group by block interaction ($F(2,3632.1) = 3.4$, $p = 0.03$) that was characterized by a decrease of US expectancy for each stimulus group decreasing from block 1 to block 2 (CS+ like: estimate = 1.44, SE = 0.171, z-ratio = 8.434, $p_{corr} < 0.001$; ambiguous: estimate = 0.99, SE = 0.11, z-ratio = 9.11, $p_{corr} < 0.001$; CS− like: estimate = 0.46, SE = 0.14, z-ratio = 3.32, $p_{corr} = 0.002$).

Smokers versus non-smokers. We found a stimulus main effect ($F(9,3614.6)$ 20.703, $p < 0.001$) with higher US expectancy for the CS+ as compared to each generalization stimulus that ranged from GS3 to CS− (estimates > 0.517) and lower US expectancy for the CS− as compared to each generalization stimulus that ranged from CS+ to GS7 (estimates < − 0.635, for details see Table S3, Fig. 2b). Furthermore, we found a main effect of block ($F(1,3613.2) = 4.983$, $p = 0.026$) with a decrease of US expectancy from block 1 to block 2 (block1–block2: estimate = 1.05, SE = 0.08, z-ratio = 13.135, $p_{corr} < 0.001$). When grouping the stimuli as CS+ like, CS− like and novel, we found an interaction for stimulus by group ($F(2,3642.4) = 3.37$, $p = 0.035$). The following post-hoc

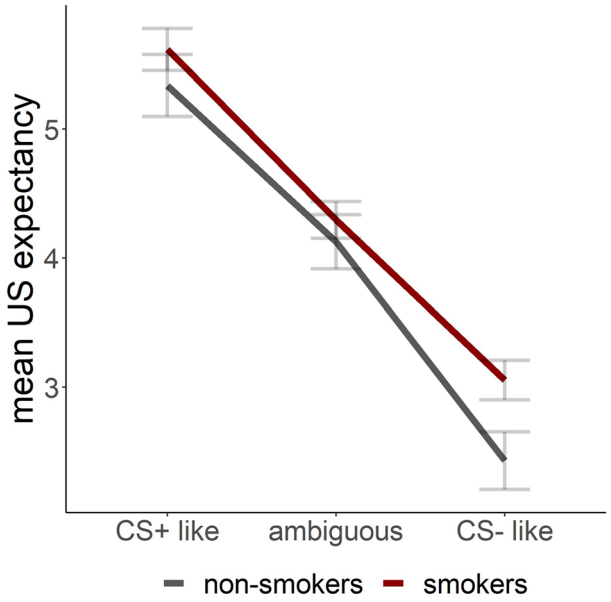

Figure 3. Day 2 mean US expectancy for grouped stimuli. Participants rated their US expectancy trialwise during the generalization test on day2 for CS+, CS− and the eight generalized stimuli in between (GS2–GS9). Rating results from the non-smoking group were used to define the grouping of stimuli. Stimuli that did not differ from CS+ were grouped as CS + like (CS+, GS2, GS3), stimuli that differed between CS+ and CS− were grouped as ambiguous (GS4–GS7) and stimuli that did not differ from CS− were grouped as CS-like (CS8, GS9, CS−). There is a trend towards a group difference between smokers and non-smokers when comparing CS− like stimuli during the generalization test on day 2. No differences were found between smokers and non-smokers for CS+ like and ambiguous stimuli.

test revealed a trend towards an increased US expectancy rating in the smoker group when compared to the non-smokers for the CS− like stimulus group (Fig. 3; estimate = − 0.624, SE = 0.271, z-ratio = 2.307, $p_{corr} = 0.063$).

Fear rating. Four groups. We found a stimulus main effect ($F(1,198) = 75.745$, $p < 0.001$) with higher fear ratings for the CS+ as compared to the CS− ($t(806) = 10.901$, $p_{corr} < 0.001$) (Fig. 2c). Furthermore, we found a main effect of time ($F(1,198) = 16.509$, $p < 0.001$) with a decrease in fear ratings from pre generalization test to post generalization test (pre–post: $t(806) = 3.35$, $p_{corr} < 0.001$). Additionally, we found a stimulus by time interaction ($F(1,198) = 11.791$, $p < 0.001$). The interaction consisted of higher differentiation between the CS+ and the CS− pre generalization test when compared to post generalization test ($t(402) = 2.6$, $p_{corr} = 0.01$).

Smokers versus non-smokers. We found a stimulus main effect ($F(1,200) = 75.013$, $p < 0.001$) with higher fear ratings for the CS+ as compared to the CS− ($t(806) = 10.901$, $p_{corr} < 0.001$). Furthermore, we found a main effect of time ($F(1,200) = 23.920$, $p < 0.001$) with a decrease in fear ratings from pre generalization test to post generalization test (pre–post: $t(806) = 3.35$, $p_{corr} < 0.001$). We found a stimulus by time interaction ($F(1,200) = 10.109$, $p = 0.002$). The interaction consisted of higher differentiation between the CS+ and the CS− pre generalization test when compared to post generalization test ($t(402) = 2.6$, $p_{corr} = 0.01$) (Fig. 2d). Additionally, we found a time by group interaction ($F(1,200) = 4.554$, $p = 0.034$). An independent samples t-test revealed that the non-smoker group rated lower fear compared to the smoker group after the experiment towards the CS− ($t(200) = -2.857$, $p_{corr} = 0.02$).

We found that smokers show an increased fear rating towards the safety cue (CS−) after fear generalization, when compared to non-smokers. Furthermore, we found a trend towards an increased US expectancy in CS- like stimuli in smokers, when compared to non-smokers. Again, we found no difference between groups that were acutely smoking before acquisition training or not.

Discussion

Our results indicate a difference in fear learning and fear generalization between smokers and non-smokers that is not affected by acute smoking before or after acquisition training. Smokers show increased fear ratings towards the CS− after the generalization test when compared to non-smokers. Additionally, we found a trend to an increased US expectancy towards stimuli that were generalized as the CS− (CS− like stimuli) in smokers, when compared to non-smokers. Our results thereby suggest that safety learning processes in smokers might be impaired and that this impairment is even transferred to novel stimuli. Interestingly, we found these differences between smokers and non-smokers in a situation that required retrieval of safety information (i.e., no presentation of the US), but not during learning (i.e., fear acquisition). Our results are in line with findings from

chronic nicotine administration in mice, which indicated increased freezing responses towards the CS−[14]. The experiments in rodents further suggest that nicotine administration throughout fear acquisition and extinction delayed safety learning and inhibition of conditioned threat responses during extinction training[18]. Additionally, the translation of this finding to humans revealed reduced CS-discrimination in smokers when compared to non-smokers, along with reduced CS-discrimination in mice[14]. Our finding that smokers show an impairment in retrieving safety information is further in line with a previous study that indicated impaired contextual inhibition in a safe context in smokers when compared to non-smokers[15]. Hence, our results pinpoint the influence of smoking to increased fear ratings and US expectancy ratings towards stimuli that have been learned as safe.

Against our hypothesis, we did not find an effect of any of our interventions on acute smoking (i.e., groups that smoked directly before or after the acquisition) and even withdrawal (i.e., control group that required a restriction of smoking for 6 h after acquisition) on fear learning or generalization. We furthermore could not find differences in aversive learning or the generalization test with respect to withdrawal symptoms or nicotine dependency between smokers (for details see supplement).

As a main effect, smokers showed generally increased fear ratings when compared to non-smokers, but we found no main group differences in the US expectancy or anxious temperament (STAI-trait anxiety). This indicates that there is no support for increased trait anxiety in smokers in our sample when compared to non-smokers (for details see supplement). Previous studies have found inconsistent results on whether smoking influences trait anxiety in humans (see for increased trait anxiety in smokers:[19]; see for no difference of trait anxiety between smokers and non-smokers:[20]).

In the generalization test on day 2, we could identify generalization of US expectancy to novel stimuli that were similar to the CS+ or CS− and hence analysed as CS+ like, CS− like or ambiguous stimuli. Interestingly smokers showed a trend towards an increased US expectancy to the CS− like stimuli, when compared to non-smokers. Both, US expectancy ratings and fear ratings decreased over time during the generalization test, which indicates extinction learning. This decrease in US expectancy was found for CS+ like and ambiguous stimuli in both smokers and non-smokers. However, the decrease of the CS− like stimuli was slightly weaker in smokers. In line with these findings for US expectancy are also the results of the fear ratings. Similarly to US expectancy, we found that smokers showed no decrease in rated fear during the generalization test to the CS−, whereas non-smokers did decrease their ratings. No differences in the decrease of fear ratings between groups were found for the CS+.

Our results suggest increased fear ratings and US expectancy in smokers when no US was present (generalization test) to a stimulus that was learned safe (CS−) when compared to non-smokers. This effect in smokers could be linked to maladaptive threat responses that are observed in individuals that suffer from pathological anxiety. Previous meta-analyses found that patients with anxiety-related disorders show an increased fear response during fear acquisition towards the CS− when compared to healthy controls[9,21]. Hence, our results in smokers resemble learning deficits in individuals with anxiety-related disorders. Thereby, our results might highlight a possible linkage between smoking and pathological anxiety: impairments in learning and retrieving safety information. Future studies are required to clarify if smoking causally leads to such learning deficits or the other way around.

One interesting aspect from our results might be relevant for the prevention and treatment of pathological anxiety: We would advocate to promote smoking cessation programs in order to reduce the risk of maladaptive threat responses in the long run. Our results support no effect of smoking restriction and withdrawal symptoms on threat responses. Hence, the process of quitting should not lead to exaggerated threat responses, per se. In fact, clinical studies that employed a smoking cessation treatment in parallel to mental health care for PTSD patients reported high rates of smoking abstinence and successful reduction of PTSD symptoms[22]. Hence, individuals that might be diagnosed with AD or exhibit other symptomatology that involves impaired safety learning might also benefit from smoking cessation programs.

Our results have several limitations. As this is an online study, there is an inherently reduced control of the participants with respect to their adherence to the study protocol (i.e. smoking manipulations). We informed the participants that there are no consequences if they for example could not fulfil the 6 h smoke break, but we encouraged them to honestly report if they managed to restrict smoking for 6 h, or not. Several participants reported that they have not followed instruction and were regrouped. This might seem like a big disadvantage of an online study, but it also provides a chance to investigate participants at home in their usual smoking environment. Most participants also followed the timeframe instructions of 24 h between day 1 and day 2 very precisely, which indicates a general adherence to instructions.

In sum, we found that smoking leads to increased fear responses towards the safety stimulus during and after the generalization test. No group differences were found during fear acquisition. Manipulations acute smoking and withdrawal at different time-points seem to make no difference in aversive learning, however the crucial point is if a person is a smoker or not. Smoking therefore might be considered a risk factor for impaired safety learning and ultimately enhances the individual development or maintenance of pathological anxiety. However, further investigations are needed to specify the causal effect of smoking and its active ingredient nicotine on fear learning in humans. Nevertheless, one clear recommendation for the reduction of the risk to develop maladaptive threat responses is to quit smoking.

Material and methods

Participants. For this online study 273 participants were recruited online. Healthy individuals between 18 and 65 years with no self-reported diagnoses of neuropsychiatric disorders, who consume less than 15 units alcohol per week and no illegal drugs could participate. The final sample for analysis consisted of 202 subjects (female: N = 125, mean age = 29.47 ± 9.81, Table 1). Participants were included into the final data set if the experiment on day 2 was started 24 h after starting day 1 (with a tolerance of 6 h before and after the start). Participants

	Mean (SD) Non-smokers Group 1	Mean (SD) Smokers Group 2	Mean (SD) Smokers Group 3	Mean (SD) Smokers Group 4	Mean (SD) Smokers (Groups 2, 3 & 4)
Sample size	64	46	46	46	138
Age [years]	29.38 (10.47)	27.96 (8.49)	30.2 (10)	30.37 (9.59)	29.51 (9.44)
Gender	Females: 43 Males: 21	Females: 31 Males: 15	Females: 27 Males: 19	Females: 24 Males: 22	Females: 82 Males: 56
Coffee consumption [cups/day]	1.198 (1.07)	1.22 (1.21)	1.34 (1.05)	1.7 (1.358)	1.42 (1.22)
Alcohol consumption [drinks/week]	1.43 (1.98)	2.58 (2.72)	2.7 (2.69)	3.28 (3.24)	2.85 (2.91)
Fagerström [sum score]	–	1.96 (1.88)	3.3 (2.51)	2.61 (2.35)	2.62 (2.33)
STAI-T [sum score]	43.14 (5.68)	44.59 (9.39)	44.80 (6.05)	44.96 (6.79)	44.78 (7.55)

Table 1. Demographics for each group separately and for all smoker groups combined. Group 1 contains only non-smokers, group 2 contains smokers that took a smoking break of 6 h after fear acquisition on day 1, group 3 contains smokers that smoked a cigarette directly after fear acquisition on day 1 and group 4 contains smokers that smoked a cigarette directly before fear acquisition on day 1. The last column contains all smokers from groups 2, 3 and 4 combined.

had to rate the US as more unpleasant than the nUS, which was defined by a mean rating of the 3 USs being at least 1 point higher than the mean rating of the 3 nUSs on a scale of 1 to 10. furthermore, participants with an incomplete data set were excluded (for an overview see Fig. S2). All participants gave written, informed consent and the experiment was approved by the local ethics committee (Ethikkommission der Ärztekammer Hamburg PV 5514). The subjects received 15€ as reimbursement for completing both days of the study. All research was performed in accordance with the relevant guidelines and regulations.

Groups. Four experimental groups were included in this study (Fig. 4c). The first group consisted of only non-smoking subjects (N = 64). The second group consisted of smoking subjects, who were instructed to have a 6-h smoking break after completing the acquisition (ACQ) on day 1 (N = 46). The third group consisted of smoking subjects, who were instructed to smoke after completing the acquisition on day 1 (N = 46). The fourth group consisted of smoking subjects, who were instructed to smoke a cigarette directly before starting the acquisition (N = 46). Subjects who were originally allocated to group 2, but did not take a 6 h smoking break were regrouped into group 3 for analyses of the final data set. Smokers were pseudo-randomly assigned to one of the smoker manipulation groups.

Stimuli material. Two black rings with a smaller and a larger diameter (CS1: 5 cm; CS2: 11.75 cm) served as conditioned Stimuli (CS). CS1 and CS2 were counterbalanced as CS+ or CS−. For the Generalization test, additional eight generalized stimuli (GS2-–GS9) with an increasing 15% ring size between CS1 and CS2 were presented[16]. A black fixation cross served as the ITI.

Three pictures from the International affective picture system (IAPS) database[23] that were rated as unpleasant ([24], #3001, #3030, #3051) were chosen as unconditioned Stimuli (US) that followed upon the CS+ . Three different pictures that were rated neutral (#7009, #7026, #7175) were chosen as neutral US (nUS) that followed upon the CS−.

Procedure. The study took place on 2 consecutive days with a temporal difference of 24 h (± 6 h). On the first day participants filled out a questionnaire containing a Fagerström test for nicotine dependence (FTND) and the State-Trait Anxiety Inventory (STAI-S/STAI-T) and gave information on age, sex, alcohol and coffee consumption and smoking habit. Questionnaires were completed on www.soscisurvey.de[25]. Directly after completing the questionnaires, participants started the behavioural experiment.

The experiment on day 1 contained a habituation phase in which the CS+ and CS− were presented once without being followed by the US/nUS. Day 1 further entailed an acquisition phase with three blocks, each consisting of four CS+ and four CS− presentations. Blocks were presented randomized. The CS+ presentation was followed by a US picture in nine out of twelve trials (75% reinforcement rate). Similarly, a nUS picture followed in 75% of the CS− trials. In 25% of the trials, the CSs faded out without US/nUS presentation. Each trial started with a 7 s CS presentation that was followed by a 3 s US/nUS presentation and ended with an ITI presentation (jittered between four to six seconds) (Fig. 4a).

Behavioural experiments were completed with PsychoPy3[26]. On the second day participants started again with a questionnaire containing STAI-S and a report on their overnight sleep. If the participant was sorted into the second group, they additionally answered if they managed to take a smoking break of 6 h after the acquisition and if not, when they started smoking again. Those participants were also asked if they noticed any withdrawal symptoms between day 1 and day 2.

Similar to the day before, participants started the behavioural experiment after finishing the questionnaires. The experiment on day 2 contained the generalization test with two blocks including one CS+ and one CS− presentation, as well as one GS2 to GS9 each. That concludes to ten trials per block, which were presented in random order. No CS or GS was followed by a US/nUS picture (Fig. 4b). The experiment ended with a CS/

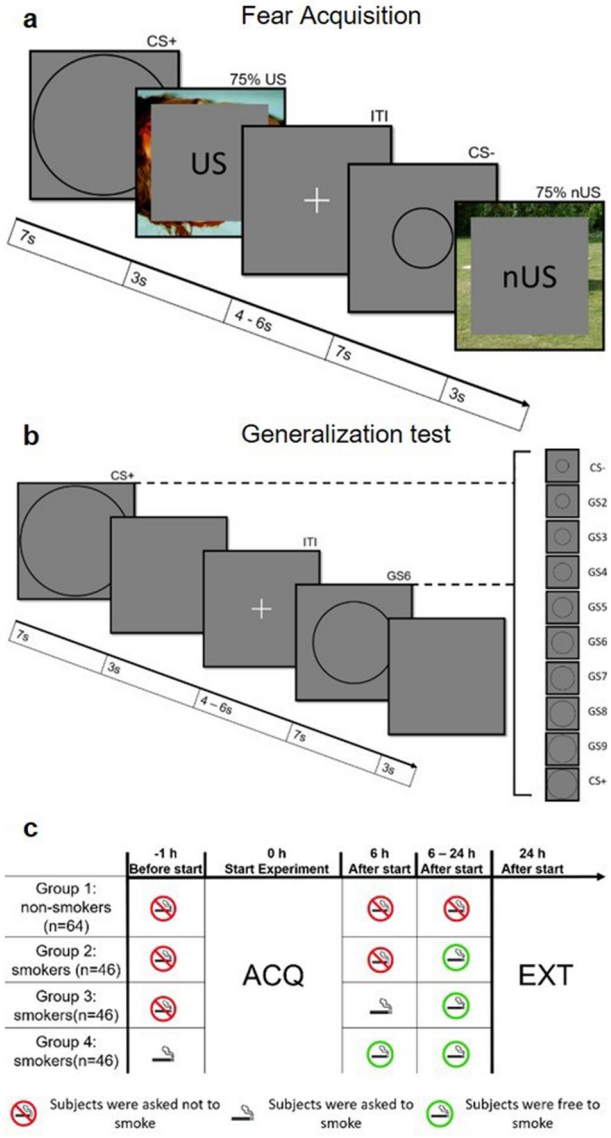

Figure 4. Experimental procedure. (**a**) Day 1: Fear acquisition. The CS + was followed in 75% of the time by an aversive picture, which served as US. The CS− was followed in 75% of the time by a neutral picture, which served as nUS. When not followed by a picture, CSs were fading into the grey background (1 s). Per trial a CS was presented for 7 s followed by a 1 s overlap of CS and US/nUS and then a 2 s only presentation of US/nUS. After each trial the ITI was presented (jittered between 4 and 6 s). Size of rings serving as CS + and CS− was counterbalanced. (**b**) Day 2: Generalization test. In addition to CS + and CS- serving as extremes, 8 rings of gradually increasing size were introduced as generalized stimuli (GS). After a 7 s presentation the CS or GS faded into the grey background. After each trial the ITI was presented (jittered between 4 and 6 s). (**c**) Group formation. The non-smoking group (group 1) did not smoke at all during the experiment. Group 2 (smokers) was asked not to smoke 1 h before and 6 h after the experiment on day 1. Group 3 (smokers) was asked not to smoke 1 h before the experiment and to smoke directly after the experiment on day 1. Group (smokers) 4 was asked to smoke directly before the experiment on day 1.

US identification where participants decided which CS or GS was paired with the US on day 1 (or if none of the stimuli were paired with the US). Participants had the possibility to stop the experiment at any time.

Ratings. Participants rated their fear when confronted with CS+ and CS− on a continuous scale between 1 (no fear/stress) and 10 (very much fear/stress) before and after the acquisition phase on day 1, as well as before and after the generalization test on day 2.

On both days, participants rated their US expectancy on each trial (two to six seconds after CS onset) on a continuous scale from 1 (I don't expect an unpleasant picture) to 10 (I definitely expect an unpleasant picture).

After generalization test and fear rating participants were asked about how unpleasant they perceived the US/nUS photos on a continuous scale from 1 (not unpleasant) to 10 (very unpleasant) (for details see Fig. S3).

Additionally, participants were asked for their avoidance (how often they looked away from the screen) and their awareness (if they noticed a connection between the CS and the US/nUS on day 1).

Data analysis. For analysis of the US expectancy we calculated linear mixed effect models in R using lme4 package[27]. The dependent variable in our model were the US expectancy ratings. Random intercepts and slope for subjects were entered in our model. The fixed effects in our model was the interaction between CS-types (CS+ and CS−), blocks (one to three) and groups (one to four; for smoker vs. non-smoker: one to two) (lmer (RatingResults ~ (1|participants) + stimuli*block*group)).Then an F-test with a Kenward-Roger approximation for degrees of freedom was performed in the form of an ANOVA type 3 calculation[28]. To further test the results estimated marginal means (EMMs) were computed using the emmeans package as post-hoc tests and p-values were corrected for multiple comparisons using Bonferroni-Holms method. Results with z-ratio are asymptotic results.

Fear ratings were analysed in Jasp using a repeated-measures ANOVA type 3 with repeated measures factors CS-type and time-point, as well as the between subject factor group. Follow-up tests were calculated by independent sample t-tests and were Bonferroni-Holm corrected.

Data availability

Data generated from this study are available from the authors upon reasonable request.

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Author contributions
M.M., S.W., J.R. and J.H. conceived and designed the study paradigm. M.M. and S.W. collected the data. M.M. and J.H. analyzed and interpreted the data. M.M. and J.H. drafted the manuscript, and S.W. and J.R. provided critical revisions. M.M. created the figures. All of the authors discussed the results, commented on the article, and approved the final manuscript for submission.

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Smokers show increased fear responses towards safety signals during fear generalization

Supplementary Material

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Supplementary Results

Table S1: Day 2 US expectancy post-hoc tests between stimuli for all groups. Results were corrected for multiple comparisons using Bonferroni-Holms method.

Stimuli	Estimate	SE	z-ratio	p_{corr}
CS+ - GS3	0.517	0.179	3.36	0.01
CS+ - GS4	0.969	0.178	5.143	<0.001
CS+ - GS5	1.35	0.18	8.083	<0.001
CS+ - GS6	1.637	0.178	10.417	<0.001
CS+ - GS7	2.333	0.179	13.959	<0.001
CS+ - GS8	2.596	0.18	14.864	<0.001
CS+ - GS9	2.93	0.178	16.995	<0.001
CS+ - CS-	2.969	0.178	16.927	<0.001
CS- - GS2	-2.777	0.178	-15.86	<0.001
CS- - GS3	-2.452	0.179	-13.46	<0.001
CS- - GS4	-1.999	0.177	-11.804	<0.001
CS- - GS5	-1.619	0.179	-8.643	<0.001
CS- - GS6	-1.331	0.178	-6.456	<0.001
CS- - GS7	-0.635	0.178	-2.92	0.032

Table S2: Day 2 US expectancy post-hoc tests between stimuli for smokers vs. non-smokers. Results were corrected for multiple comparisons using Bonferroni-Holms method.

stimuli	Estimate	SE	z-ratio	p_{corr}
CS+ - GS3	0.517	0.179	2.881	0.04
CS+ - GS4	0.969	0.178	5.451	<0.001
CS+ - GS5	1.35	0.18	7.506	<0.001
CS+ - GS6	1.637	0.178	9.184	<0.001
CS+ - GS7	2.333	0.179	13.06	<0.001
CS+ - GS8	2.596	0.18	14.425	<0.001
CS+ - GS9	2.93	0.178	16.47	<0.001
CS+ - CS-	2.969	0.178	16.632	<0.001
CS- - GS2	-2.777	0.179	-15.533	<0.001
CS- - GS3	-2.452	0.179	-13.682	<0.001
CS- - GS4	-1.999	0.177	-11.268	<0.001
CS- - GS5	-1.619	0.179	-9.018	<0.001
CS- - GS6	-1.331	0.179	-7.484	<0.001
CS- - GS7	-0.635	0.178	-3.564	0.005

Statistics Smoker manipulation groups without non-smoking individuals

In order to test the robustness of the statistical results, we examined the effect of smoking manipulations between smoking individuals, only.

Fear Acquisition

US expectancy results were analysed with the mixed-model in R.

We found a main effect of stimulus ($F(1,2979.87)=42.43, p<0.001$) with higher US expectancy for the CS+ as compared to the CS- (CS+-CS-:estimate=3.76,SE=0.097,z-ratio=38.790,$p_{corr}<0.001$). Furthermore, we found a main effect of block ($F(2,2981.64)=13.88, p<0.001$) with an increasing US expectancy from block 1 to block 2 and block3 (block1–block2:estimate=-0.5612,SE=0.119,z-ratio=-4.726,$p_{corr}<0.001$; block1–block3: estimate=-0.6474,SE=0.119,z-ratio=-5.44,$p_{corr}<0.001$) and a stimulus by block interaction ($F(2,2977.7)=40.878, p<0.001$). The interaction consisted of higher differentiation between the

CS+ and the CS- in block 2 when compared to block 1 ($t(1344)=12.06, p_{corr}<0.001$) as well as in block 3 when compared to block 2($t(1344)=3.11, p_{corr}=0.002$). Additionally, the analyses revealed a block by group interaction (block*smoker groups: $F(4,2977.63)=3.06, p<0.0158$), but follow-up post-hoc tests revealed no group differences.
Fear Ratings were analysed in jasp. We found a stimulus main effect ($F(1,134)=41.04, p<0.001$) with higher Fear Ratings for the CS+ as compared to the CS- ($t(548)=6.725, p_{corr}<0.001$). Furthermore, we found a main effect of time ($F(1,134)=53.486, p<0.001$) with an increase in Fear Ratings from pre ACQ to post ACQ (pre–post:$t(548)=5.79, p_{corr}<0.001$) and a stimulus by time interaction ($F(1,134)=69.198, p<0.001$). The interaction consisted of higher differentiation between the CS+ and the CS- post ACQ when compared to pre ACQ ($t(274)=7.144, p_{corr}<0.001$).

Generalization test
We found a stimulus main effect ($F(9,2431.4)=16.759, p<0.001$) with higher US expectancy for the CS+ when compared to the generalization stimuli from GS3 to CS- (minimal t-value: $t(2432)=2.969, p_{corr}=0.042$) and lower US expectancy for the CS- as compared to CS+ to GS6 (maximal t-value: $t(2432)=-4.032, p_{corr}=0.002$).

Table S3: Day 2 US expectancy post-hoc tests between stimuli for smoker manipulation groups without non-smoking individuals. Results were corrected for multiple comparisons using Bonferroni-Holms method.

stimuli	t	df	p_{corr}
CS+ - GS3	2.969	2432	0.042
CS+ - GS4	3.649	2432	0.005
CS+ - GS5	6.667	2432	<0.001
CS+ - GS6	8.982	2432	<0.001
CS+ - GS7	11.371	2432	<0.001
CS+ - GS8	11.651	2432	<0.001
CS+ - GS9	13.39	2431	<0.001
CS+ - CS-	13.083	2432	<0.001
CS- - GS2	-12.288	2432	<0.001
CS- - GS3	-10.021	2432	<0.001
CS- - GS4	-9.43	2431	<0.001
CS- - GS5	-6.233	2432	<0.001
CS- - GS6	-4.032	2432	0.002

We found a stimulus main effect ($F(1,135)=46.204, p<0.001$) with higher Fear Ratings for the CS+ as compared to the CS- ($t(550)=8.266, p_{corr}<0.001$). Furthermore, we found a main effect of time ($F(1,135)=4.983, p=0.016$) with a trend towards a decrease in Fear Ratings from pre EXT to post EXT (pre–post:$t(505)=1.846, p_{corr}=0.065$). Additionally, we found an interaction of stimulus by time ($F(1,153)=7.237, p=0.008$). The interaction consisted of higher differentiation between the CS+ and the CS- pre ACQ when compared to post ACQ ($t(274)=2.137, p_{corr}=0.034$).

Statistics Fagerström
We checked both US expectancy and fear ratings for an effect of nicotine dependence with the Fagerström test for nicotine dependence (FTND). The score of the FTND was included into the model (lmer (RatingResults~(1|participants) +stimulus*time*group+fagerström)). The group factor that is included in the model includes the three groups with smokers. We found no main effect of the Fagerström score for US expectancy rating on day 1 ($F(9,125.12)=0.409, p=0.928$) or day 2 ($F(1,133.91)=1.213, p=0.273$). Also we found no main effect of the Fagerström score for fear rating on day 1 ($F(1,133.38)=0.958, p=0.3295$) or day 2

(F(1,134)=1.837,p=0.178). When checking for interactions of the FTND with our fixed effects in the model, we found a trend towards a stimulus by group by FTND interaction (F(2,2964.54)=2.532,p=0.08). For further analysis, we calculated correlation coefficients between the differential US expectancy rating on day 1 and the FTND for each group separately. As the data shows no normal distribution, we used spearman's rho for correlation analysis. Only group 4 showed a correlation between US expectancy rating and FTND (rho=0.163,p<0.011). No correlation between US expectancy on day 1 and the FTND was found for group 2 and group 3 (group 2:rho=-0.049,p=0.282; group 3:rho=-0.016,p=0.722).

Statistic Withdrawal

We checked both US expectancy and fear ratings for an effect of withdrawal. The score of withdrawal symptoms was included into the model (lmer (RatingResults~(1|participants) +stimuli*time*group+withdrawal)). The group factor that is included in the model includes the participants of group 2 and the regrouped participants into group 3. Statistics has only been calculated for the Generalization test, as participants were only asked to take a smoking break between day 1 and day 2 and there should be no effect of this smoking manipulation on day 1. We found no main effect of the withdrawal for US expectancy rating (F(1,66.99)=0.945,p=0.334) or fear rating (F(1,67)=0.121,p=0.729) on day 2.

Statistic sex

We checked both US expectancy and fear ratings for an effect of sex of participants. Sex was included into the model (lmer (RatingResults~(1|participants) +stimuli*time*group+sex)). The group factor that is included in the model are smoker vs. non-smoker.

We found no main effect of sex for US expectancy rating for day 1 (F(1,198.9)=0.238,p=0.626) and day 2 (F(1,198.8)=0.027,p=0.871). We found a main effect of sex for the fear rating on day 1 (F(1,199.07)=4.094,p=0.044) with an increased fear rating by females, when compared to males (t(199)=2.023,p_{corr}=0.044). There was no main effect of sex on the fear rating on day 2 (F(1,199)=0.964,p=0.327).

Statistics reaction time

We checked US expectancy ratings on both days in regard of the participant's reaction time. We included reaction time as dependent variable into our model. Stimulus, block and group (smoker vs. non-smoker) were included as fixed effects into the model (lmer (reactiontime~ stimulus*block*group)). On day 1 we found a trend towards a main effect of stimulus (F(1,4383.5)=3.10,p=0.078), but follow up post-hoc tests showed no difference of reaction time between CS+ and CS-. Additionally we found a main effect of block (F(2,4384.90)=11.667,p<0.001). Follow up post-hoc tests revealed that subjects were rating faster over time (block 1–block 2: estimate=0.316,SE=0.0346,z-ratio=9.118,p_{corr}<0.001; block 2 – block 3:estimate=0.063,SE=0.0346,z-ratio=1.805,p_{corr}=0.071). On day 2 we found no differences regarding the reaction time.

Statistic STAI-T

We checked trait anxiety with the State-Trait Anxiety Inventory (STAI-T). An ANOVA showed no effect of group for the smoker manipulation groups (F(3,198)=0.805,p=0.492). An independent sample t-test revealed also no differences for smokers vs. non-smokers (t(200)=1.541,p=0.125).

Supplementary Material & Methods

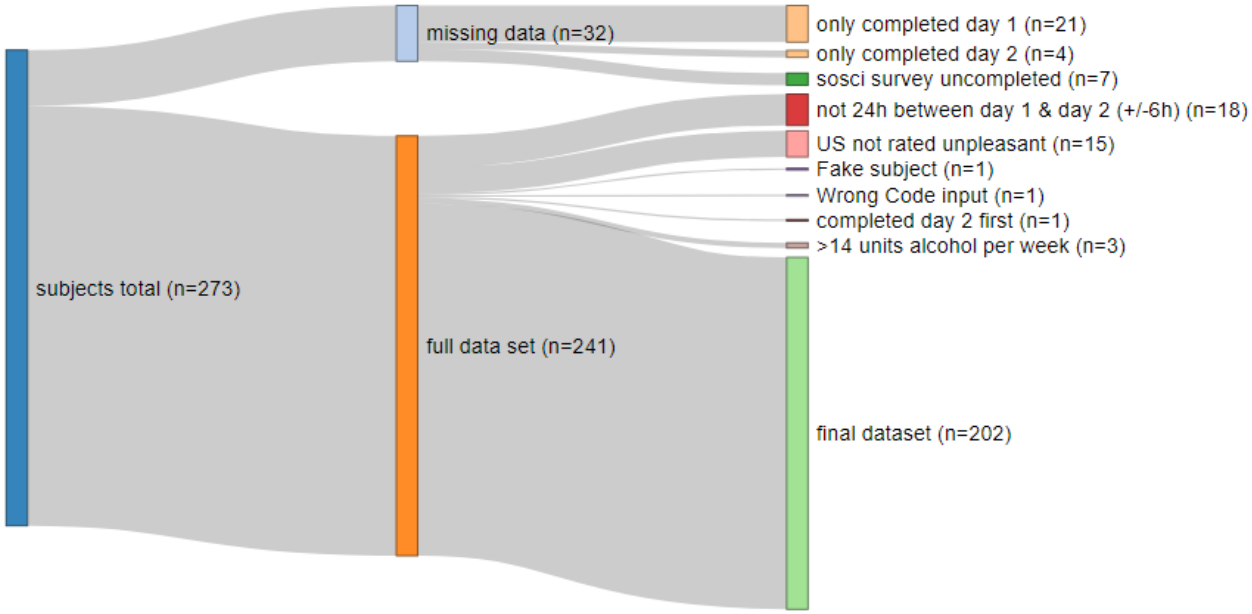

Figure S1: Participant exclusion. From the total of 273 subjects that had been recruited, 241 generated a full data set and finally 202 could be included into the final data set. Main reasons for exclusion by numbers of participants were missing data, participation outside of set time frame and participants that rated the US as not unpleasant.

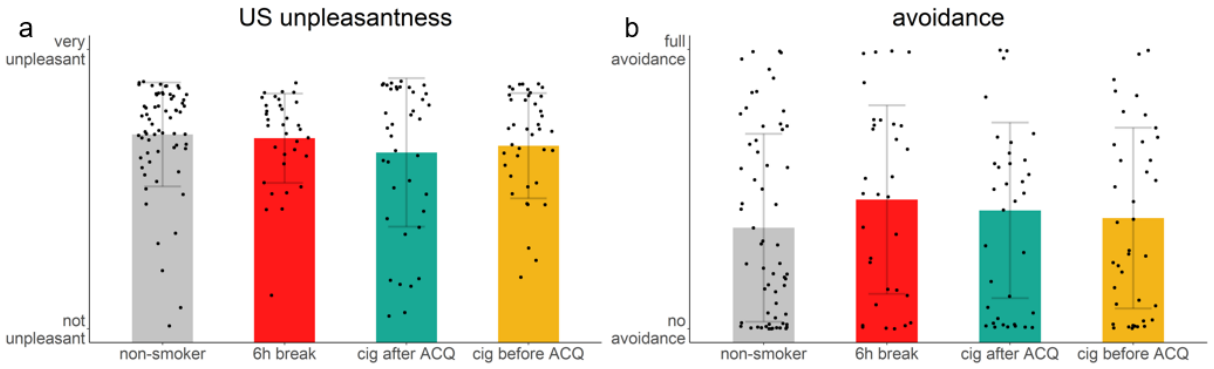

Figure S2: a) US unpleasantness per group. Individual representations in this figure indicate the difference between the mean of the three US and nUS ratings. Subjects with a difference ≤ 1 are already excluded. No difference between the experimental groups were found regarding their rating of US unpleasantness. b) US avoidance per group. Subjects were asked if they avoided to look at the screen when the US was presented on a scale from full avoidance to no avoidance. No difference between the experimental groups were found regarding their rating of US avoidance.

STUDY II

Nicotine reduces discrimination between threat and safety by reduction of hippocampal activations (in preparation)

Madeleine Mueller, Tahmine Fadai, Jonas Rauh & Jan Haaker

Nicotine reduces discrimination between threat and safety by reduction of hippocampal activations

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Abstract

Maladaptive fear learning is linked to the development of anxiety disorders. Nicotine has been shown to impair Fear Acquisition and Extinction in animals, but a detailed investigation of the causal pharmacological effect of acute nicotine on fear learning in humans is still lacking.
Therefore, we conducted this fMRI study to investigate the effect of acute nicotine on Fear Acquisition (ACQ) and a 24h subsequent Extinction training (EXT) with a Return of Fear (RoF) manipulation in healthy non-smokers (n=88). Participants were pseudo-randomly and double-blinded assigned to one of three experimental groups: 1. nicotine administration before Fear Acquisition, 2. nicotine administration before Extinction training or 3. placebo control group.
Our results show that nicotine administration before both ACQ and EXT lead to decreased discrimination between threat and safety in self-reported fear. This effect is mirrored by decreased differential hippocampal activation during ACQ in the group that received nicotine, when compared to placebo controls. Furthermore, we could identify a network (Hippocampus-Accumbens-ventral tegmental area (VTA)) that is typically active when novel stimuli are processed and stored in long-term memory to be impaired by nicotine administration.
Discrimination between threat and safety is essential for coping with a changing environment, but is impaired in patients suffering from anxiety disorders. Nicotine intake in healthy individuals results in a similarly impaired discrimination. Therefore we highlight nicotine as a risk factor for the development of pathological anxiety.

Introduction

A key mechanism for coping with a threatening environment is aversive learning. Adaptive aversive learning enables individuals to discriminate between what is dangerous and what is safe in their surroundings. Maladaptive learning is characterized by impaired discrimination between threats and safety, for example in individuals that are diagnosed with an anxiety disorder (AD)[1]. One risk factor for maladaptive aversive learning is smoking, as indicated in higher smoking rates in patients suffering from AD, when compared to healthy individuals[2]. Furthermore, a prospective longitudinal study found that smoking in healthy individuals results in a higher risk for the onset of a panic attack[3]. But the key question is how nicotine, the psychoactive ingredient in cigarette smoke, might interfere with aversive learning, leading to impaired discrimination between safety and danger.
One possibility is that nicotine impacts the storage of aversive learning in humans. In animals, there is evidence that nicotine affects learning and associative memory formation. On the cellular level, nicotine binds to β2-containing nicotinic acetylcholine receptors (nAChR)[4] and has a direct influence on synaptic plasticity such as hippocampal long term potentiation (LTP) [5,6]. The hippocampus is important to process new and salient information about threats and

storing memories that results from aversive learning[7]. Such aversive learning is commonly examined in laboratory protocols of fear conditioning. During Fear Acquisition a conditioned stimulus (CS+) is learned as a danger signal, based on the prediction for an aversive, unconditioned stimulus (US, e.g. an electric stimulus). Another conditioned stimulus (CS-) is learned as a safety signal and predictive for the absence of the US. Subjects learn to discriminate between CS+ and CS- and develop differential (i.e., higher for the CS+ as compared to the CS-) conditioned responses (CR).
Chronic nicotine intake, such as smoking seems to impair discrimination of threat and safety (but see [8]) in fear conditioning protocols. As such, a chronic schedule of nicotine administration in rats, and smoking in humans, has shown to decrease the discrimination between CS+ and CS- [9]. Similarly, experiments in rodents revealed that the discrimination between threat and safety (contexts) is dose-dependently impaired by acute nicotine administration [10]. Supporting this finding, acute infusion of nicotine into the dorsal HC disrupted safety learning in rodents [11]. These findings are related to results in humans, showing that smokers have an impaired discrimination between threat and safe contexts, when compared to non-smokers, indicated by lower discrimination in subjective fear, US expectancy and SCR [12]. A similar impairment was further found in smokers, which revealed less discrimination in self-reported fear towards learned threat and safety signals, compared to non-smoking individuals [13].
While smoking seems to impair discrimination of threat and safety, it is unclear if there is a causal pharmacological effect by which nicotine affects the discrimination of threat and safety. Such a pharmacological effect could aid to explain maladaptive learning that is observed in smokers. The main hypothesis of this study is therefore that acute nicotine reduces discrimination between threat and safety during aversive learning in humans.

Material & Methods

Participants

For this study 88 healthy, non-smoking participants between 18 and 40 years were recruited. Individuals confirmed to have no diagnoses of neuropsychiatric disorders, to consume less than 15 units of alcohol per week and no illegal drugs (see Table 1). Additionally subjects had to be suitable for fMRI measurements. All participants were non-smokers, which was defined by not being an active smoker during the time of the data acquisition and having smoked less than 200 cigarettes during their lifetime. Two participants had to be excluded from the analysis due to false statements, resulting the final sample to consist of 86 subjects (55.8% female). All participants gave written, informed consent to participate after an educational talk to an fMRI-physician and received 120€ reimbursement for completing both experimental days. The study was approved by the local ethics committee (Ethikkommission der Ärztekammer Hamburg PV 5514).

Groups

To test the effect of acute nicotine on Fear Acquisition and Extinction training, as well as the reinstatement test, we compared three groups. Group 1 (Nic1) received 1mg nicotine before Acquisition on day 1 and then placebo before the Extinction training on day 2. Accordingly group 2 (Nic2) received a placebo before the Acquisition on day 1 and then 1mg nicotine before the Extinction training on day 2. Group 3 (Pla) as the control group received placebo before both days. The study was double-blinded.

Table 1: Demographics per group. Nic1 received nicotine before Fear Acquisition, Nic2 received nicotine before Extinction training and the control group received placebos before both days.

	Nic1	Nic2	Pla
	Mean (sd)	Mean (sd)	Mean (sd)
Sample size	30	29	27
Age [years]	24.79 (4.31)	25.83 (4.77)	25.52 (4.20)
Gender	56.67 % female	55.17 % female	55.56 % female
Coffee consumption [cups/day]	0.89 (0.97)	1 (1.22)	0.67 (0.75)
Alcohol consumption [drinks/week]	1.62 (2.07)	0.9 (1.17)	2.04 (3.79)
STAI-T	40.2 (4.25)	40.41 (4.39)	43.44 (3.14)
STAI-S [day 1]	42.9 (3.74)	42.55 (3.84)	42.74 (3.99)
STAI-S [day 2]	43.13 (3.75)	42.67 (4.83)	43.2 (3.78)
Attention test - Day 1 [concentration performance score]	252.43 (46.06)	257.68 (38.88)	266.74 (38.31)
Attention test - Day 2 [concentration performance score]	272.03 (31.38)	263.25 (37.89)	273.24 (33.26)
Body Mass Index	23.25 (2.70)	23.67 (3.70)	22.92 (3.17)

Stimulus Material

We used a two day context-dependent cue conditioning paradigm. The context was a virtual room that was presented on a screen (Source Engine, Valve Corporation, Bellevue, USA [14]). Three different contexts were used, where context A and context B were both virtual offices but differed in their set-up and context C was a mixture between context A and context B. Each context was presented from two different viewpoints.

The cue was a coloured light that was illuminating the room in either yellow or blue (as described in [15]). One cue (CS+, duration of 6sec) was predictive for an aversive electric stimulus (US, 5.5sec after CS+ onset) that was presented on the right hand of the participant, whereas another cue (CS-, duration of 6sec) was not reinforced. Cues were counterbalanced. Context-presentations without cues were used as inter-trial intervals (ITIs, duration range between 7-11 sec). Visual stimuli were presented using Presentation® software (Version 20.3, Neurobehavioral Systems, Inc., Berkeley, CA, www.neurobs.com).

Unconditioned stimulus

The electrotactile stimulus that served as a US consisted of a train of 3 pulses, each with a duration of 2ms and an interval of 50ms. It was delivered via a surface electrode (Specialty Developments, Bexley, UK) on the right dorsal hand using a DS7A electrical stimulator (Digitimer, Welwyn Garden City, UK). The US intensity was individually adjusted prior to acquisition training to a threshold that was perceived as very unpleasant but not hurtful (mean=2.28mA, sd=4.09, min=0.4mA, max=37mA).

Experimental Procedure

The two-day paradigm (see Figure 1) consisted of a Fear Acquisition on the first day, followed approximately 24h later by an Extinction training with a Return of Fear manipulation in form of a reinstatement. Both experimental days were conducted in the fMRI scanner. Depending on the pseudo-randomly assigned group, either 1mg nicotine or 1mg placebo was administered

double-blinded 15min before participants were placed in the scanner and started the experiment.
The Acquisition was executed on the first day. Four trials of stimulus habituation without reinforcement were followed by 3 blocks with each 8 CS+ and 8 CS- presentations. The reinforcement rate for the CS+ was 75%. On the second day, 24 hours later, the extinction training and the subsequent reinstatement were executed. The extinction training consisted of two blocks with each 8 CS+ and 8 CS- presentations. The reinforcement rate was now 0%. Subsequently, the reinstatement took place, where four electric stimuli were presented without any context or cue information. Participants saw a black screen. That was followed by a reinstatement test with one block with 8 CS+ and 8 CS- presentations. Again the reinforcement rate was 0%. During Acquisition participants saw context A and during the extinction training participants saw context B. In the reinstatement test, participants saw a mixture of context A and context B.

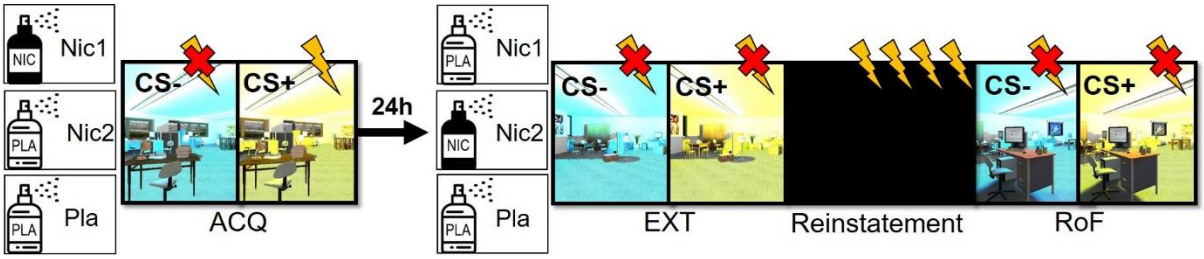

Figure 1: Experimental Procedure. The group Nic1 received nicotine before the Fear Acquisition on day 1 (ACQ), all remaining subjects received Placebo. After 24h the group Nic2 received nicotine and the remaining subjects received Placebo. Following that, subjects performed the Extinction training (EXT) with a subsequent Return of Fear (RoF) manipulation in form of a Reinstatement. CS-colours were counterbalanced.

Pharmacological intervention
Participants received 1mg nicotine as oral-spray (Nicorette® Spray, Johnson & Johnson GmbH) and 1mg placebo as oral-spray (St. Severin Cayenne Pepper Spray®, HECHT Pharma GmbH, 27432 Bremervörde), or placebo on both days. The dose was tested in a prior pilot study and proofed to be effective with little side effects. Typical side effects (dry mouth/skin, blurry vision, inertia, nausea, vertigo and headache) as well as self-reported side effects were documented after both experimental days. Participants rated their perception on a scale from 0 (not side effect) to 6 (extreme side effect). No difference of side effects were found between participants receiving nicotine and participants receiving a placebo ($mean_{Nic}$=0.34, sd_{Nic}=0.86; $mean_{Pla}$=0.25, sd_{Pla}=0.76).

Outcome measures
Ratings
Participants rated how much fear/stress they felt towards the CSs and the ITIs on a visual Analogue Scale [VAS, 0 (none) – 100 (maximally)] before and after the acquisition as well as before and after the extinction training and after the reinstatement test. Additionally subjects rated their US expectancy as a binary choice (yes/no) trialwise for all CSs on both experimental days.

SCR
Skin conductance responses were measured on the hypothenar on the left hand of the participant and recorded with a BIOPAC MP-100 amplifier (BIOPAC® Systems Inc, Goleta, California, USA). Raw data was scored manually. The response must begin between 1000ms and 4000ms after stimulus onset. The response should last between 0.5 s and 5 s. The amplitude of the response should be larger than 10 nS. Null-reactions were defined as responses taking place later than 4000ms after stimulus onset or when there was no response. A missing response was defined as a response that starts before 1000ms after stimulus onset

or if the signal quality was too bad to identify the response. Scored data was normalized for each day and participant (logarithmized and range-corrected).

fMRI scan sequence

MRI data were obtained on a 3T Magnetom-PRISMA System (Siemens, Erlangen, Germany) using a 64-channel head coil. fMRI measurements were performed using single-shot echo-planar imaging with parallel imaging (GRAPPA, in-plane acceleration factor 2) and simultaneous multi-slice acquisitions ("multiband", slice acceleration factor 2). Echo planar multiband images were acquired with 42 continuous axial slices (1.5 mm thickness, 0.5 mm gap) in aT2*-sensitive sequence (TR= 1493 ms, TE= 30 ms, flip angle = 60°, field of view = 225 × 225 mm_2). Moreover, high-resolution T1-weighted structural brain image (MP-RAGE sequence, 1 mm isotropic voxel size, 240 slices) were obtained.

ROIs fMRI

Regions of interest (ROI) were defined as key structures in emotional processing and fear, such as the Amygdala, the Hippocampus, Insular cortex, dACC and vmPFC, and dopaminergic innervated key structures, such as the Nucleus Accumbens and the ventral tegmental area (VTA/SN). These structures were defined by Harvard-Oxford probability maps for the Amygdala, the Hippocampus, the Insular cortex and the Nucleus Accumbens. For the dACC and vmPFC were no anatomical masks available, therefore we defined both ROIs as in previous studies. The vmPFC ROI was defined as a box of 20 × 16 × 16 mm at x=0 y=42 z=-12. The dACC ROI was defined as a box of 20 × 16 × 16 mm at x=0 y=28 z=26 [15]. The VTA/SN complex was defined by [16]. Correction for multiple comparisons within these ROIs was performed by using family-wise error correction based on the Gaussian Random Fields as implemented in SPM.

Questionnaires

Participants gave information on age, gender, alcohol and coffee consumption, as well as their smoking background. Furthermore, participants completed the State-Trait Anxiety Inventory (STAI-S/STAI-T). Questionnaires were filled out on www.soscisurvey.de [17]. To check if nicotine has a general effect on attention, participants completed the d2 Test of attention after each experimental day.

Sensitivity analyses

Sensitivity analyses calculated for a repeated-measures ANOVA (within and between subject interactions) for US expectancy indicated a sufficial sample size of 78 participants in total (26 per group) to detect an effect size = 0,229 and a critical F = 3.1186421 assuming a power (1-β error probability) of 0.95 and an α error probability of 0.05 (G*Power 3.1.9.4). Ten additional measurements of participants were conducted for the data acquisition, as the drop-out rate is quite high for pharmacological studies, as well as for fMRI studies. This concluded to a total of 88 participants after data acquisition.

Data Analysis

Rating/SCR. The analysis of the fear and US expectancy ratings, as well as the SCR was calculated using linear mixed effect models in R with the lme4 package [18]. As preregistered, we focused the analysis on the last Block/post Rating of the ACQ and EXT. The dependent variable in the models were the ratings, or respectively the SCR. Additionally we implemented random intercepts and slope for subjects. To test the paradigm, we analysed the interaction between stimuli-types (CS+/CS-) in the post rating for Fear Ratings and during the last block for US expectancy ratings/SCR and the groups (ACQ: placebo (Nic2 and Pla) /nicotine (Nic1); EXT: nicotine before day 1 (Nic1)/ nicotine before day 2 (Nic2)/ placebo (Pla)): RatingResults/SCR~(1|participants)+stimulus*group). The Return of Fear analysis was performed similarly to the EXT, but we included the last three trials of the EXT, as well as the

first three trials after reinstatement as blocks (model: lmer(rating~(1|sub)+stim*block*group (2 stimuli (CS+/CS-), 2 blocks (US expectancy and SCR: block 1: 3 trials before reinstatement, block 2: 3 trials after reinstatement; Fear Ratings: block 1: post EXT rating, block 2: post RoF rating), 3 groups (nic1/nic2/pla)). To further investigate group effects across outcome measures, we used the same model, but the stimulus discrimination (CS+ - CS-) was calculated as dependent variable: StimulusDifference~(1|participants)+group (additional *block in RoF analysis). Regarding the differential post Fear Ratings, we used jasp to calculate an ANOVA with the dependent variable of the stimulus difference and calculated follow up post-hoc tests. Additional analyses of the other blocks/pre Ratings we performed accordingly.

fMRI. Preprocessing and statistical analysis of functional MRI data was carried out using SPM12 (Statistical Parametric Mapping, http://www.fil.ion.ucl.ac.uk/spm) running under Matlab2021b (The MathWorks, Inc., Natick, Massachusetts, United States). Before preprocessing, the first five volumes of each time series were discarded to account for T1 equilibrium effects. Remaining images were unwarped, realigned to the first image, coregistered to the individual high resolution T1 structural image, normalized (using DARTEL) and smoothed. Following statistical analyses were performed by using a general linear model (GLM) at the single-subject level as standard approach for fMRI implemented in the SPM software. Experimental conditions (i.e., CS+, CS−, (omitted) US, introductions, ratings, and button presses) were defined as separate regressors modelling the predicted time courses of experimentally induced brain activation changes as a stick function. Subsequently a full-factorial analysis was calculated on the group level. As preregistered, we focused our analyses on the last block of ACQ and EXT, but added analyses of the other blocks. Additional analyses on both days were performed in which the US expectancy of each participant was used as parametric modulator (i.e., expectation of no US > expectation of a US).

Connectivity analyses. To investigate functional connectivity differences towards the US between groups, we employed psycho-physiological interactions (PPI, SPM12 standard approach) for the whole ACQ. As seed region served the VTA/SN as described in our ROIs. We then used the PPIs of each participant as regressor in an individual GLM, including movement as regressor. Finally, calculated estimates we contrasted on group level.

Results

Acquisition

Paradigm

To show that participants discriminated between CS+ and CS- at the end of Acquisition training (last block/post Rating; pre-registered contrast), we examined Fear Ratings, US expectancy and SCR towards the CS+, when compared to the CS- across all groups.

A stimulus discrimination after the ACQ was found in all three outcome measures. Specifically, we found a stimulus main effect in the Fear Ratings post ACQ ($F(1,83)=23.65$, $p<0.001$), where participants rated higher fear towards the CS+, compared to the CS- ($t(83)=9.59$, $p_{corr}<0.001$). Similarly, analysis of US expectancy ratings during the last block revealed a stimulus main effect ($F(1,1246.6)=334.18$, $p<0.001$) with a higher US expectancy towards the CS+, when compared to the CS- ($t(1247)=28.97$, $p_{corr}<0.001$). This stimulus effect was also found in SCRs during the last block ($F(1,928)=12.049$, $p<0.001$), with higher SCR towards the CS+ when compared to the CS- ($t(928)=3.159$, $p_{corr}=0.002$). Analyses regarding the first/middle block can be found in the supplement.

Our predefined regions of interest (ROI) for fMRI-data analysis also revealed CS-discrimination, indicated by higher hemodynamic responses to the CS+, as compared to the CS- during the last block of the ACQ within the bilateral Insula (left: MNI xyz: -32,20,4; $T=9.32$, $p_{FWE}<0.001$; right: MNI xyz: 39,16,2; $T=9.11$, $p_{FWE}<0.001$), dACC (MNI xyz: 3,22,27; $T=6.47$,

p_{FWE}<0.001), left Ncl. Accumbens (MNI xyz: -14,15,-6; T=3.37, p_{FWE}=0.009), as well as in the VTA/SN (MNI xyz: 12,-22,-10; T=5.02, p_{FWE}<0.001) across all groups.

Group effects

In order to test if acute nicotine administration reduces CS-discrimination at the end of Fear acquisition training, we compared CS-responses between the group that received nicotine (nic1) with individuals that received placebo on day 1 (pla). Our preregistered analysed focused on the last block during acquisition training (8 trials of each CS), based on our pilot data.

Fear Rating

According to our hypothesis, we found a trend towards a stimulus*group interaction (F(1,83)=3.112, p=0.081), which consisted of a trend-wise lower differential Fear rating (CS+>CS-) after the acquisition training in the group that received nicotine, when compared to the placebo group (t(83)=1.764, p_{corr}=0.081). This effect was driven by lower fear ratings towards the CS+ in the group that received nicotine, compared to the placebo group (t(164)=14.29, p_{corr}=0.024; see Figure 2). There was no group effect in the analysis of the Fear Ratings before acquisition training (F(1,83)=0.043, p=0.836).

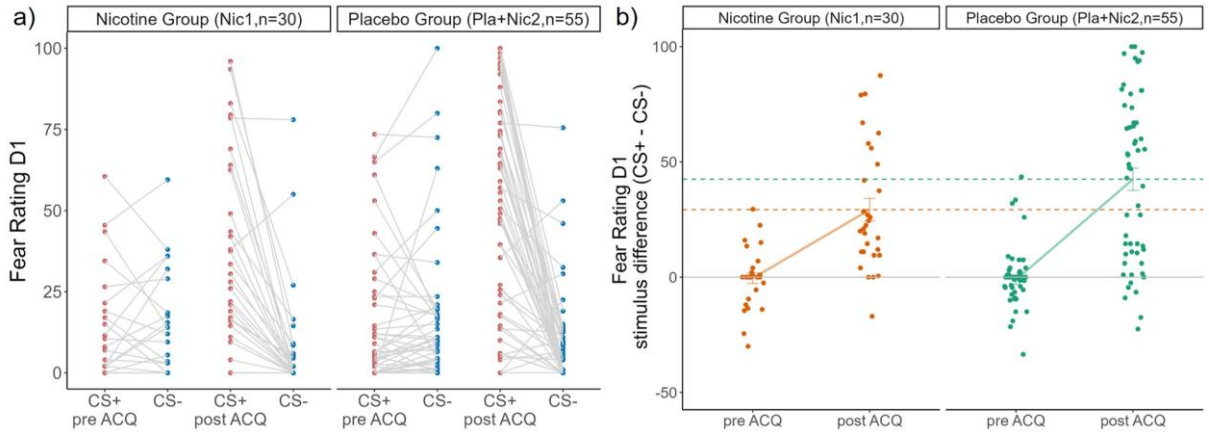

Figure 2: Weaker stimulus discrimination after ACQ in the group that received nicotine (nic1), when compared to the Placebo group in Fear Ratings. a) Individual Fear Ratings pre and post ACQ per group. b) Differential Fear Ratings (CS+ - CS-) per group. Single subject responses are shown as scatterpoints, mean differential Fear ratings are depicted as lines with standard error. Dashed lines represent the mean differential Fear Ratings per group post ACQ.

US expectancy

We found no group effect on US expectancy in the last block of the ACQ (F(1,83)= 0.032, p=0.859; Figure S1a).

SCR

We found a stimulus*group interaction (F(1,928)=6.182, p=0.013, Figure S3), with higher differential skin conductance response in the group that received nicotine, when compared to the placebo group (t(60.1)=2.292, p_{corr}=0.025; see figure S3a). This enhancement in differentiation by nicotine was contrary to our pre-registered hypothesis and comparison of CS-specific responses between groups revealed no differences.

fMRI

First, we aimed to delineate the neural effect of reduced CS-differentiation by nicotine on within our ROIs, as already indicated by the reduced differentiation in Fear Ratings. This analysis revealed reduced differential (CS+ > CS-) responses in the left (and trend-wise in the right) hippocampus in the group that received nicotine, compared to the Placebo group (left HC MNI xyz: -27,-39,-4; T=3.85, p_{FWE}=0.012; right HC (MNI xyz: 28,-9,-28; T=3.18, p_{FWE}=0.089; see Figure 3). This reduction in discriminatory responses between the CS+ and the CS- in the hippocampus thereby mirrored the reduced discrimination in rated fear in the group that

received nicotine, compared to Placebo controls. The inverse contrast (i.e., enhanced discriminatory responses in the Nicotine, vs. the Placebo group) revealed no voxel within our ROIs. An exploratory follow-up correlation between each individual estimate for the HC activation (CS+>CS-) and the Fear ratings post ACQ and pre EXT showed no significant results for either group.

In a second, exploratory analysis, we compared the differential responses (CS+>CS-) between groups in the first and second block of ACQ. We found a similar decrease in the differential activation of the left HC (left HC: MNI xyz: -26,-33,-10; T=3.55, p_{FWE}=0.030) and trendwise in the right HC (right HC: MNI xyz: 27,-30,-9; T=3.36, p_{FWE}=0.054) in the group that received nicotine, compared to the placebo group the first block (see table S1). Interestingly, we additionally found decreased differential responses in the left AMY in the group that received nicotine, compared to the placebo group in the first block of the ACQ (left AMY: MNI xyz: -24,0,-22; T=3.68, p_{FWE}=0.009) and in the second block (left AMY: MNI xyz: -32,0,-18; T=3.08, p_{FWE}=0.049; see table S2). We also found a similar effect in the left Ncl. Accumbens (MNI xyz: -8,6,-8; T=3.10, p_{FWE}=0.020). As illustrated in Figure 5, the differentiation between CS+ and CS is reflected in the hippocampal, amygdala and Ncl. Accumbens responses within the Placebo group, but is impaired after administration of nicotine.

Parametric Modulations. In order to follow the individual learning of threat contingencies, we entered individual US expectancy ratings (if US expected: 1, if US not expected: -1) as a modulator for the activity towards the CS+. Hence, the activation in this analysis would reflect responses in a brain region that is responsive when an individual expects a US during CS+ presentation (this analysis was similar to the preregistered analysis for the EXT, including the whole ACQ time-course). Here, we found that the activity in SN/VTA was following the timecourse of the US expectancy in the group that received nicotine, whereas responses in the SN/VTA decreased in the placebo group within increasing US expectancy (MNI xyz: -10,-21,-12; T=4.74, p_{FWE}<0.001; see Figure 4). In other words, the activity in the Placebo group decreased with increasing expectancy in the SN/VTA, whereas nicotine administration led to constant responses when participants expected a US.

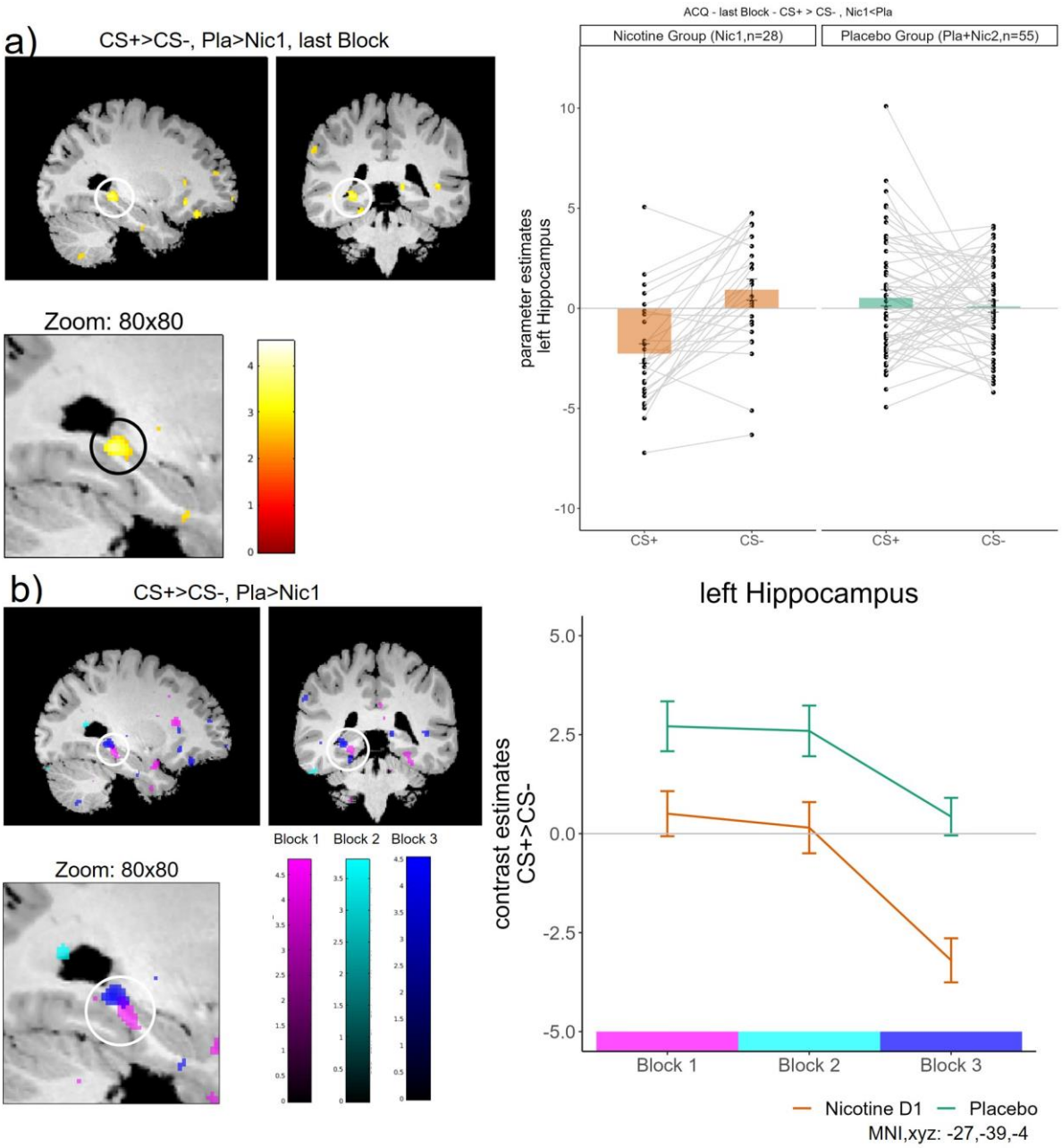

Figure 3: Acute nicotine administration, compared to placebo, reduces differential responses to the CS+ and the CS- during a) the last block of ACQ in the left Hippocampus. Scatterpoints represent single subject parameter estimates to each CS. Bars represent means across each group with standard error. We found the same effect represented in the Fear ratings. b) This effect is robust over all three blocks of the ACQ in the left HC.

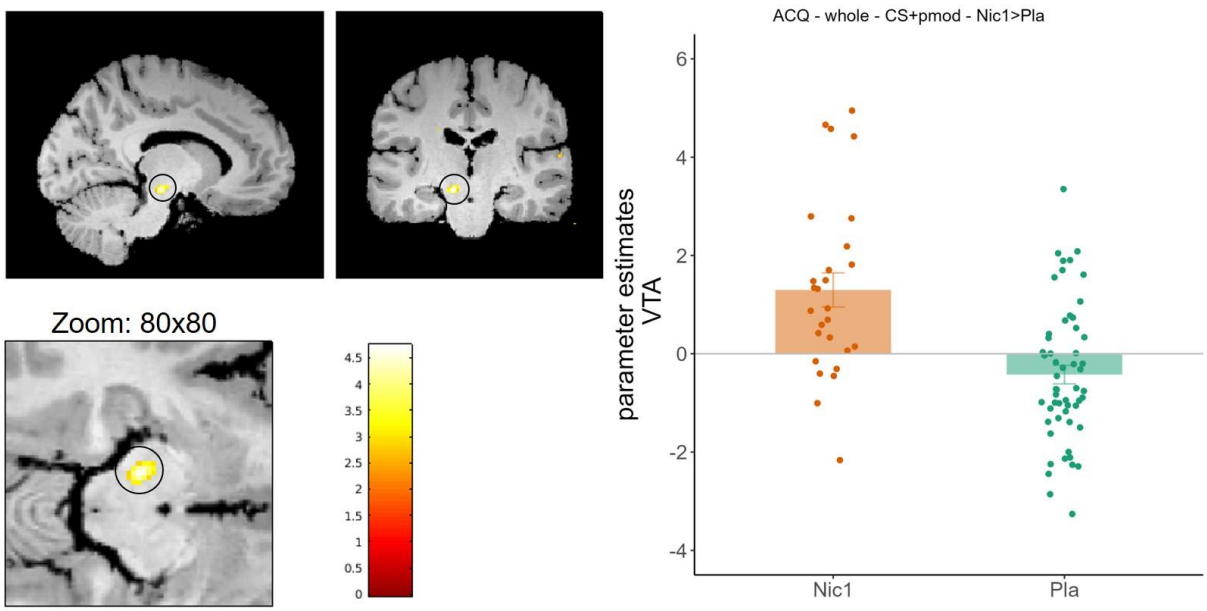

Figure 4: Stronger activation in the VTA in the Nicotine group, when subjects expected the US, when compared to the Placebo group over the whole ACQ. Scatterpoints represent single subject contrast estimates in the VTA, bars represent means with standard error.

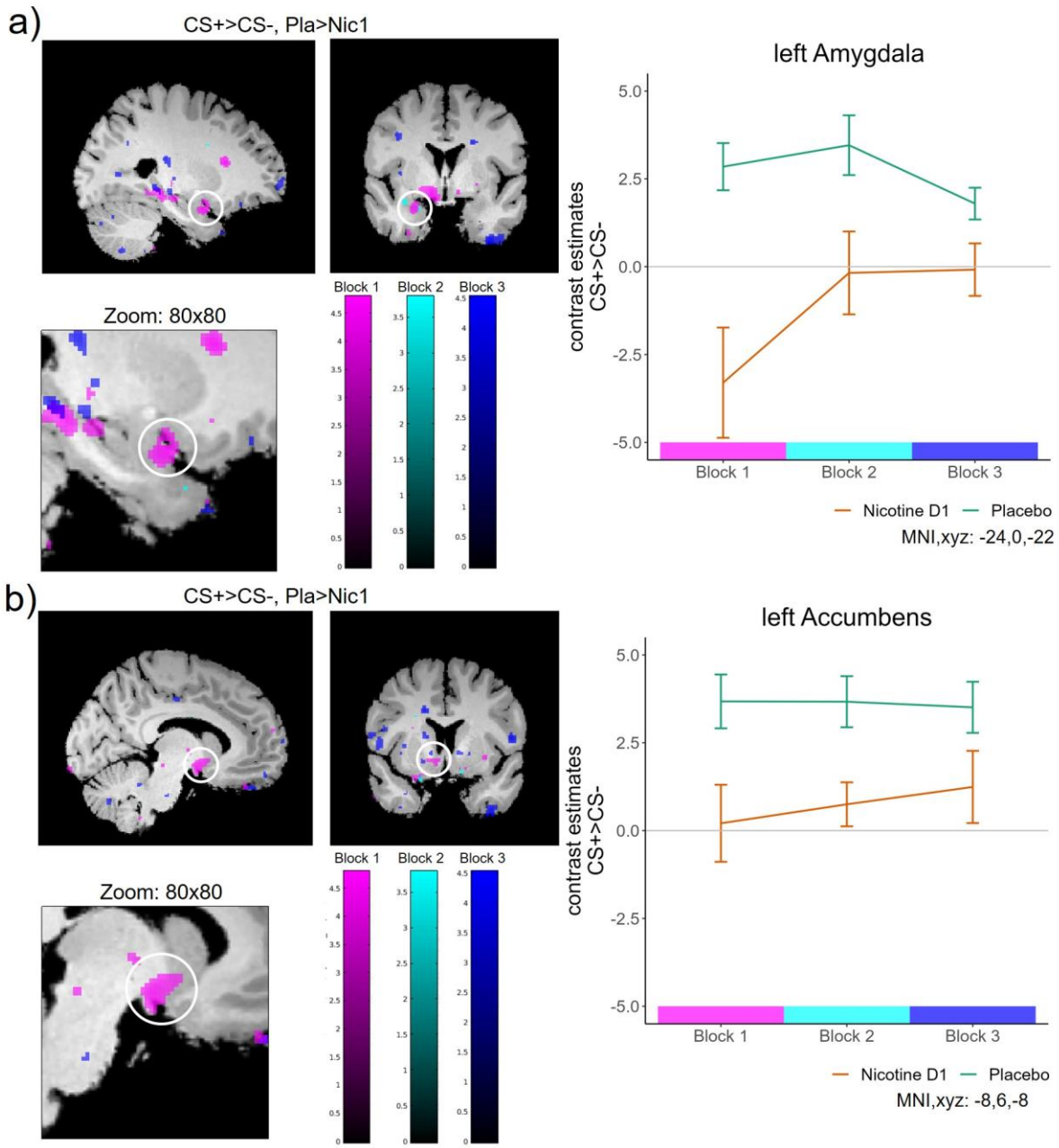

Figure 5: Stimulus discrimination during Fear Acquisition, fMRI results over all blocks in the a) left AMY and b) left Acc. The group that received nicotine (Nic1) shows a lower activity in the ROIs over time, when compared to the Placebo group.

US

In order to explore, if acute nicotine administration not only reduced activation that are reflecting the differentiation between learned threat (i.e., CS+) and safety (i.e., CS-) signals, but also activation to aversive outcomes, we compared response estimates to the US. We found decreased activations in multiple ROIs in the nicotine, compared to the placebo group (Nic1<Pla) to the US in the (whole) ACQ, such as the bilateral HC (left HC: MNI xyz: -27,-33,-12; T=4.01, p_{FWE}=0.009, right HC: MNI xyz: 24,-26,-12; T=4.65, p_{FWE}=0.001), bilateral INS (left INS: MNI xyz: -39,-2,3; T=4.25, p_{FWE}=0.005; right INS: MNI xyz: 36,8,3; T=3.27, p_{FWE}=0.084), bilateral Ncl. Accumbens (left Ncl. Acc: MNI xyz: -6,12,-4; T=3.5, p_{FWE}=0.007; right Ncl. Acc: MNI xyz: 12,14,-8; T=3.15, p_{FWE}=0.016), VTA (MNI xyz: 10,-24,-16; T=3.30, p_{FWE}=0.033) and trendwise in the dACC (MNI xyz: 2,24,18; T=3.48, p_{FWE}=0.055) (Figure 6). Hence, nicotine administration reduced neural responses to the aversive outcome, which are thought to drive learning of threat signals. Importantly, we found no group difference in the US valence ratings

(rated after the last ACQ block (t(81)=-0.239, p=0.811), which thereby speaks against a general devaluation of the US by nicotine administration.
Additional exploratory Pearson correlations to investigate the relationship between the neural US response and differential Fear Ratings post ACQ showed no effects in both groups.

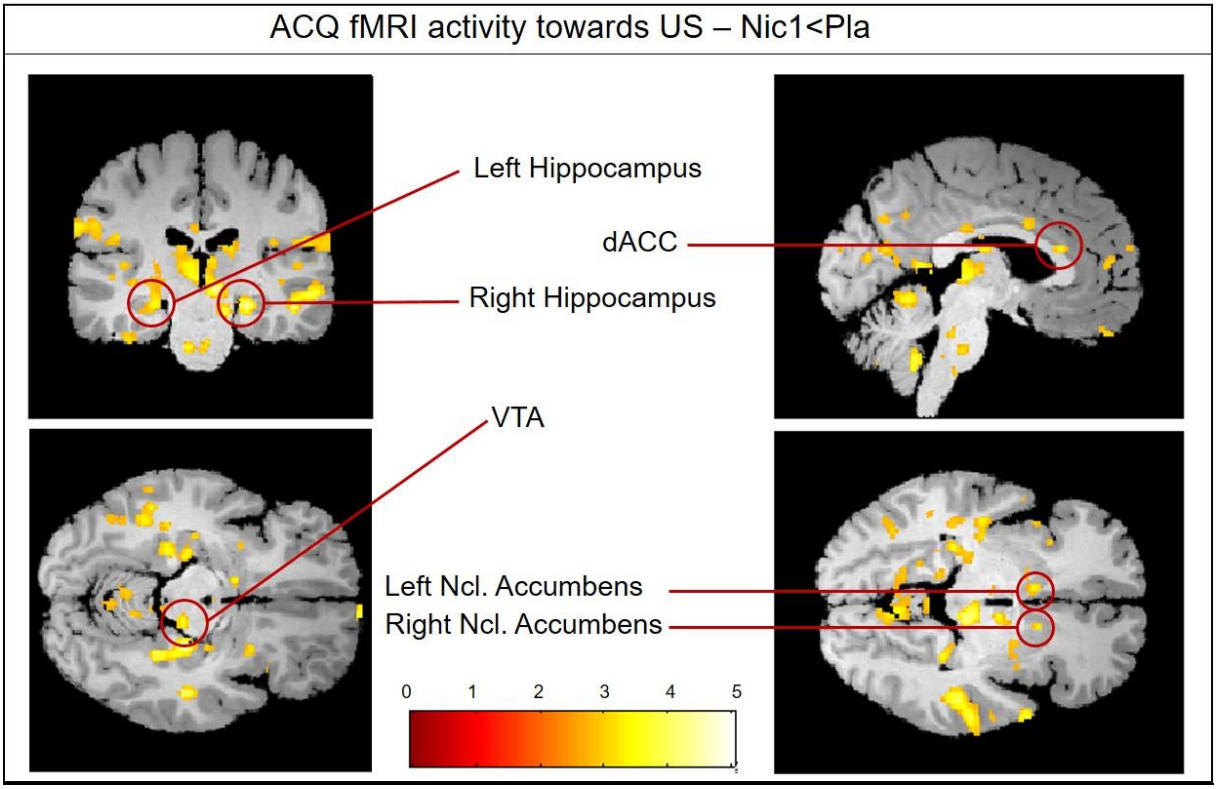

Figure 6: The Nicotine group showed decreased activation, when confronted with the US over the whole ACQ in multiple ROIs, as compared to the Placebo group.

PPI

To further investigate the findings of multiple ROIs being linked to decreased activation towards the US in the nicotine group, we employed a functional connectivity analyses in form of a psycho-physiological interaction (PPI). Our PPI analyses with the VTA/SN as a seed region was calculated for the whole ACQ. We found decreased functional connectivity in the group that received nicotine before ACQ towards the left Hippocampus (MNI xyz: -30,-9,-22; T=2.67, p_{FWE}=0.005), as well as in the bilateral Ncl. Accumbens (left Acc: MNI xyz: -8,6,-9; T=3.31, p_{FWE}=0.001; right Acc: MNI xyz: 10,12,-10; T=2.72, p_{FWE}=0.004).

Extinction

Paradigm

On day 2, we still found CS-discrimination at the end of extinction across groups, indicated by higher Fear Ratings, US expectancy and trend-wise higher SCRs towards the CS+, when compared to the CS- (Fear Rating post EXT: stimulus effect F(1,79)=8.795, p=0.004; CS+>CS-: t(79)=2.811, p_{corr} =0.006; US expectancy last Block EXT: (F(1,1219)=84.176, p<0.001; CS+>CS-: t(1219)=12.203, p_{corr}<0.001; SCRs last Block EXT: F(1,717)=3.065, p=0.080, CS+>CS-: no difference in post-hoc tests; for results of the first block, see Supplement) . The differential CS responses were furthermore reflected in the fMRI-data, were we found increased activation in multiple ROIs in the differential stimulus contrast CS+>CS- (Table 2) across all groups.

Table 2: Extinction training (last Block) fMRI-results of ROIs of the whole sample.

EXT – last Block	CS+>CS-		
	T	P(FWE)	MNI, xyz
Left HC	3.30	0.070	-32,-15,-21
Left INS	6.44	<0.001	-42,12,0
Right INS	4.98	<0.001	34,22,-3
dACC	4.01	0.012	-3,21,24
vmPFC	4.24	0.006	8,50,-20
VTA/SN	3.03	0.067	-8,-24,-14

Group effects

In order to examine if prior nicotine administration during acquisition training (Nic1), as well as acute nicotine administration during extinction (Nic2) resulted in diminished CS-discrimination, as compared to placebo (Pla), we compared responses between all three groups (Nic1/Nic2/Pla) in the last EXT block (i.e., eight trials; see preregistered analysis/methods).

<u>Fear Rating</u>

Against our hypotheses, we found no effect of nicotine on the differential Fear Ratings at the end of EXT. However, our secondary analyses revealed a stimulus*group interaction before the EXT ($F(2,79)=4.949$, $p=0.009$) with higher differential Fear rating of the placebo group before the extinction, when compared to both nicotine groups (pla-nic1: $t(52)=2.734$, $p_{corr}=0.009$; pla-nic2: $t(50)=2.472$, $p_{corr}=0.017$). Hence, these group differences suggest that acute nicotine during extinction, as well as prior nicotine during acquisition of threat responses, reduces the memory retrieval that allows the differentiation between threat and safety cues (i.e., CS+ and CS-, respectively). See Figure 7.

Additionally, our analysis revealed a main effect of acute nicotine administration on extinction of threat responses ($F(2,124.48)=4.465$, $p=0.0134$), indicating trendwise increased Fear Ratings across both CSs in the group that received acute nicotine before extinction (nic2), when compared to the placebo group ($t(79)=2.237$, $p_{corr}=0.084$).

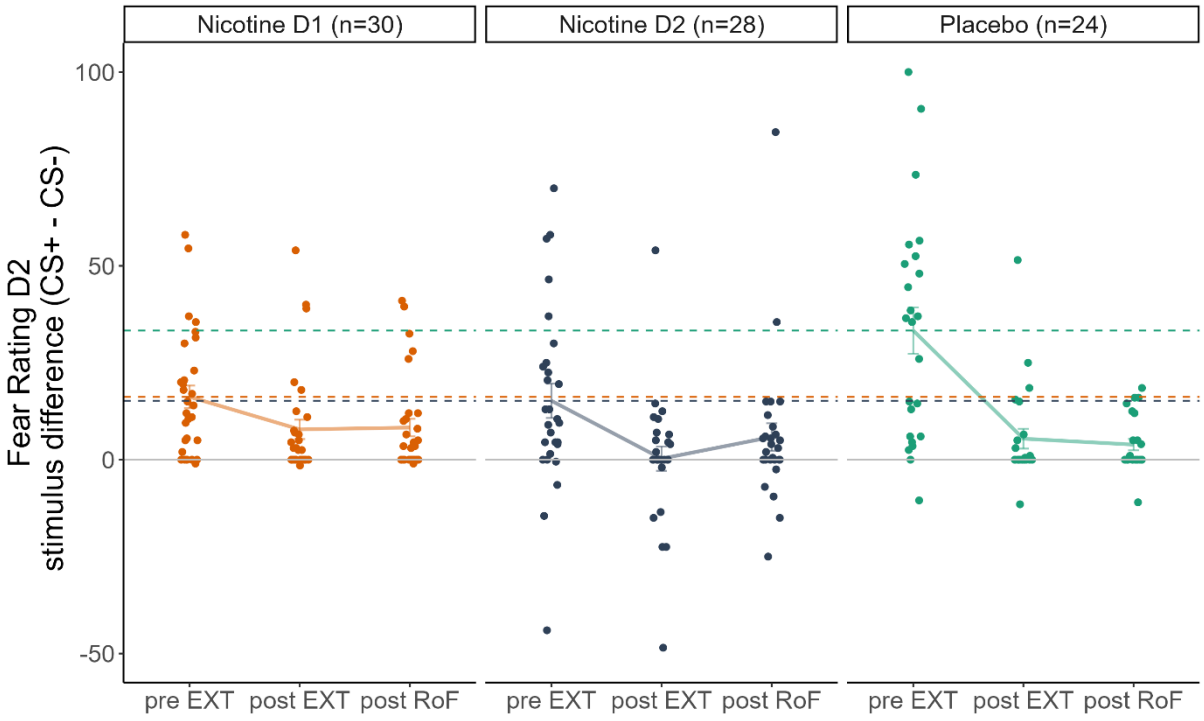

Figure 7: Nicotine (administered during ACQ or during EXT) weakened discrimination between CS+ and CS- in Fear Ratings. Differential Fear Ratings on day 2 per group illustrate reduced differential fear in both nicotine groups (i.e., Nic1 received nicotine during acquisition training; nic2 received nicotine before EXT), compared to the placebo group. No group differences in the differential Fear Ratings were found after the EXT. For Fear Ratings D2 both stimuli per group, see Figure S2. Single subject responses are shown as scatterpoints, mean differential Fear Ratings are depicted as lines with standard error.

US expectancy

We found a stimulus*group interaction ($F(2,1219.09)=4.656$, $p=0.01$, see Figure S1b), but post-hoc comparisons of CS specific responses between groups revealed no differences.
Furthermore, we found no such group effect on US expectancy in the memory retrieval during the first block of EXT ($F(2,1213.15)=1.632$, $p= 0.196$).

SCR

The analysis of SCRs revealed a stimulus*group interaction ($F(2,717)=4.922$, $p=0.008$, Figure S3b) in the last block of the EXT, which consisted of higher SCR towards the CS+ in the group of nicotine administration during acquisition (Nic1), when compared to the placebo group (Nic1 – pla: $t(70)=2.695$, $p_{corr}=0.026$; and compared to acute nicotine administration Nic1 – Nic2: $t(70)=2.359$, $p_{corr}=0.042$). These results are against our hypothesis, but mirror the SCR results from day 1. We found no effects of nicotine administration on SCR during memory retrieval in the first block.

fMRI

Next, we aimed to examine the effect of prior nicotine during acquisition (Nic1) and acute nicotine administration during extinction (Nic2) on hemodynamic responses during extinction training (last block, as preregistered), both compared against placebo (Pla).
Similar to our findings from acquisition training, this analysis revealed that nicotine administration during acquisition of threat responses (Nic1), still led to reduced differential responses (CS+ > CS-) in the left HC during the last block of EXT (MNI xyz: -21-34,-6; $T=3.94$, $p_{FWE}=0.010$), when compared to the placebo group. This effect mirrors the effect of nicotine on patterns of hippocampal activity, as well as the Fear ratings, during acquisition of threat responses. Additionally, we found that nicotine during acquisition reduced differential responses in extinction training within the bilateral INS (left: MNI xyz: -39,-3,0; $T=4.60$, $p_{FWE}=0.001$; right INS: MNI xyz: 42,0,-4; $T=3.82$, $p_{FWE}=0.012$) and right Ncl. Accumbens (MNI xyz: 8,12,-9; $T=2.85$, $p_{FWE}=0.036$), when compared to the placebo group.
The acute effect of nicotine on extinction training was reflected by reduced differential responses in the left INS (MNI xyz: -39,-2,-2; $T=3.75$, $p_{FWE}=0.021$; see table 3 and figure S4), as compared to placebo controls.
Exploratory analysis of the first block of the EXT, indicated a trendwise reduced discrimination after acute nicotine administration (Nic2), as compared to placebo in the left Ncl. Accumbens (MNI xyz: -9,10,-12; $T=2.67$, $p_{FWE}=0.065$; figure 8). This effect mirrors the reduced discrimination in Fear ratings in this group.

Table 3: Extinction training (last Block) fMRI-results of ROIs. Group comparisons.

EXT – last block	Nic1>Pla		
	T	P(FWE)	MNI, xyz
Left AMY	3.40	0.024	-30,0,-22
Right HC	3.27	0.078	27,-14,-20
	Nic1<Pla		
dACC	3.44	0.062	-6,33,28
vmPFC	4.05	0.010	8,42,-20
	Nic2>Pla (like Fear Rating)		
Left INS	3.65	0.029	-36,-10,9
	(CS+>CS-, Nic1&Nic2) < (CS+>CS-, Pla)		

Left HC	3.20	0.092	-20,-38,-3
Right HC	3.18	0.099	22,-30,-8
Left INS	4.64	0.001	-39,-3,0
Right INS	3.28	0.083	38,-2,-2
	(CS+>CS-, Nic1) < (CS+>CS-, Pla)		
Left HC	3.94	0.010	-21,-34,-6
Left INS	4.60	0.001	-39,-3,0
Right INS	3.82	0.012	42,0,-4
Right Ncl. Acc	2.85	0.036	8,12,-9
	(CS+>CS-, Nic2) < (CS+>CS-, Pla)		
Left INS	3.75	0.021	-39,-2,-2

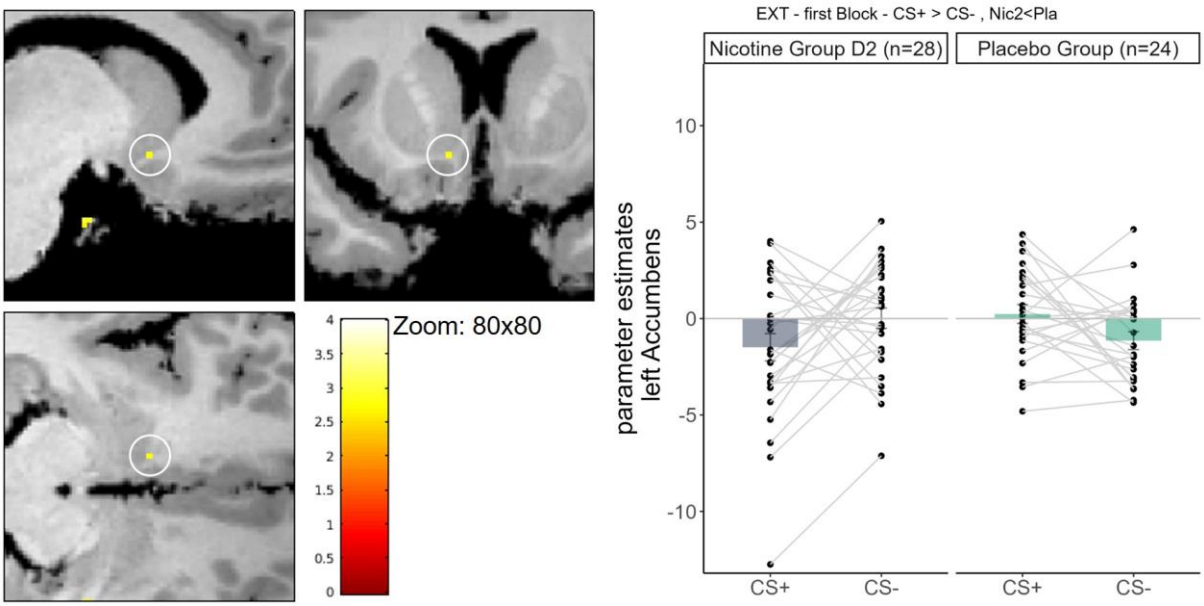

Figure 8: Stronger discrimination in left Ncl. Accumbens in placebo group, when compared to Nic2. Bars represent means across each group with standard error.

Return of Fear

The Return of Fear was employed in this experiment by an application of reinstatement US after extinction training, which was followed by CS presentation in a context that was a mixture of the acquisition and extinction context. We expected an increase of Fear Ratings, US expectancy and SCR following the reinstatement.

The analysis of paradigm confirmed that US expectancy increased after reinstatement, when compared to before reinstatement. US expectancy analyses indicated a stimulus by block interaction ($F(1,877.29)=3.895$, $p=0.049$). We found that both stimuli showed an increase in US expectancy after the reinstatement (CS+, pre-post reinstatement: $t(878)=6.363$, $p_{corr}<0.001$; CS-, pre-post reinstatement: $t(878)=2.049$, $p_{corr}=0.041$). Comparing the stimulus discrimination before and after reinstatement (CS+>CS-, RoF>EXT), we found a trendwise effect in the SCR analysis, but no effect in Fear Ratings or fMRI (for details see Supplement).

The analysis of group effects revealed only an effect in the Fear Ratings. Here, we found a block by group interaction ($F(2,79)=3.558$, $p=0.033$), which consists of an increase in differential Fear Ratings in Nic2 after the RoF, when compared to after the EXT ($t(79)=2.945$, $p_{corr}=0.013$). When testing which stimulus was crucial for this effect, we found that the Fear Ratings after the RoF in Nic2 towards the CS+ are higher, compared to the CS- ($t(237)=5.857$, $p_{corr}=0.044$). There was no effect in Nic1 ($t(79)=0.255$, $p_{corr}=0.8$) or Pla ($t(79)=-0.742$, $p_{corr}=0.921$). For a detailed report see Supplement.

Discussion

Our study departed with the hypothesis that nicotine leads to decreased discrimination between danger and safety stimuli, when compared to the Placebo group. Our analyses of the Fear Ratings and fMRI results confirmed our hypotheses for both, acquisition and extinction of conditioned threats. During Fear Acquisition, we found a decreased discrimination of rated fear in the group that received nicotine, when compared to the placebo group. Reflecting the Fear Ratings, the nicotine group also showed a lower differential (CS+ > CS-) hippocampal activation than the placebo group. Nicotine administration before Fear Acquisition further led to a decreased discrimination in subjective fear between the CS+ and CS- during memory retrieval within the early Extinction training on day 2. Furthermore, the deficit in threat and safety discrimination in hippocampal activity by nicotine administration during ACQ was still evident during the end of Extinction training, when compared to the placebo group. In striking similarity to these effects, acute nicotine administration before Extinction training also resulted in decreased differential fear ratings between the CS+ and CS-, accompanied by decreased differentiation in Insular activity. The Return of Fear manipulation turned out to be relatively robust against nicotine effects over outcome measures.

There is strong evidence from rodent and human studies that chronic nicotine disrupts discrimination of threat and safety learning[9,10,13]. Here we could show that already a small dose of acute nicotine leads to similar changes in human learning processes. The Hippocampus has been found to play a crucial role in fear learning and extinction[19]. Our results highlighted decreased differential hippocampal activation after nicotine administration, which is leading to maladaptive discriminatory learning.

The effect of nicotine was not restricted to changes in neural activity to the conditioned cues, but also to the US. Nicotine administration decreased neural activity in multiple ROIs towards the US, which however does not simply represent a dampening effect on the aversiveness, since the valence ratings towards the US did not differ between groups. Instead, we found that nicotine administration reduced activation and connectivity of a network consisting of a hippocampal-Accumbens-VTA loop. This network is thought to process novelty-dependent input of information into long-term memory[20]. Furthermore, the VTA of participants that received nicotine before ACQ showed increased activation towards the CS+, when participants expected the US, compared to placebo controls, but US expectancy did not differ between groups. Assuming that this mechanism is impaired by nicotine, it would mean that salient new information about CS-US contingencies is restrained to enter long-term memory. This may therefore lead to impaired discrimination between threat and safety, reflected in subjective fear and hippocampal activity. This impairment in discrimination is thought to reflect clinically relevant maladaptive aversive learning, as the same effect is found in patients with Anxiety disorders (AD)[21]. In line with the impairments induced by nicotine are findings in patients suffering from post-traumatic stress disorder (PTSD), which showed decreased hippocampal activation during extinction recall, when compared to healthy controls[22].

The analysis of the US expectancy ratings, which rather reflects cognitive understanding of the paradigm, was not effected by nicotine on either day, in line with a prior study. Analyses of skin conductance responses on the other hand revealed group effects that were contradictory to our hypotheses. We found a stronger stimulus discrimination in the group that received nicotine on day 1 during the last block in both ACQ and EXT then in the placebo group. As we find this effect only in the last block, nicotine does not simply increase skin conductance, but seems to lead to decreased habituation over time. These results were not reflected by any other outcome measures.

The limitations of our study include that every participant regardless of their body weight received the same dose of nicotine (1mg) for practical reasons. Nevertheless, we recruited participants with average BMI, which did not differ between groups. Additionally, we could only rely on self-reported non-smoking status, as well as drug and psychiatric disorder history. But

we informed participants on a possible drug test before the experiment and the subsequent exclusion in case of a positive result.

In conclusion, our study gave deep insights into the effect of nicotine on fear learning and extinction processes in humans, which has been a lack of knowledge to this point. We found that nicotine impairs the acquisition of novel information, such as the discrimination between threat and safety and its subsequent storage in the long term memory. This is likely due to decreased activation in the VTA and its network including the Ncl. Accumbens and importantly, the Hippocampus after nicotine administration. We found this to be reflected in self-reported fear. This study confirms that nicotine administration leads to maladaptive aversive learning in humans and provides new evidence of smoking as risk factor for the development of pathological anxiety.

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Supplement
Nicotine reduces discrimination between threat and safety by reduction of hippocampal activations

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Results
Acquisition
Analysis of the first and second block during ACQ
Additional analysis to our preregistered findings included the examination of Fear Ratings, US expectancy and SCR towards the CS+, when compared to the CS- over all groups during the first and second block.
Regarding the **Fear Ratings**, we found no stimulus main effect pre ACQ ($F(1,83)=0.036$, $p=0.851$). There was no group effect in the analysis of the Fear Ratings before acquisition training ($F(1,83)=0.043$, $p=0.836$). The analyses of the **US expectancy** ratings revealed a main effect of stimulus in the first block ($F(1,1228.48)=184.57$, $p<0.001$), showing an increased US expectancy towards the CS+, when compared to the CS- ($t(1227)=21.014$, $p_{corr}<0.001$). The same effect was found in the middle block ($F(1,1239.12)=397.92$, $p<0.001$; CS+ > CS-: $t(1239)=32.03$, $p_{corr}<0.001$). No group effect was found in the first block ($F(1,160.56)=0.001$, $p=0.974$) or in the middle block ($F(1,165.37)=0.035$, $p=0.851$). The analyses of the **SCR** showed a stimulus main effect in the first block of the ACQ ($F(1,928)=7.074$, $p=0.008$), resulting from an increased SCR towards the CS+, when compared to the CS- ($t(928)=3.964$, $p_{corr}<0.001$). Regarding the middle block, we found a trendwise stimulus main effect ($F(1,928)=3.049$, $p=0.081$), again with an increased SCR towards the CS+, when compared to the CS- ($t(928)=2.116$, $p_{corr}=0.035$). No group effects were found in the first block ($F(1,115.21)=0.001$, $p=0.976$) or in the middle block ($F(1,94.8)=0$, $p=0.996$).
Further analyses of the **fMRI** data were made regarding the differential contrast (CS+>CS-) between groups (Nic1< Pla) concerning the first and second block of the ACQ. Similar to the results from the analysis of the last block, we found reduced differential responses in the left HC (left HC: MNI xyz: -26,-33,-10; $T=3.55$, $p_{FWE}=0.030$) and trendwise in the right HC (right HC: MNI xyz: 27,-30,-9; $T=3.36$, $p_{FWE}=0.054$) in the first block. Additionally, we found reduced differential responses in the left AMY in the same contrast in the first block of the ACQ (left AMY: MNI xyz: -24,0,-22; $T=3.68$, $p_{FWE}=0.009$), as well as during the middle block of the ACQ (left AMY: MNI xyz: -32,0,-18; $T=3.08$, $p_{FWE}=0.049$). We also found an activation in the left Accumbens for the same contrast (left Acc: MNI xyz: -8,6,-8; $T=3.10$, $p_{FWE}=0.020$). Additional main effect of group results of ROI activations during the first Block of the ACQ can be found in table S1 and for the middle block of the ACQ in table S2.
Additionally, for the contrast (Nic1<Pla; main effect of group) during the middle block of the ACQ, we only found an activation in the right AMY (right AMY: MNI xyz: 32,-3,-21; $T=3.15$, $p_{FWE}=0.049$).
We furthermore compared responses across both CSs between group within all three blocks. we found reduced responses in the right HC (MNI xyz: 24,-27,-12; $T=3.69$, $p_{FWE}=0.020$), bilateral Ncl. Acc (left: MNI xyz: -6,14,-4; $T=3.10$, $p_{FWE}=0.020$; right: MNI xyz: 12,12,-6; $T=3.06$,

p_{FWE}=0.018), VTA/SN (MNI xyz: 10,-24,-16; T=2.98, p_{FWE}=0.068) and the bilateral AMY (left AMY: MNI xyz: -20,-4,-12; T=3.06, p_{FWE}=0.052; right AMY: MNI xyz: 21,-9,-12; T=3.84, p_{FWE}=0.007).

Analysis of the main effects during the last block of ACQ

Comparing **fMRI** data across both, the CS+ and CS-, we additionally found decreased activation in the nicotine, when compared to the Placebo group, within in the right HC (MNI xyz: 26,-27,-15; T=3.90, p_{FWE}=0.011), bilateral Ncl. Acc (left: MNI xyz: -8,12,-6; T=3.21, p_{FWE}=0.015, right: MNI xyz: 6,9,-6; T=3.38, p_{FWE}=0.008) and SN/VTA (MNI xyz: 12,-22,-10; T=3.97, p_{FWE}=0.004).

Table S1: Fear Acquisition (first block) fMRI-results of ROIs. Group comparisons.

ACQ – first Block	Nic1<Pla		
	T	P(FWE)	MNI, xyz
Left AMY	3.06	0.052	-20,-4,-12
Right AMY	3.84	0.007	21,-9,-12
Left HC	3.71	0.019	-27,-15,-20
Right HC	3.69	0.020	24,-27,-12
Left INS	3.97	0.009	-38,0,0
Right INS	3.25	0.080	38,-2,-3
Left Ncl. Acc	3.10	0.020	-6,14,-4
Right Ncl. Acc	3.06	0.018	12,12,-6
VTA/SN	2.98	0.068	10,-24,-16
	(CS+>CS-, Nic1) < (CS+>CS-, Pla)		
Left AMY	3.68	0.009	-24,0,-22
Left HC	3.55	0.030	-26,-33,-10
Right HC	3.36	0.054	27,-30,-9
Left Ncl. Acc	3.10	0.020	-8,6,-8

Table S2: Fear Acquisition (middle block) fMRI-results of ROIs. Group comparisons.

ACQ – middle Block	Nic1<Pla		
	T	P(FWE)	MNI, xyz
Right AMY	3.15	0.049	32,-3,-21
	(CS+>CS-, Nic1) < (CS+>CS-, Pla)		
Left AMY	3.08	0.049	-32,0,-18

Table S3: Fear Acquisition (last Block) fMRI-results of ROIs of the whole sample.

ACQ – last Block	CS+>CS-		
	T	P(FWE)	MNI, xyz
Left INS	9.32	<0.001	-32,20,4
Right INS	9.11	<0.001	39,16,2
dACC	6.47	<0.001	3,22,27
Left Ncl. Acc	3.37	0.009	-14,15,-6
VTA/SN	5.02	<0.001	12,-22,-10

Extinction

Analysis of the first block of EXT

Further examinations of our paradigm included the discrimination between CS+ and CS- during Extinction training, we examined Fear Ratings, US expectancy and SCR towards the CS+, when compared to the CS- over all groups during the first block/pre Rating.

Before the first stimulus presentation we found a stimulus main effect in the pre EXT **Fear Rating** ($F(1,79)=14.783$, $p<0.001$). Post-hoc tests revealed that participants rated higher fear towards the CS+, when compared to the CS- ($t(79)=8.427$, $p_{corr}<0.001$). There was no main effect of group before the EXT ($F(2,135.44)=2.168$, $p=0.118$). Stimulus by group interactions are described in the main manuscript. Regarding the **US expectancy**, we found a stimulus main effect over all groups during the first block of the EXT ($F(1,1212.08)=180.112$, $p<0.001$). This effect stems from increased US expectancy towards the CS+, when compared to the CS- ($t(1213)=21.853$, $p_{corr}<0.001$). There was no group effect in the first block of the EXT ($F(2,118.62)=1.352$, $p=0.263$). No main effects of stimulus ($F(1,717)=0.751$, $p=0.386$) or group ($F(2,73.61)=0.705$, $p=0.497$) during the first block was found in the **SCR**.

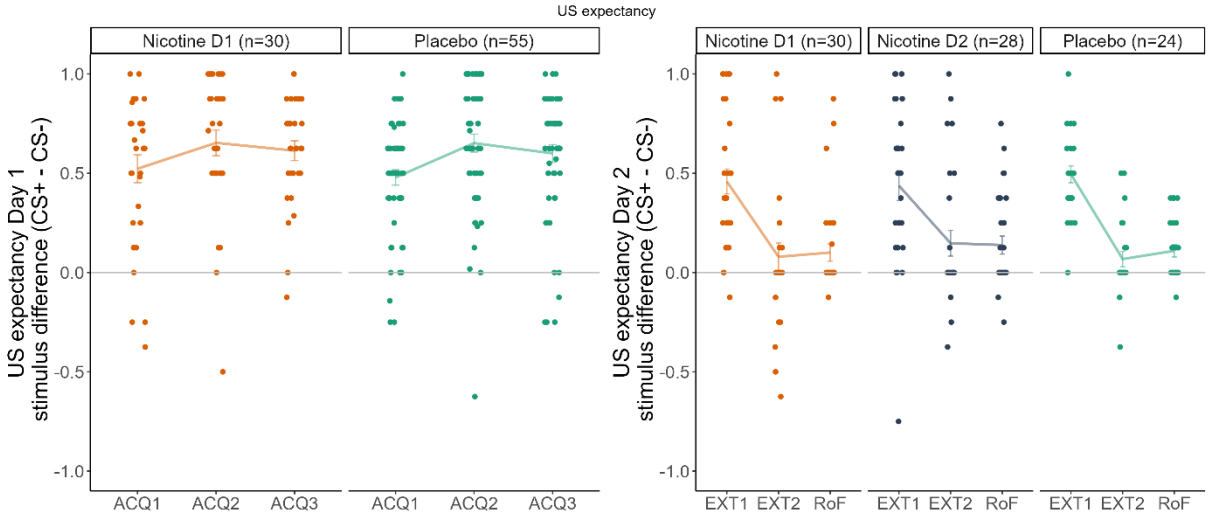

Figure S1: No group differences were found a) during the last ACQ3 or b) EXT2 in the US expectancy.

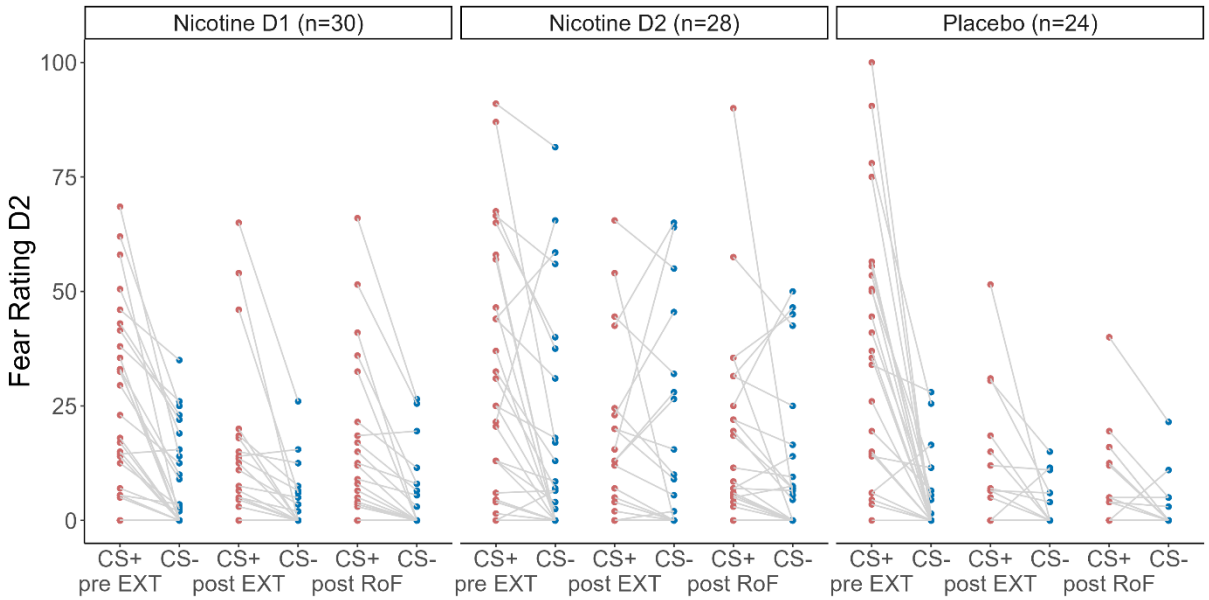

Figure S2: Nicotine (administered during ACQ or during EXT) weakened discrimination between CS+ and CS- in Fear Ratings. Single subject responses are shown as scatterpoints.

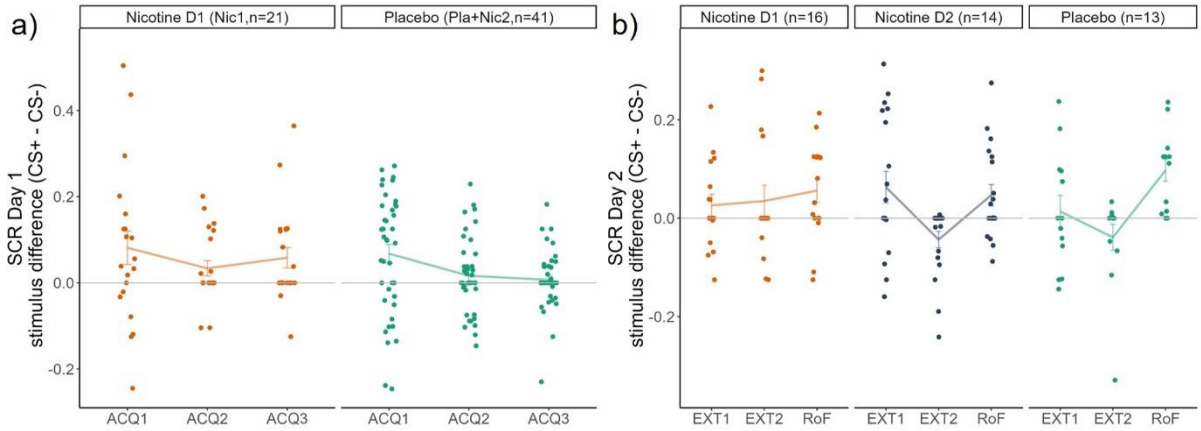

Figure S3: Differential Skin conductance Responses a) during the day 1 and b) during day 2. a) During the last block the differential SCR is higher in the Nicotine group, when compared to the Placebo group. b) During EXT2 the group that received nicotine on day 1 showed a trendwise increased SCR, when compared to the Nicotine group on day 2. There were no group effects concerning RoF.

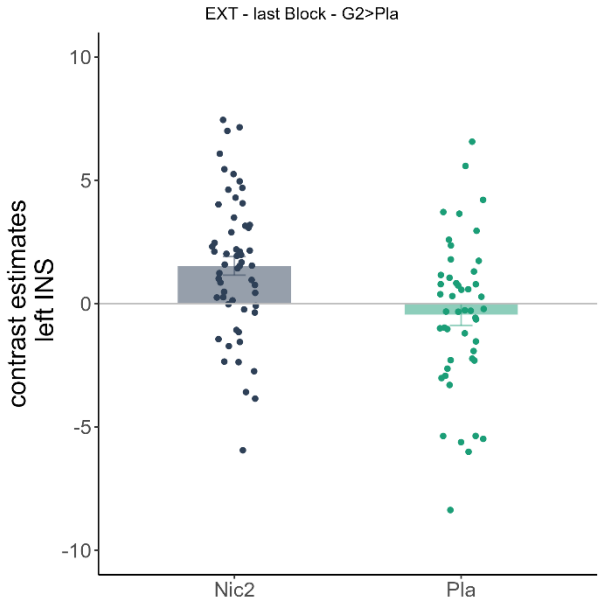

Figure S4: The Nicotine group D2 showed increased activity in the left INS, when compared to the Placebo group during the last block of the EXT. Bars represent means across each group with standard error.

Analysis of the main effects during the last block of EXT

Furthermore, exploratory **fMRI** analysis across both CSs during the last block of extinction revealed increased activity in the group that received nicotine during acquisition (nic1) in the left AMY (MNI xyz: -30,0,-22; T=3.40, p_{FWE}=0.024), as well as trendwise in the right HC (MNI xyz: 27,-14,-20; T=3.27, p_{FWE}=0.078), when compared to placebo controls. Within the opposite contrast (Nic1<Pla), we found enhanced activity in the vmPFC (MNI xyz: 8,42,-20; T=4.05, p_{FWE}=0.010) and a trendwise in the dACC (MNI xyz: -6,33,28; T=3.44, p_{FWE}=0.062) for the placebo group, when compared to nic1. The same analysis of acute effects of nicotine on extinction training revealed increased neural activity in the left INS (MNI xyz: -36,-10,9; T=3.65, p_{FWE}=0.029; see figure 7a), which is in line with increased Fear ratings in nic2.

Return of Fear

Group effects

fMRI

Regarding only the RoF block, we found an increased differential activation in the group that received nicotine before ACQ (Nic1), when compared to the Placebo group in the vmPFC (MNI xyz: 9,50,-10; T=2.68, p_{FWE}=0.004). This is driven by increased activation towards the CS- in the Placebo group (see Figure S4).

Combining the last block of the EXT and the RoF block, we found increased activation in the right Ncl. Accumbens in Nic2, when compared to Nic1 (MNI xyz: 10,-10,-10; T=2.84, p_{FWE}=0.033) and furthermore trendwise increased activation in the bilateral INS (left INS: MNI xyz: -33,18,-2; T=3.35, p_{FWE}=0.058; right INS: MNI xyz: 42,-6,6; T=3.30, p_{FWE}=0.067). Additionally, we found a trendwise increased activation in the last EXT block and the RoF block in Nic2, when compared to Pla in the left INS (MNI xyz: -36,-10,10; T=3.17, p_{FWE}=0.094). Furthermore we found trends towards an increased discrimination (CS+>CS-) in the RoF block in Nic2, when compared to Nic1 (right AMY: MNI xyz: 30,-6,-18; T=2.91, p_{FWE}=0.089; MNI xyz: 30,-14,-20; T=3.13, p_{FWE}=0.097). These data can be found in table S4.

When comparing the discrimination between (CS+>CS-) in the last block of EXT to the Reinstatement Test, we found increased activations in the left HC (MNI xyz: 34,-21,-18; T=3.87, p_{FWE}=0.010), right INS: (MNI xyz: 38,-12,14; T=3.46, p_{FWE}=0.043), dACC (MNI xyz: -3,21,24; T=3.36, p_{FWE}=0.069) and vmPFC (MNI xyz: 4,34,-20; T=3.75, p_{FWE}=0.021, see table 3).

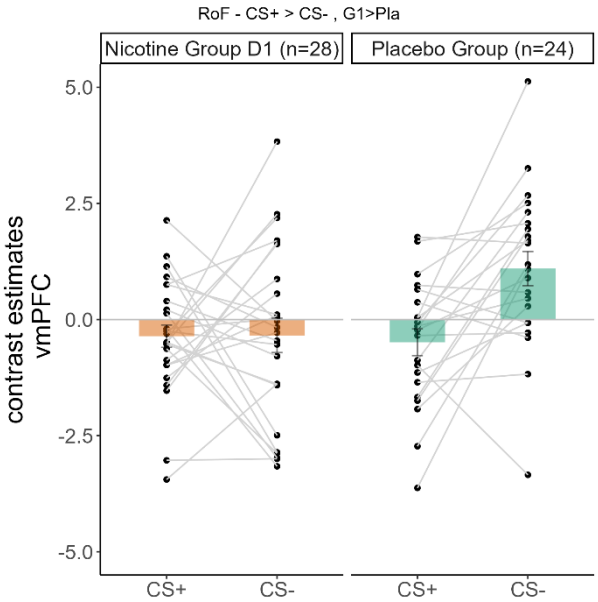

Figure S5: The Nicotine group D1 showed increased differential activity in the vmPFC, when compared to the Placebo group during the RoF, because of increased activation towards the CS- in the Placebo group. Bars represent means across each group with standard error.

Table S4: Return of Fear fMRI-results of ROIs. Group comparisons.

RoF	Nic1<Nic2 (RoF+EXT)		
	T	P(FWE)	MNI, xyz
Left INS	3.35	0.058	-33,18,-2
Right INS	3.30	0.067	42,-6,6
Right Ncl. Acc	2.84	0.033	10,-10,-10
	Pla < Nic2 (RoF+EXT)		
Left INS	3.17	0.094	-36,-10,10
	(CS+>CS-, Nic2) < (CS+>CS-, Nic1), (RoF)		

Right AMY	2.91	0.089	30,-6,-18
Right HC	3.13	0.097	30,-14,-20
	CS+>CS-, RoF<EXT		
Left HC	3.87	0.010	34,-21,-18
Right INS	3.46	0.043	38,-12,14
dACC	3.36	0.069	-3,21,24
vmPFC	3.75	0.021	4,34,-20

Other analyses

Finite Impulse responses

Nicotine has vasoconstrictive effects. To ensure that this does not interfere with our analysis of hemodynamic brain responses, we calculated the activity in different ROIs over a time period of 10sec (10 time bins; Figure S6). The activity did not differ between groups.

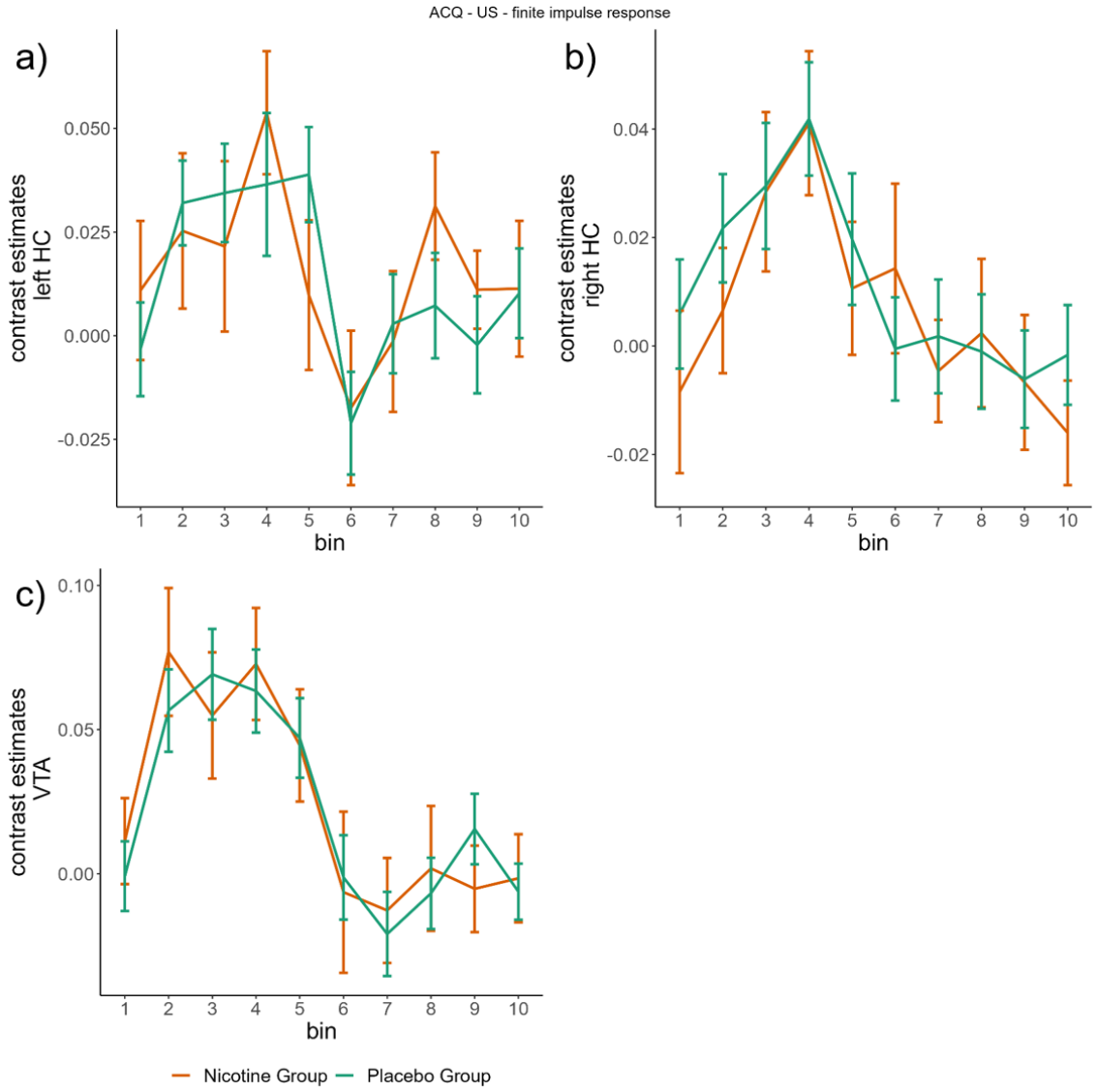

Figure S6a-c: Finite impulse response. The activity pattern of both groups on day 1 over the time of 10sec after US presentation in the bilateral HC (a) and b)) and c) the VTA/SN.

D2 – Test of attention

To test the general effect of nicotine on attention, subjects underwent a test of attention on both days. A rmANOVA revealed a main effect of day ($F(1,5989.33)=27.207$, $p<0.001$). Post-hoc tests showed that subjects achieved better results on day 2 ($t(84)=5.199$, $p_{corr}>0.001$), likely because it was the second time they underwent the test and already had routine. Furthermore we found a trend towards a day*group interaction ($F(2,1304.57)=2.963$, $p=0.057$), but post-hoc tests showed no difference.

To check the nicotine influence of nicotine for each day individually, we performed an independent samples t-test comparing the results of the attention test between the group that received nicotine on that respective day and the other two placebo groups (day 1: Nic1 vs Pla; day 2: Nic2 vs Nic1&Pla) and found no effect of nicotine on the attention of the subjects (day 1: $t(84)=-1.02$, $p_{corr}=0.313$; day 2: $t(84)=-1.148$, $p_{corr}=0.254$).

STUDY III

Acquisition of threat responses are associated with elevated plasma concentration of endocannabinoids in male humans, *Neuropsychopharmacol.* **47**, 1931–1938 (2022)

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Acquisition of threat responses are associated with elevated plasma concentration of endocannabinoids in male humans

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Endocannabinoids (eCBs) are involved in buffering threat and stress responses. Elevation of circulating eCBs in humans was reported to strengthen inhibition (i.e., extinction) of threat responses and to reduce effects of stressors. However, it remains unclear whether the acquisition of threat responses involves a physiological change in circulating eCBs. Here, we demonstrate in male human volunteers that the plasma concentration of the eCB N-arachidonoylethanolamine (AEA) and its metabolite arachidonic acid (AA) are increased during acquisition of threat responses. Furthermore, elevated responses to a learned threat cue (e.g., rating of fear) were associated with individual increases in plasma concentration of the eCB 2-arachidonoylglycerol (2-AG). In complementing these observations, we found individual increases in AEA associated with elevated neural responses during threat learning in the amygdala. Our results thereby suggest that physiological increases in circulating eCB levels are part of a response mechanism to learned threats.

Neuropsychopharmacology; https://doi.org/10.1038/s41386-022-01320-6

INTRODUCTION

Endocannabinoids (eCBs) have emerged as a promising target for the pharmacological treatment of anxiety and stress-related disorders. Specific interest in a buffering effect on stress and threat responses by eCB signaling is based on findings derived from experiments in rodents and humans that demonstrated how the enhancement of eCBs dampens behavioral threat responses and their underlying neural processes [1, 2]. eCBs entail N-arachidonoylethanolamine (AEA, anandamide) and 2-arachidonoylglycerol (2-AG), which are both synthesized on demand from arachidonic acid (AA)-containing membrane precursors. AA itself is also a product of eCB degradation. AEA and 2-AG are endogenous ligands to the CB1 and CB2 receptors [3], whereby the presynaptic CB1 receptor is involved in the modulation of behavioral responses by suppression of neurotransmitter release [4].

As such, pharmacological enhancement of eCBs has emerged as an interesting treatment option for individuals suffering from exaggerated threat responses after traumatic experiences. In these individuals, altered concentrations of circulating eCBs have been found [5–7], albeit mixed evidence for enhanced or decreased eCBs, when compared with control cohorts. To understand pathological states and develop pharmacological treatments, information about the physiological response during threat responses of circulating eCBs in humans is needed. In particular, it has not been investigated, whether the concentration of circulating eCBs changes in response to acquisition of threat responses and whether such a change in plasma eCBs is associated with behavioral, physiological, and neurophysiological threat responses.

Laboratory threat responses are commonly examined within fear conditioning models in which humans or other animals undergo acquisition training. This involves the presentation of a neutral stimulus (conditioned stimulus, CS) that is predictive of the occurrence of an aversive, potentially threatening stimulus (unconditioned stimulus, US, e.g., electric shock). As a result, the presentation of the CS elicits a conditioned threat response (CR). Repeated presentation of the CS without the US, so-called extinction training [8], reduces the CR by inhibition of the previously learned CS-US association [9–11]. Acquisition of threat associations has been linked to neural activation in the dorsal anterior cingulate cortex (dACC), the anterior insula (AI), and the amygdala [12–16]. Comprehensive experimental research has confirmed that eCBs are involved in buffering threat responses [4, 17, 18]. In humans, several studies examined the peripheral and central elevation of AEA via a functional polymorphism within the gene coding for a major metabolizing enzyme of AEA, the fatty acid amid hydrolase (FAAH [19–21]). Such an enhancement of circulating AEA plasma concentration was found to dampen the reactivity to negative affect (e.g., threatening images), reduced effects of stressors (e.g., psycho-social stress task) and augmented threat extinction learning [20–24]. These findings align with a recent study in male humans ($N = 51$) exploring the association between circulating AEA levels and neural brain activation during fear extinction. Here, plasma concentration of baseline AEA (start of extinction) was positively correlated to the decrease

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Table 1. Demographics. The final sample consisted of 45 healthy male volunteers (for exclusion criteria of each outcome measure see Supplementary Methods and Results).

Demographics					
Sample ($N = 45$) healthy male volunteers					
Age [years] Mean (SD)	Height [cm] Mean (SD)	Weight [kg] Mean (SD)	Education	Stai-T Mean (SD)	Stai-S Mean (SD)
26.9 (4.2)	181.9 (7.1)	81.4 (11.4)	44,4% university degree 46.7% students without degree	32.7 (7.3)	33.4 (4.9)

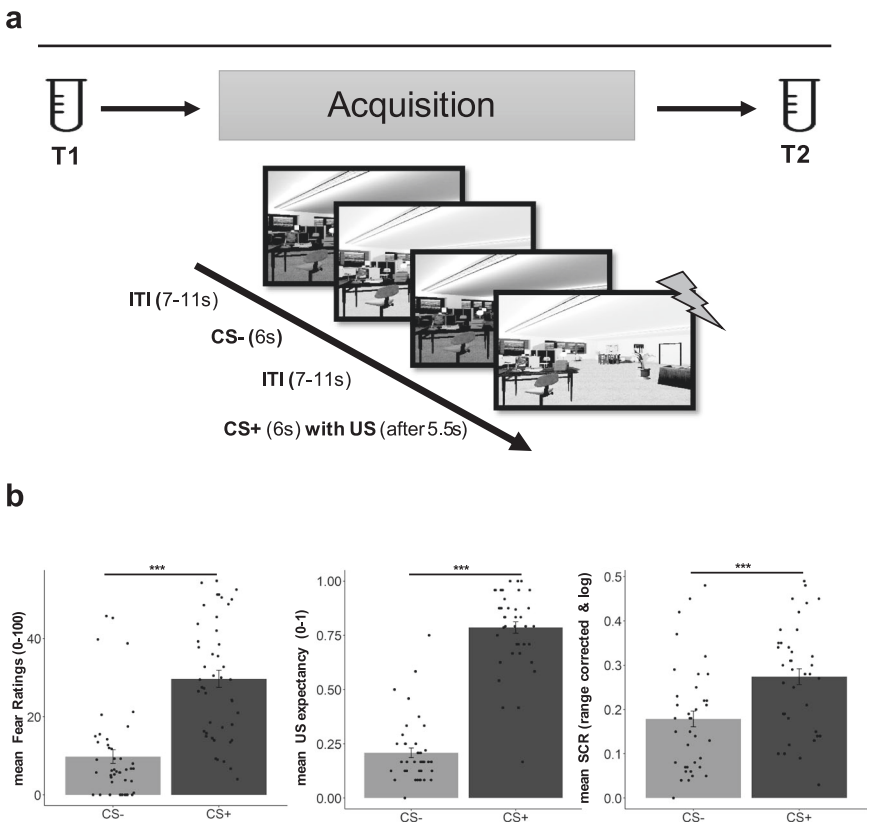

Fig. 1 Design and plasma sampling overview. a Illustration of the plasma sampling of AEA, 2-AG and AA (T1-T2) during acquisition phase of the context-dependent cue conditioning paradigm. Plasma concentration were sampled before (T1) and directly after (within 5 min) (T2) acquisition training ($N = 45$). Participants also underwent extinction training on Day2 (including eCB sampling) and a retention-test, as well as a reinstatement procedure on Day3, which was part of another study [31] (see Supplementary Methods). **b** Illustration of the mean responses to the CS + and the CS- during acquisition training measured as fear ratings, US expectancy, and skin conductance response (SCR). RmANOVAs of each outcome measure indicated a differentiation between the CS+ and the CS-, with higher responses to the CS + as compared to the CS- (Bonferroni-Holm corrected post-hoc tests all $p_s < 0.001$; see Table S1). Analyses of fear ratings and US-expectancy, but not SCRs, further revealed an interaction between CS-type and time (fear ratings ($N = 45$), $p < 0.001$; US-expectancy ($N = 41$), $p < 0.001$; SCR ($N = 42$), $p = 0.364$) reflecting a steeper increase in responses to the CS+, as compared to the CS- during acquisition (see Table S1).

(exponential decay to the CS+) in neural signaling within brain regions activated during threat acquisition, namely dACC and right AI [25].

While previous evidence supports a role for central and peripheral eCBs in the extinction of threat responses, it has not been investigated in humans whether plasma eCB levels are affected by the acquisition of threats in the first place. Such effect of eCBs on threat acquisition is supported by experiments in rodents, demonstrating a release of AEA in response to aversive electrical foot shocks (compared to low intensity and no shocks) within brain regions that process threats, namely the amygdala, hippocampus, periaqueductal gray and dACC [17, 26]. Thereby, eCB release upon foot shock is assumed to contribute to the conditioned analgesia that is found as a defensive threat response in rodents [27]. Additionally, it was shown that the enhancement of circulating AEA by pharmacological blockade of the FAAH (by URB597) in rodents strengthens acquisition training measured by freezing behavior, when compared to non-shock and saline controls [28]. However, other studies failed to show such an effect of enhanced acquisition training by elevated circulating AEA when comparing genetic polymorphisms of genes coding for the FAAH or pharmacological FAAH inhibition in animals, including humans [20–22, 24].

The function of 2-AG on threat responses seems different from AEA, since increased circulating 2-AG plasma levels by blockade of degrading enzyme monoacylglycerol lipase in rodents has been found to impair fear extinction [29] and to promote fear expression [30]. However, decreases in threat acquisition after elevation of circulating 2-AG plasma levels were also reported in rodents [28]. It is suggested that an optimal 2-AG level is necessary for adaptive threat responses and either too high or too low concentration impairs expression of threat responses [4].

In sum, previous studies that used pharmacological interventions and examined genetic polymorphisms within the eCB system could not reveal consistent effects on the dynamic fluctuations of circulating eCBs during threat acquisition across species. Hence, the key question remains: Do circulating eCB levels change in response to threat acquisition in humans?

To this end, this study examined circulating plasma concentration of the eCBs AEA and 2-AG, as well as AA, before and after a context-dependent threat acquisition that was combined with functional magnetic resonance imaging (fMRI) within a sample of 44 male participants. We hypothesized that circulating eCB and AA concentrations change during the acquisition of fear and that the individual changes in eCB and AA concentration were related to affective ratings of fear, US expectancy, peripheral physiological responses (skin conductance response, SCR), and neural responses (fMRI).

METHODS

Participants

Fifty healthy male participants gave written informed consent and were reimbursed for participation. Five participants were excluded for the analyzes of eCB and AA plasma concentrations and one additional participant was excluded for fMRI analyses (see Table 1 and Supplementary Methods for details and sensitivity analysis). The study was approved by local ethics committee in Hamburg (Ärztekammer Hamburg).

Procedure

Participants performed a context-dependent cue conditioning paradigm with acquisition training in context A (ACQ, Day1), extinction training in context B (EXT, Day2) and a retrieval-test within a 50:50 mixture of context A and B (generalization context [31], Day3), including a reinstatement procedure. Analyses focused on the ACQ phase, since participants received L-DOPA or placebo (double-blind randomized) before EXT on Day2 (L-DOPA effects, but not eCB analysis is part of a different study [31]). To examine the plasma concentration of AEA, 2-AG, and AA during ACQ, blood samples were taken directly before the ACQ (T1) and directly after the ACQ training (T2), see Fig. 1a. On the second day, blood samples were taken before drug administration (1 h before extinction training, T3), directly before extinction training (T4), and directly after finishing extinction training (T5). No blood sample was taken on the third day (see Fig. S1).

The acquisition training was preceded by a habituation phase (two presentations of each of the CSs within context A and B) without any US. Acquisition training consisted of 24 trials for each CS (duration:6 s), consisting of blue or yellow illuminated rooms (see Supplementary Methods). The CS + was followed by a US in 75% of the trials (5.5 s after CS + onset), consisting of an aversive electrotactile stimulation to the right hand (see Supplementary Methods), whereas the CS- was never followed by a US (see Fig. 1a). Participants were not informed about the conditioning contingencies or the learning element beforehand. Colors of the CS+ and CS- were counterbalanced across participants. Approximately 24 h after conditioning, participants returned to the fMRI laboratory. US electrode was attached, as on day 1 and 24 trials were presented for each CS, while no US was administered. Day 3 was conducted in the psychophysiological laboratory and the US-electrode was attached. The retrieval test consisted of eight unreinforced trials of each CS within a generalization context (50/50-mixture of context A and B). The retrieval test was followed by four unsignaled reinstatement-USs (interval range 10–15 s), while participants were exposed to a black screen. For the reinstatement-USs, the same individual electrical stimulation intensity was used as determined on day 1. 6–10 s after the last reinstatement-US, a second retrieval test (reinstatement-test) was employed, including 16 trials (with no US) of each CS.

Analyses of AEA, 2-AG, and AA

Blood samples were analyzed for the plasma concentration of N-arachidonoylethanolamine (AEA), 2-arachidonylglycerol (2-AG), and arachidonic acid (AA) as described in [25] and expressed as pmol/mL. Blood samples were collected by repeated venous punctures and immediately centrifuged at 4 °C for 10 min at 2000 *g*. 50 µL of the obtained plasma was aliquoted, frozen immediately, and stored at −80 °C.

Outcome measurements

Fear ratings. Participants rated fear/stress/tension for each CS before and after acquisition training on a computerized Visual Analogue Scale [VAS, 0 (none)–100(maximal)], confirmed by key press.

US-expectancy. On each CS trial, participants were instructed to rate their US-expectancy as binary choices by pressing the upper (1 = expectancy of a US) or lower key (0 = no expectancy of a US). No scale was presented to the participants to ensure undivided attention (for CS-US contingency awareness see Supplementary Methods).

Skin conductance. SCR was measured via self-adhesive Ag/AgCl electrodes, placed on the palmar side of the left hand on the distal and proximal hypothenar. Phasic SCRs to the onsets of each CS were manually scored as the largest response occurring 0.9 to 4.0 s after CS onset. Amplitudes were logarithmized and range-corrected (SCR/SCRmax CS [day]) for separate days to account for inter-individual variability (see Supplementary Methods).

Statistical analyses

Analyses of the main effects of task were employed by means of repeated measures ANOVAs, using JASP Team (JASP(Version 0.9.1)[Computer software], 2018). In all analyses, an α-level of $p < 0.05$ was adopted and sphericity correction (Greenhouse-Geisser) was applied, if necessary. *P* values were corrected using the Bonferroni-Holm method for independent observations (i.e., plasma concentration of three independent eCBs for each outcome measure). Changes in plasma concentration of AEA, 2-AG, and AA were tested via paired *t* tests between the concentration before (T1) and after (T2) acquisition training, as well as before (T4) and after (T5) extinction training. Association between main effects of task (e.g., CS+- CS- in block2 – block1 on Day1) and changes in eCBs and AA concentration (e.g., difference between T1 and T2) were examined by Pearson correlational analyses (see Supplementary Methods for analysis of extinction and retrieval). In order to control the effects of acquisition for the influence of the circadian rhythm and baseline concentration of eCBs and AA, regression models including these control variables were performed (see Supplementary Methods). Additionally, we examined changes in plasma concentrations within a similar time window (i.e., 60 min) approximately twenty-four hours after acquisition training (for details see Supplementary Methods).

fMRI acquisition and preprocessing

Task-relevant functional data were obtained on day 1 and day 2 at a 3 T Magnetom-PRISMA System, Siemens, Erlangen, Germany with echo planar multiband imaging with a resolution of 1.5 mm and a 0.5 mm gap. Preprocessing and statistical analysis were employed in SPM12 (Statistical Parametric Mapping, http://www.fil.ion.ucl.ac.uk/spm) including unwarping, realignment, and was coregistered to individual high-resolution structural images. Statistical analyses involved a general linear convolution model at the single-subject level, including onsets for the CS+, CS−, US, introduction, ratings, and button presses. Furthermore, we defined a parametric time modulation of linearly changing responses to the US regressor in order to examine neural responses that decrease as a function of US presentations. Resulting estimate images of interest were then normalized to a sample-customized DARTEL template [32]. Normalized first-level beta-maps were smoothed with an isotropic full-width at half-maximum Gaussian kernel of 4 mm.

Regression models of responses estimates were performed entailing the changes in eCBs and AA concentration and neural responses as the 1) contrast estimates for CS+ > CS- or 2) linearly changing responses to the US. Regions of interest were defined by the main effect during acquisition (without considering the influence of the eCBs and AA), including anatomical masks for the bilateral amygdala and insula [33], as well as the peak voxel in the dACC (MNI (coordinate system of the Montreal Neurological Institute and hospital),$x;y;z = 0;28;26$) with a surrounding box (20 × 16 × 16 mm). To examine the vmPFC as a key structure for safety learning, defined at the coordinates (MNI:$x;y;z = 0;42;-12$) with surrounding box (20 × 16 × 16 mm) as in previous experiments [34].

RESULTS

Participants acquired conditioned responses as evident from fear ratings, trial-wise US expectancy, and SCR during acquisition training

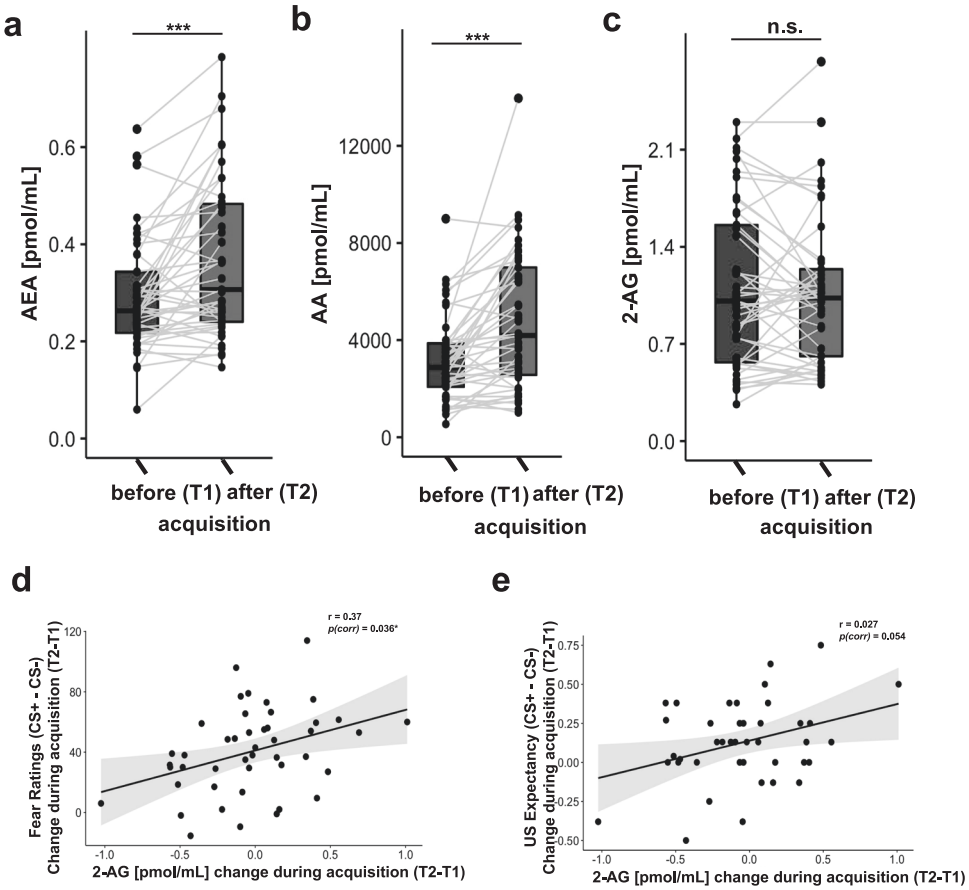

Fig. 2 Changes in eCB concentrations during acquisition training. Pair-wise comparisons between **a** AEA, **b** AA, and **c** 2-AG plasma concentration before the acquisition of threat responses (T1, baseline) and after acquisition training (T2). Boxplots illustrate the group concentration average, as well as individual concentration (black point) and their inter-individual change from T1 to T2 (gray lines). Positive correlations reflecting association between the individual increase (from T1 to T2) in differential (CS + -CS-) ratings of fear **d** and expectancy of the US **e** with the increases (from T1 to T2) in plasma 2-AG concentration during acquisition of conditioned threat responses.

(see Table S1; for main effects of extinction training and retrieval see Supplementary Results and Table S11, S13, respectively).

Increasing AEA and AA plasma concentration during acquisition and extinction training

Next, we examined the hypothesized changes in eCB and AA plasma concentrations during acquisition training by comparing the within-subject concentrations before the acquisition (T1) with concentrations after acquisition training (T2). Two-sided paired sample t-tests revealed an increase in AEA and AA concentration during acquisition training ($N = 45$; $p_s < 0.001$, see Fig. 2a, b and Table S3). We found no statistical support for a difference between time points in 2-AG levels ($p = 0.655$, see Fig. 2c and Table S3).

Importantly, the control analyses for the influence of the circadian rhythm revealed no evidence for a change in the plasma concentration of AEA within a similar time window 24 h later (Two-sided paired t tests: $t_s(20) < 1$, $p_s > 0.3$, see Table S4 and Fig. S2a). Hence, it is unlikely that the increase during acquisition training reflects a mere passage of time.

During extinction ($n = 21$, placebo subjects, see *procedure*) we found a general increase in AEA and AA concentration (see Supplementary Results).

Association between conditioned threat responses and changes in 2-AG plasma concentration during acquisition and extinction training

While our initial analyses revealed an increase in AEA and AA during acquisition of conditioned threat responses, we further examined if individual changes in eCB and AA levels were associated with the individual expression of threat responses. To this end, individual changes in eCB and AA plasma concentration during acquisition training (T2–T1) were tested for association with conditioned threat responses (i.e., block2 – block1 on Day1 of the differential responses to CS+ and CS-). The analyses revealed a positive correlation between 2-AG concentrations and fear ratings, as well as US expectancy, indicating that increasing 2-AG plasma concentration during acquisition training (from T1 to T2) was associated increasing differential fear ratings (CS + -CS-) from beginning to the end of acquisition training (T1 to T2). These findings were mirrored for US-expectancy, albeit lower statistical evidence after correction for multiple comparisons (two-sided Pearson correlation: fear ratings ($N = 45$): $r = 0.37$, $p_{uncorr} = 0.012$, $p_{corr} = 0.036$; US-expectancy ($N = 41$):$r = 0.35$, $p_{uncorr} = 0.027$, $p_{corr} = 0.054$, see Fig. 2d, e and Table S5 for separate correlation with each CS). However, we found no support for an association between changes in 2-AG and SCRs ($N = 42$, $p_{uncorr} > 0.5$), as well as no correlation between changes in AEA or AA with any of the outcome measurements (all $p_{uncorr} > 0.14$, see Table S5). Importantly, none of the eCB or AA changes was associated with the intensitiy or valence of the US (see Table S7). The achieved power for the reported association between fear ratings and changes of 2-AG was moderate 0.72 (see sensitivity analyses in the Supplement).

During extinction, we found a negative correlation, which indicated an association between individual increase in 2-AG concentration and differential decrease in US expectancy from beginning to end of extinction that however not survived correction for multiple comparison ($r = -0.461$, $p_{uncorr} = 0.036$,

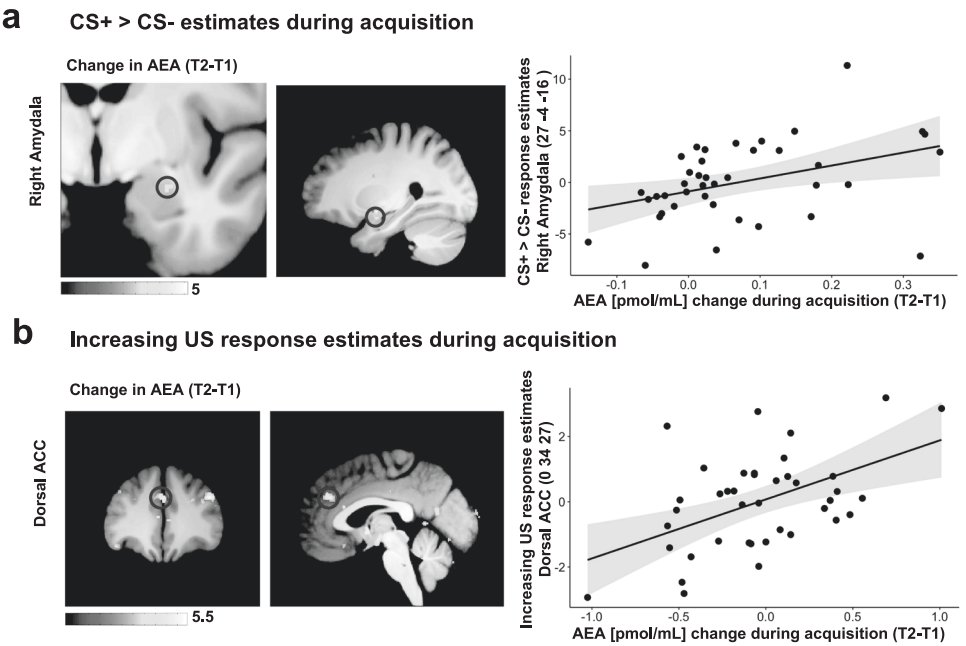

Fig. 3 Association between changes in eCB concentrations and neural responses during acquisition training. a The regression analysis of neural responses to the CS+, compared to the CS- in the right amygdala revealed a positive association with the changes in AEA plasma concentration during acquisition training (for statistics see Table S9). **b** The regression model of neural responses that increase to the US during acquisition training revealed that an increase in activity in the dorsal ACC was associated with increase in AEA concentration (for statistics see Table S10). T-maps are displayed on an averaged image across the whole sample with a threshold of $p_{uncorr} < 0.005$ for illustrative purposes.

$p_{corr} = 0.108$, see Table S12). We found no association between changes in eCB or AA concentration during extinction and responses during the retrieval-test (see Table S14).

Regression models including control variables support association between conditioned threat responses and changes in plasma concentration

In a second step, we aimed to verify the association between 2-AG and conditioned threat responses within a regression model including baseline concentrations of AEA, 2-AG, and AA, since baseline concentration of AEA has been reported to influence baseline anxiety [35]. To further control for the influence of the circadian rhythm on AEA and 2-AG [36, 37], we included the anticipated change of AEA and 2-AG based on the daytime of T2 as regressors into the model. The regression models for US expectancy and fear ratings revealed again that the individual acquisition of differential conditioned responses (CS+-CS-) was associated with changes in plasma concentration of 2-AG. Hence, a stronger acquisition of conditioned responses was accompanied by a higher increase in 2-AG during acquisition training of fear ratings ($p = 0.008$) and trend-wise for US-expectancy ($p = 0.056$), but not of SCRs. Furthermore, the baseline levels of AEA independently correlated negatively with the acquisition of differential conditioned responses (CS+-CS-) in all outcome measures, indicating that higher baseline AEA levels were associated with lower conditioned responses (all $p_s < 0.096$). This is in line with a previous study showing a negative association between baseline AEA concentration and anxiety within a (stress) experiment [35]. Importantly, the circadian rhythm of the eCBs (i.e., daytime of the sampling), which was included in each regression model, did not mitigate these effects (see Supplementary Results).

Regression models on association between neural responses and changes in eCB and AA plasma concentration during acquisition of threat responses

Our results already indicated a general increase in AEA and AA during acquisition training, as well as an increase in 2-AG plasma concentration that correlated with differential conditioned responses (fear and US-expectancy ratings). Therefore, we tested via regression models, if the increase in eCB or AA levels is associated with activation in brain regions that reflect the discrimination of learned threat responses (i.e., CS+ > CS-) during acquisition training (main effects: Table S8). Analysis revealed a positive association between the differential response (CS+-CS-) in the right amygdala and increasing AEA plasma concentration (T1–T2) during acquisition training (MNI:$x;y;z = 27;-4;-16$; $t = 3.89$; $p_{FWE} = 0.03$; $p_{uncorr} < 0.001$, see Fig. 3a and Table S9).

Next, we tested for changes in the eCB system related to neural processes while adapting to the aversive US. Therefore, linear temporal dynamics of neural responses to the US during acquistion training were modeled. Temporal response estimates were then included in a regression model including changes in eCB and AA plasma concentrations (T1 to T2) as regressors (see Table S10). During acquisition training, we found that a linear increase in activation in the left hippocampus was accompanied by increasing plasma concentration of 2-AG (MNI:$x;y;z = -15;-9;-20$; $t = 4.7$; $p_{FWE} = 0.008$; $p_{uncorr} < 0.001$). Similarly, we found that linearly increasing activation in the dorsal ACC to the US was associated with increasing plasma concentration of AEA during acquisition training (MNI:$x;y;z = 0;34;37$; $t = 4.89$; $p_{FWE} = 0.008$; $p_{uncorr} < 0.001$, see Fig. 3b). Hence, activation in brain regions that are involved in the acquisition of conditioned threat responses, namely the amygdala, hippocampus, and dACC, are associated with increased plasma concentration of 2-AG and AEA.

DISCUSSION

In this study, we provide support for an overall increase in peripheral concentration of AEA and AA in the blood plasma during the acquisition of conditioned threat responses in male human volunteers. Importantly, no overall increase of 2-AG concentration during acquisition of threat responses was observed, but an association between changes in plasma concentration of 2-AG with the individual expression of

conditioned threat responses in fear ratings and a trend in US expectancy. This association of 2-AG change was confirmed in regression models including control factors for the influence of eCB baseline levels [35] and circadian rhythm of eCB concentration [36, 37]. Furthermore, calculated regression analyses of neural responses revealed an association between differential CS responses (i.e., CS + > CS-) in the amygdala and individual increases in AEA concentration during acquisition of threats. In addition, regression analyses revealed an association between individual elevation of AEA and 2-AG concentration and neural responses increasing across US presentations in the hippocampus, as well as the dACC.

Previous experiments in humans and rodents have provided evidence that enhancement of circulating AEA seems to buffer threat responses, in particular during extinction learning [20–24]. In line with these findings are recent results, indicating that plasma levels of AEA before extinction training were associated with decreasing neural responses in brain regions, such as the dACC and the insula, [25]. Our results extend recent findings by showing that the concentration of circulating AEA and its precursor and metabolite AA is already increasing during the acquisition of threat responses, potentially as a normal, physiological function in healthy male humans. Moreover, our results align with previous experiments in rodents, indicating an increase of eCB levels in brain regions such as the amygdala, hippocampus, periaqueductal gray, and mPFC when acquiring threat responses [26, 38]. These studies suggested that the generation of eCBs is a part of a defensive response, which might contribute to conditioned analgesia to foot shocks. Due to the fact that measured blood plasma concentration in humans does not directly reflect the concentration of eCBs in the brain, it is not fully understood from which source circulating eCBs arise and how they specifically reflect eCB driven neural responses [39].

Nevertheless, our results still associate circulating eCBs with processes of aversive learning and related neural responses in the brain. It could well be that the acquisition of differential conditioned threat responses (i.e., higher responses to the CS + as compared to the CS-) probes adaptive response to cope with threats. In fact, individuals suffering from anxiety-related disorders often fail to differentiate between the CS + and the CS- [40, 41]. Hence, the increase in eCBs might be related to the adaptive discrimination between a CS + that predicted the occurrence of the US in comparison to a safe cue as a defensive (coping) response to threats. In parallel, our results might suggest that eCBs are involved in aversive learning in general, since we found elevated plasma concentration during threat acquisition and extinction.

While our results suggest that learning to predict threats is related to increases in eCBs and AA, other factors, such as stress and general arousal (e.g., prior knowledge that aversive stimulation will be applied, positioning in the fMRI environment, etc.) might have additionally contributed to the elevation of eCB concentrations. Prior studies found acute stress related to a decrease in AEA concentration in the rodent brain, whereas mixed results (decreasing, increasing, and no change) in concentration of peripheral AEA were reported in humans [21, 24, 35, 42]. Further studies in rodents reported that acute stress level amplified 2-AG concentration in the amygdala [43], whereas evidence for changes in circulating 2-AG in humans were indecisive (decreasing/no change [35, 39]). However, potential effects of acute stress would rather have affected the general change in eCB concentration and consequentially would neither explain the association between 2-AG and the conditioned threat responses, nor the association between differential (controlled for activation to the CS-) neural activations associated with the individual increase in AEA plasma concentration. Our findings of an association between 2-AG and conditioned threat responses is furthermore in line with a recent study in humans which reported that higher 2-AG concentrations after a traumatic injury predicted greater symptoms of depression 6 months later [44]. This study already suggests that changes in the physiological concentration of eCBs are relevant to the adaption of future behavior.

Seemingly in contrast to our results, other studies in humans did not show an effect of enhanced eCB levels on the performance during acquisition training using polymorphisms of genes coding for the FAAH or pharmacological inhibition of the FAAH [20, 24, 45]. However, an absence of enhanced behavioral or physiological measures in acquisition by pharmacologically augmented eCB level does not necessarily contradict that physiological responses of the eCB system are involved during acquisition of threats. We advocate for a better understanding how the eCB system is involved during the acquisition of threat responses. These insights might aid to understand disturbances of the eCB system in individuals that experienced traumatic events [6, 7, 42] and provide a basis to develop new treatments for trauma and stress-associated disorders.

Our findings are limited by the investigation of male volunteers only. Future studies are warranted to delineate the eCB responses to threats in female populations, given that females are overrepresented in populations that suffer from anxiety-related disorders [46].

Our results indicate that acquisition of threat responses is reflected in dynamic changes of eCB plasma concentrations, by elevated plasma concentration of AEA and its metabolite AA during acquisition of threats. We further provide initial evidence for an association between increased 2-AG plasma concentration with fear ratings as well as between increases in AEA concentration and elevated activity in the amygdala. Hence, our results provide a novel perspective of how physiological changes in circulating eCBs are involved in aversive learning. We further suggest future studies to reveal the potential of eCBs in adaptive and maladaptive coping with threats and thereby advancing pharmacological treatment that focuses on balancing eCB plasma in patients with anxiety-related disorders.

DATA AVAILABILITY

Source data for the analyses and figures are available at:
https://osf.io/vq3bs/?view_only=ea7da978554f43bca27b89059e04d7e6

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AUTHOR CONTRIBUTIONS

JH, RE, JF, and BL conceived and designed the study paradigm. RE and JF collected the data. SW analyzed and interpreted the behavioral and physiological data. JR, MM, and SW analyzed the fMRI data. SW and JH drafted the initial manuscript, and BL, JF, and MM provided critical revisions. All of the authors discussed the results, commented, and revised the article, and approved the final manuscript for submission.

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Competing interests

The authors declare no competing interests.

ADDITIONAL INFORMATION

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Supplementary materials

Acquisition of threat responses are associated with elevated plasma concentration of endocannabinoids in humans

Supplementary methods

Participants

Fifty healthy, right-handed male adults without any self-reporteded life-time psychiatric or neurological diagnoses (age: range: 20 – 38 years, *M*=26.9, *SD*=4.2), were recruited in this study. One subject had to be excluded after illegal drug-screening test (positive drug urine test), carried out prior to acquisition training on day 1 (M-10/3-DT; Diagnostik Nord). Four additional subjects were excluded for the analyses of eCB and AA plasma concentrations (incidental finding of a brain abnormality N=1, not following the instructions N=1, accidental press of the emergency bell N=1, missing blood samples N=1). The final sample for the analyses of blood and behavioral data included forty-five participants. One additional subject had to be excluded for fMRI analyses, because of movement-related artefacts in the scanner. The final sample included in fMRI analyses therefore contained fourty-four subjects. Participants gave written informed consent and were reimbursed with 120 EUR.

The participants were recruited to participate at a pharmacological intervention during extinction [1], as well as examination of eCB and AA concentration during acquisition training.

Sensitivity analyses

Post-hoc calculated sensitivity analyses of a two-sided, paired comparison for plasma concentration indicated a sufficial sample size of 45 participants to detect an effect size dz = 0,4941242 and a critical t =2,0153676 assuming a power (1-β error probability) of 0.90 and an α error probability of 0.05 (G*Power 3.1). Similar analyses for correlation analyses indicated that the sample of 45 participants would be sufficient to detect correlation with a ρ=0,46 and a critical r=0,29 assuming a power (1-β error probability) of 0.90 and an α error probability of 0.05 (G*Power 3.1).

Stimulus material

Conditioned stimuli. Computer-generated pictures showing office-rooms (Source Engine, Valve Corporation, Bellevue, USA) were used to indicate contexts (context A or context B) that were visible from two different perspectives. The inter-trial intervals consisted of context picture presentations (ITIs, duration range 7-11 sec, mean 7.8) while illumination of the context in either a blue or a yellow light (duration of 6 sec) served as a conditioned stimuli (CS). Depicted contexts and applied colors signaling the CSs were counterbalanced across

participants. The visual stimulus material was presented on a computer screen using Presentation® software (NeuroBehavioral Systems, Albany California, USA).

Unconditioned stimulus. An electrotactile stimulus consisting of a train of 3 square-wave pulses of 2 ms duration each (interval 50 ms) served as the unconditioned stimulus (US), always following 5 sec after CS+ onset. The US was delivered by a DS7A electrical stimulator (Digitimer, Welwyn Garden City, UK) connected to a surface electrode with platinum pin (Specialty Developments, Bexley, UK), which was placed on the right dorsal hand. The US intensity was individually adjusted to a level of tolerable pain before starting the fear conditioning paradigm on day 1 within the scanner environment (*M*=8.2 mA, *SD*=3.4 mA and range: min=2.5 mA, max=21mA). Therefore, participants were asked to rate the aversiveness of the US between 0 ("I feel nothing") and 10 ("maximally unpleasant") (rating: *M*=7.1, *SD*=0.9 and range: min=4, max=8 mA) after each electrotactile stimulation presented in increasing intensity (calibration start at 2.5 mA). No correlation between US-intensity or US-valence with measures of acquisition was found (see table S7).

Procedure

Participants performed a context-dependent cue conditioning paradigm including acquisition training in context A (ACQ, Day 1), extinction training in context B (EXT, Day 2) and a retention-test within a mixture of context A and B (generalization context [2]), which included a reinstatement procedure (Day 3). Here, we focused our analyses on the ACQ phase before any administered pharmacological intervention, since participants received L-Dopa or placebo (double-blind randomized) before EXT on day 2. Day 1 and 2 took place in the fMRI scanner, whereas Day 3 was conducted in a behavioral laboratory. To examine the plasma concentration of AEA, 2-AG and AA, blood samples were taken at several time points on Day 1 and 2 (see figure S1 *Experimental design*).

Acquisition training (Day 1). Prior to conditioning, adjustment of the US (see *stimulus material*) was performed separately for each subject. Then, the experiment started with a habituation phase, including two presentations of each of the CSs within context A and B without any presentation of the US. Following, acquisition training consisted of 24 trials for each CS. The CS+ was followed by a US in 75% of the trials, whereas the CS- was never followed by a US. Participants were not informed about the conditioning contingencies or the learning element at any time of the experiment.

Extinction training (Day 2). Approximately 24 hours after conditioning, participants returned to the fMRI laboratory. US electrodes were attached the same as on day 1. During extinction training 24 trials were presented for each CS and no US was administered.

Retention test (Day 3). Day 3 was conducted in the psychophysiological laboratory and US were attached. The experiment started with a retrieval test consisting of 8 unreinforced

trials of each CS within a generalization context (50/50-mixture of context A and B). Retrieval test was followed by 4 unsignaled reinstatement-USs (interval range 10-15 sec) while participants were exposed to a black screen. For the reinstatement USs the same individual electrical stimulation intensity was used as individually determined during acquisition training. 6-10 sec after the last reinstatement-US, a second retrieval test (reinstatement-test) was employed, including 16 trials (with no US) of each CS. The order of CS+ and CS- after the reinstatement US was counterbalanced across subjects.

At the end of the experiment, CS-US contingency awareness was assessed using a semi-structured interview [3] and based on these results 37 participants were classified as aware and 5 were classified as unaware of CS contingency.

Blood samples. Blood samples were taken at five time-points during the experiment (see figure S1, upper hand). On the first day, blood samples were taken directly before and after the acquisition training (T1 and T2). On the second day, blood samples were taken before drug administration (one hour before extinction training, T3), directly before extinction training (T4) and directly after finishing extinction training (T5). No blood sample was taken on the third day.

Analyses of AEA, 2-AG and AA

Blood samples were immediately centrifuged at 4°C for 10 min at 2000g. 50 µL of the obtained plasma was aliquoted, frozen immediately and stored at -80°C. For all blood samples plasma concentration of anandamide (AEA), 2-arachidonylglycerol (2-AG) and arachidonic acid (AA) were quantified as described in [4]. All the values are reported as plasma concentrations in pmol/mL.

Outcome measurements

Fear ratings. At the beginning and end of each experimental day, participants were asked to rate the fear/stress/tension level experienced by each context and CS. Same context and CS pictures were additionally rated after the habituation phase and before any CS presentation. Ratings were performed on a computerized Visual Analogue Scale [VAS, 0 (none) – 100 (maximal)], using keys with the right hand and rating values had to be confirmed by a key press or otherwise treated as missing data [excluded participants: N(day1)=0, resulting sample size N(day1)=45]. For fear ratings, acquisition learning was measured by subtracting the difference between the CS+ and the CS- before acquisition from the rating after acquisition $((CS+ - CS-)_{before\ ACQ} - (CS+ - CS-)_{after\ ACQ})$.

US-expectancy. On each CS trial presentation, participants were instructed (before the experiment) to rate their US-expectancy as binary choices by pressing the upper key (1=expectancy of a US) or lower key (0=no expectancy of a US) of a four-key cross. No scale

was presented to the participants. Participants were excluded from the analyses (day-wise) if more than one third of all data points were missing [excluded participants: N(day1)=4, resulting sample size N(day1)=41]. US-expectancy ratings were averaged over 8 trials (as one block), resulting in 3 blocks on each day. Acquisition learning was calculated by subtracting the difference between the CS+ and the CS- US-expectancy ratings at the first block (first 8 trials of acquisition) from the last block (last 8 trials of acquisition, $(CS+ - CS-)_{early\ ACQ}$ - $(CS+ - CS-)_{late\ ACQ}$)).

Skin conductance. Skin conductance responses (SCR) were measured via self-adhesive Ag/AgCl electrodes placed on the palmar side of the left hand on the distal and proximal hypothenar. Data were recorded with a BIOPAC MP-100 amplifier (BIOPAC Systems Inc, Goleta, California, USA) using AcqKnowledge 4 software. Then, data were down sampled using a custom-made computer program (EDAview, Version 1.0) to 10 Hz. Phasic skin conductance responses (SCRs) to the onsets of each CS were manually scored defined as the largest response occurring within a time window between 0.9 to 4.0 s after CS onset. Non reactions were scored as zeros and trials with obvious electrode artefacts were scored as missing data. Amplitudes were logarithmized and range-corrected (SCR/SCRmax CS [day]) separately for consecutive days to account for inter-individual variability. SCR data from a limited number of participants revealed insufficient data quality (as judged by two researchers; due to signal-disturbances by the fMRI acquisition) and were consequentially excluded (day-wise) prior to data analyses [excluded participants: N(day1)=3, resulting sample size N(day1)=42]. SCRs were averaged over 8 trials (as one block), resulting in 3 blocks for each day. Acquisition learning for SCRs was calculated similar to US-expectancy ratings by subtracting the difference between the mean for CS+ and for CS- across the first block (first 8 trials of acquisition) from the last block (last 8 trials of acquisition, $(CS+ - CS-)_{early\ ACQ}$ - $(CS+ - CS-)_{late\ ACQ}$)).

Statistical analyses

Main effects of task. Analyses of the main effects during acquisition training regarding fear ratings, US-expectancy and SCR were employed by repeated measures ANOVAs (rmANOVA). RmANOVAs included a within-subject factor for the CS-type (CS+ and CS-) and the effect of time (fear ratings: 2 levels that include ratings before and after acquisition training, SCR and US-expectancy: 3 levels for each block that represent an average across 8 trials) and were calculated using the statistic software JASP Team (2018, JASP (Version 0.9.1) [Computer software]. In all analyses, an α-level of $p<0.05$ was adopted and sphericity correction (Greenhouse-Geisser) was applied, if necessary.

Main effects in AEA, 2-AG and AA plasma concentration. A potential change in plasma concentration of AEA, 2-AG and AA during acquisition training, was tested by means of

separate paired t-tests. For this purpose, individual differences between the concentration before (T1) and after (T2) acquisition training were compared. Similar comparisons were calculated for extinction training and were quantified as the difference between AEA, 2-AG and AA concentration before (T4) and after (T5) extinction training. The exploratory analyses of AEA, 2-AG and AA changes during extinction training were performed solely in the placebo group.

Association between main effects of task and changes in AEA, 2-AG and AA *concentration.* Analyses applied focus on changes in AEA, 2-AG and AA during acquisition training and a potential association with the main effect of task for each outcome measure. Therefore, indices reflecting the main effect of task (e.g., CS+ - CS- in T2-T1, see above) were tested for a correlation with changes in AEA, 2-AG and AA (difference between T1 and T2). Again, an α-level of $p<0.05$ was applied and p-values were corrected using the Bonferroni-Holm method for three independent observations (i.e., plasma concentration of three independent eCBs for each outcome measure). Additional data for the analyses of the association between the AEA, 2-AG and AA and the CS+ and CS- responses (instead of the CSs difference), separately calculated for each outcome measure can be found in supplementary analyses (Table S5).

Regression models including control variables for changes in AEA, 2-AG and AA concentration. Indices reflecting the main effect of task (e.g., CS+ - CS- in T2-T1, see above), were entered into linear regression models (separately calculated for each outcome measure) including changes in for AEA, 2-AG and AA as a separate regressors. Linear regression models used backward selection of regressors with an α-level of $p<0.1$. Furthermore, regressors for the baseline concentration of AEA, 2-AG and AA (T1) reflecting the expected impact of circadian rhythmic on the concentration of AEA and 2-AG were included in regression models.

Daytime of blood sampling. Previous studies in humans have shown that eCB plasma concentration follows a circadian rhythm and hence, changes during the day-time. Hanlon et al. (2020) reported a differential circadian rhythmic for 2-AG and AEA documenting the relative level of both in percentage respective to the 24-hour mean over the day [5]. The time of blood sampling in our study was coded for 2-AG (values from 1.00 to 1.60 in steps of 0.05) and AEA (values from 0.80 to 1.30 in steps of 0.05), by interpolation steps of 30 min that would reflecting the anticipated, relative changes in individual concentrations from the individual 24-hr mean, using previously reported results [5,6]. To control for the circadian rhythm, these anticipated influences of daytime for the sample T2 was entered as two regressors (one for AEA, one for 2-AG) into each regression model.

Baseline level of AEA, 2-AG and AA. Studies have shown that inter-individual differences in baseline levels of eCBs exist and that these baseline level might correlate with

anxiety during the experiment [7]. To examine the effect of baseline levels of AEA, 2-AG and AA (i.e., T1 on day 1) on dependent measures, baseline levels were entered as regressors in each regression model.

fMRI analyses. MRI data were obtained on a 3T Magnetom-PRISMA System (Siemens, Erlangen, Germany) using a 64-channel head coil and parallel single-shot echo-planar imaging (GRAPPA, in-plane acceleration factor 2) [8]and simultaneous multi-slice acquisitions ("multiband", slice acceleration factor 2 [9,10] as described in [11]). Image reconstruction algorithm was provided by the University of Minnesota Center for Magnetic Resonance Research. Echo planar multiband images were acquired with 42 continuous axial slices (1.5 mm thickness, 0.5 mm gap) using a T2*-sensitive sequence (TR = 1493 ms, TE = 30 ms, flip angle = 60°, field of view = 225 × 225 mm^2). Slice arrangement was individually adjusted in order to cover the following areas: dorsal anterior cingulate cortex, ventral medial prefrontal cortex, nucleus accumbens, amygdala, and midbrain SN/VTA. Moreover, high-resolution T1-weighted structural brain image (MP-RAGE sequence, 1 mm isotropic voxel size, 240 slices) were obtained. To account for T1 equilibrium effects, the first five functional images of the time series collected during acquisition training (day 1) were discarded. During preprocessing, images were unwarped, realigned to the first image and coregistered to the individual high resolution T1 structural image. In a next step, subject- and regressor-specific parameter estimate images of interest were normalized to a sample-customized DARTEL template [12] and smoothed with an isotropic full-width at half-maximum Gaussian kernel of 4 mm.

Following statistical analyses were performed using SPM12 (Statistical Parametric Mapping, http://www.fil.ion.ucl.ac.uk/spm) running under Matlab2017a (The MathWorks, Inc., Natick, Massachusetts, United States). Using a standard approach for fMRI implemented in the SPM software, involving a general linear convolution model (GLM) at the single-subject level and a random-effects analysis on group level. Individual linear modeling included relevant experimental conditions (i.e., ITI, CS+, CS−, US, introduction slides, ratings, and button presses), defined as separate regressors and predicted time course of experimentally induced brain activation changes was modeled as a stick function. Furthermore, we defined a parametric time modulation of linearly changing responses to the US regressor in order to examine neural responses that decrease as a function of US presentations over time.

To examine associations between neural responses in regions that were responding to the main effects of task with the changes in AEA, 2-AG and AA, individual contrast estimate maps for higher responses to the CS+ as compared to the CS- were included into group analysis using one sided t-test models, as implemented in SPM. We employed four separate regression models that all included individual changes in AEA, 2-AG and AA concentration as well as an intercept as regressors. Hence, we are able to predict neural responses that were

either 1) the contrast estimates for CS+>CS- or 2) linearly changing responses to the US presentations.

Regions of interest (ROI) were defined as key structures in acquisition learning, such as the bilateral Insula, bilateral Amygdala, dACC and vmPFC. The bilateral insula and bilateral amygdala were defined by Harvard-Oxford probability maps [13]. Since there is no anatomical mask available for the dACC, we defined this ROI by its peak voxel for the main effect of task [NMI, x=0, y=28, z= 26] and a surrounding box with the dimensions of 20 × 16 × 16 mm. To examine the vmPFC for threat buffering and safety learning, defined by box with the dimensions of 20 × 16 × 16 mm at the coordinates (NMI) x=0 y=42 z=-12 [14]. Correction for multiple comparisons within these ROIs was performed by using family-wise error correction based on the Gaussian Random Fields as implemented in SPM.

Supplementary results

Acquisition training

Association between trait and state anxiety and changes in AEA, 2-AG and AA plasma concentration

Post-hoc calculated explorative analyses testing for a potential association between individual anxiety level (State and Trait Anxiety Inventory, STAI) and eCB related blood plasma concentration changes according to acquisition training revealed a positive correlation between trait anxiety and the changes of AA plasma concentration (r=0.33, p_{uncorr}=0.025, see Table S6) during the acquisition training (T2-T1) and, albeit lower statistical support, also for the changes of AEA plasma concentration (r=0.29, p_{uncorr}=0.055, see Table S6) during acquisition training. The results might indicate that individuals with higher trait anxiety scores showed a stronger increase in AEA and AA plasma concentration during acquisition. Importantly, we did not correct these analyses for multiple comparisons, since these analyses were exploratory. We found no support of an association between baseline levels of AEA, 2-AG or AA with trait or state anxiety (p_{uncorr}>0.05, see Table S6). Taking together, there is initial support for an association between trait anxiety and the changes in AA concentration (and to some extend for AEA) during acquisition (T2-T1).

Main effects of neural brain responses during acquisition of threat responses

Fear Ratings. In line with single correlation analysis, regression analysis revealed a significant model for the change in differential fear ratings across acquisition (CS+ - CS- in T2-T1, N=45; F(3, 41)=4.51, p=0.008, adjusted R^2=0.193) included a positive association with the change of 2-AG during acquisition (T2-T1; t=2.84, p=0.007) and a negative association with the baseline levels of AEA (t=-2.41, p=0.021). This indicates that individuals with higher

baseline plasma concentration of AEA reported lower differential fear during the acquisition training. A similar association has been reported in an experiment of psychosocial stress in humans [7]. Our regression model furthermore included a positive association between the baseline level of AA (t=2.16, *p*=0.036), which indicates that individuals with higher baseline plasma concentration of AA reported higher differential fear during acquisition training.

US-expectancy. Similar to the fear ratings, a significant regression model (N=41; $F(3, 37)=3.71$, $p=0.015$, adjusted $R^2=0.231$) for the differential US-expectancy across acquisition (T2-T1) included a positive association with the change of 2-AG during acquisition (T2-T1; t=1.98, *p*=0.056). In line with fear ratings, the model for US-expectancy further suggests support for a negative association with the AEA baseline level (t=-1.71, *p*=0.096). Even though both results just barely missed significance, results are similar to findings in fear ratings. The regression model further provided support for a negative association of the anticipated circadian changes in 2-AG concentration (t=- 2.253, *p*=0.030).

SCR. We found a trend towards a regression model for change in differential SCRs during acquisition training $F(1, 36)=6.63$, $p=0.014$, adjusted $R^2=0.159$). In line with the correlational analyses, we found no statistical support for an association between SCRs and the changes in 2-AG. However, a negative association between SCRs and baseline concentration of AEA (t=-2.57, *p*=0.014) was found. Hence a lower behavioral fear response is associated with increased baseline concentration levels of AEA, which mirrors the regression models for fear ratings and US-expectancy.

Extinction training

Post-hoc calculated exploratory analyses of changes in AEA, 2-AG and AA during extinction learning included a reduced number of participants on day 2 (plasma concentration: N=21, fear ratings: n=21, US-expectancy: n=21, SCR: n=17, i.e. only participants in the placebo group; no effect of placebo pill on eCB plasma levels, see figure S2 and table S4).

Main effect of task

Over the time course of extinction training, participants still exhibited conditioned responses, measured as fear ratings, US-expectancy and SCR during extinction training, indicated by a main effect of CS-type in the rmANOVAs (main effect of CS-type: fear ratings(N=21): $F(1, 20)=12.14$, $p=0.002$, $\eta^2=0.38$; US-expectancy(N=21), $F(1, 20)=30.53$, $p<0.001$, $\eta^2=0.60$; SCR(N=17): $F(1, 16)=4.49$, $p=0.050$, $\eta^2=0.22$, see table S11 for full statistics), with higher responses to the CS+ as compared to the CS- (Bonferroni-Holm adjusted post- hoc tests $p<0.001$). Analyses of all outcome measures further revealed an interaction between CS-type and time (fear ratings(N=21): $F(1, 20)= 22.64$, $p<0.001$, $\eta^2=0.53$; US-expectancy(N=21): $F(1.26, 25.28) = 12.18$, $p=0.002$, $\eta^2=0.34$; SCR(N=17): $F(0.49, 0.38 =$

20.44, $p<0.001$, $\eta^2=0.56$), which reflected a steeper increase in responses to the CS+ during extinction.

Main effects of eCB and AA change

Exploratory rmANOVAs were conducted (placebo group only, N=21) to test for an effect of blood plasma concentration changes of AEA, 2-AG and AA during the time course of extinction training (before extinction, before intake of the placebo pill: T3, before extinction, after the intake of the placebo pill: T4, after extinction: T5). The analyses revealed a main effect of time for AEA ($F(1.44, 28.88)=4.42$, $p=0.018$, $f=0.47$) and AA ($F(1.54, 30.89)=22.96$, $p<0.001$, $f=1.07$). Bonferroni-Holm adjusted post hoc tests further showed an increase ($p<0.01$) for plasma concentration of AEA and AA when comparing concentration before and after extinction training (from T4 to T5; AEA (T4: $M=0.320$, $SD=0.148$, T5: $M=0.379$, $SD=0.141$) and AA (T4: $M=3677$, $SD=2248$, T5: $M=6410$, $SD=3732$). Furthermore an increase ($p<0.01$) in AEA and AA plasma concentration was found, when comparing baseline levels on day 2 (T3, before placebo administration) with plasma concentration after extinction training (from T3 to T5, AEA (T3: $M=0.296$, $SD=0.122$, T5: $M=0.379$, $SD=0.141$) and AA (T3: $M=2167$, $SD=2248$, T5: $M=6410$, $SD=3732$). There was no evidence for an increase ($p>0.05$) from baseline on day 2 to the time-point before extinction training (Mean difference (T4-T3): AEA=0.003, AA=-410.3). Consistent with results for acquisition training, we found no support for changes in 2-AG plasma concentration ($F(1.22, 24.48)=0.34$, $p=0.612$, $f=0.13$).

In summary, we found an increase in AEA and AA plasma concentration during extinction training. Due to the small sample size (N=21) no correlation analysis between individual reduction of conditioned threat responses during extinction and changes in AEA, 2-AG or AA was performed (see sensitivity analyses).

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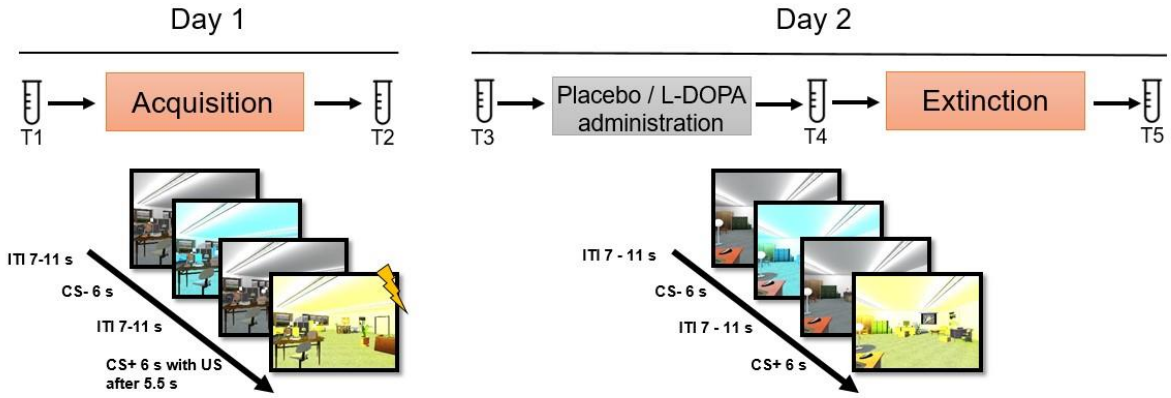

Figure S1. Experimental design: Timeline of blood plasma sampling of AEA, 2-AG and AA (T1-T5). Plasma concentrations were sampled before (T1) and after (T2) acquisition training (N=45) on the first day. Participants underwent extinction training on Day 2 (including eCB sampling T3-T5; part of another study). The acquisition and extinction training consisted each of 24 trials per CS (reinforcement rate of CS+: acquisition training 75%, extinction training 0%). Presentation times for ITIs and CSs are depicted next to the pictures.

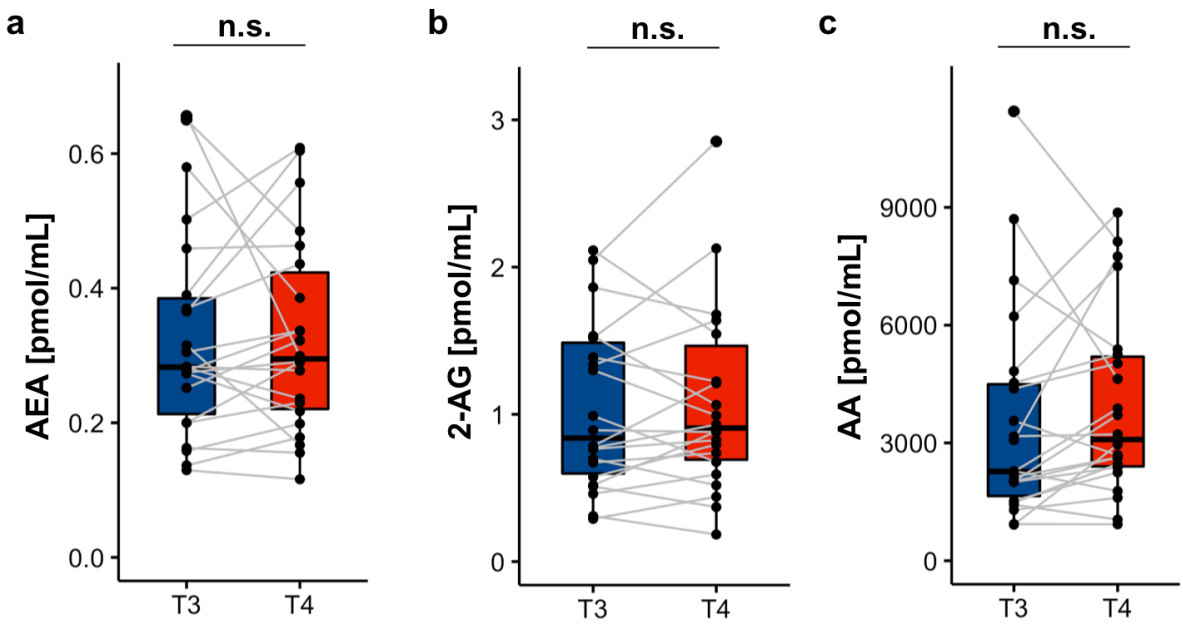

Figure S2. Illustration of pair-wise comparisons examining plasma concentration of **a)** AEA, **b)** 2-AG and **c)** AA before ingestion of a placebo pill (T3) and after (i.e., 60min) ingestion of a placebo pill (T4) twenty-four hours after acquisition training. n.s.=not significant, i.e. $p>0.05$

Table S1. Main effect of task during acquisition. Repeated-measures ANOVA showing conditioned responses of fear ratings, trial-wise US-expectancy ratings and skin conductance responses (SCR) during acquisition training.

Main effects of task during acquisition					
effect	**measure**	**N**	**F(df)**	***p***	**η^2**
CS-Type	Fear Ratings	45	(1, 44) = 74.47	<0.001***	0.63
	US-expectancy	41	(1, 40) = 184.83	<0.001***	0.82
	SCR	42	(1, 41) = 47.41	<0.001***	0.54
Time	Fear Ratings	45	(1, 44) = 77.24	<0.001***	0.64
	US-expectancy	41	(1.87, 74.96) = 0.66	.511	0.02
	SCR	42	(1.42, 58.09) = 6.16	0.009**	0.13
Time * CS	Fear Ratings	45	(1, 44) = 93.81	<0.001***	0.68
	US-expectancy	41	(1.83, 73.22) = 12.18	<0.001***	0.23
	SCR	42	(1.96, 80.27) = 1.02	0.364	0.02

*Note. The label * marks results if p < .05, the label ** marks results if p < .01, the label *** marks results if p < .001*

Table S2. Descriptives Day 1. Means and standard errors of fear ratings, trial-wise US-expectancy ratings and skin conductance responses during acquisition training (fear ratings include ratings before and after acquisition training; SCR and US-expectancy: each block represents an average across 8 trials).

Descriptives Day 1				
measure	**Time**	**CS**	***M***	***SE***
Fear Ratings	B1	CS+	9.78	11.95
		CS-	10.09	12.55
	B2	CS+	49.59	24.70
		CS-	9.50	15.34
	Mean Day 1	CS+	29.68	14.94
		CS-	9.79	11.83
US-expectancy	B1	CS+	0.75	0.24
		CS-	0.27	0.19
	B2	CS+	0.83	0.18
		CS-	0.17	0.16
	B3	CS+	0.79	0.21
		CS-	0.19	0.16
	Mean Day1	CS+	0.79	0.18
		CS-	0.21	0.15
SCR	B1	CS+	0.29	0.13
		CS-	0.22	0.14
	B2	CS+	0.258	0.145
		CS-	0.152	0.131
	B3	CS+	0.228	0.145
		CS-	0.132	0.122
	Mean Day1	CS+	0.255	0.127
		CS-	0.166	0.119

Table S3. Paired comparison of eCBs and AA during acquisition. Two-sided paired sample t-tests revealed an increase in AEA and AA concentration during acquisition training.

Paired comparison of eCBs and AA during acquisition							
plasma concentration (N=45)	timepoint	*M [pmol/mL]*	*SE [pmol/mL]*	T	df	*p*	*d*
AEA	T1	.2945	0.017	-4.151	44	<0.001***	-0.619
[pmol/mL]	T2	.3667	0.024				
2-AG	T1	1.101	0.086	0.45	44	0.655	0.067
[pmol/mL]	T2	1.075	0.0801				
AA	T1	3190.63	259.56	-5.03	44	<0.001***	-0.749
[pmol/mL]	T2	4789.24	418.45				

*Note. The label * marks results if p < .05, the label ** marks results if p < .01, the label *** marks results if p < .001*

Table S4. Paired comparison of eCBs and AA between T3 and T4. Control analysis of plasma concentrations twenty-four hours after acquisition training when participants were waiting for 60min (similar time window as between T1 and T2). Paired comparisons of AEA, 2-AG and AA plasma (N=22) before ingestion of a placebo pill (T3) and 60min after ingestion of a placebo pill (T4) with means and standard deviation.

Paired comparison of eCBs and AA between T3 and T4							
plasma concentration (N=22)	timepoint	*M [pmol/mL]*	*SE [pmol/mL]*	T	*df*	*p*	*d*
AEA	T3	0.285	0.020	0.10	20	0.919	0.02
[pmol/mL]	T4	0.285	0.019				
2AG	T3	1.157	0.154	0.61	20	0.548	0.13
[pmol/mL]	T4	1.080	0.109				
AA	T3	3062.55	339.19	-0.96	20	0.349	-0.21
[pmol/mL]	T4	3224.07	304.35				

Note. The label * marks results if $p < .05$, the label ** marks results if $p < .01$

Table S5. Correlation analysis of eCBs and AA with acquisition effect. Pearson correlation between AEA, 2-AG and AA plasma concentrations during acquisition training (T2-T1) and fear ratings, US-expectancy ratings and SCR (CS+-CS-) during acquisition. Each p-value for each plasma concentration is corrected for three measurements of the conditioned response using the Bonferroni-Holmes method.

Correlation analysis of eCBs and AA with acquisition effect											
Acquisition (T2-T1)			Endocannabinoids								
			AEA			2-AG			AA		
measure	CS	N	*r*	p_{uncorr}	p_{corr}	*r*	p_{uncorr}	p_{corr}	*r*	p_{uncorr}	p_{corr}
Fear Ratings	CS+-CS-	45	0.198	0.193	0.193	0.372	0.012*	0.036*	0.222	0.142	0.184
	CS+	45	0.232	0.126		0.306	0.041*		0.315	0.035*	
	CS-	45	0.014	0.926		-0.188	0.216		0.106	0.490	
US-expectancy	CS+-CS-	41	- 0.043	0.791	>0.99	0.346	0.027*	0.054	-0.030	0.850	0.850
	CS+	41	0.017	0.916		0.250	0.115		0.019	0.905	
	CS-	41	0.093	0.565		-0.280	0.076		0.074	0.645	
SCR	CS+-CS-	42	-0.058	0.732	0.732	-0.100	0.556	>0.99	-0.070	0.679	>0.99
	CS+	42	-0.221	0.189		-0.311	0.061		-0.232	0.167	
	CS-	42	-0.056	0.744		-0.208	0.216		-0.029	0.863	

*Note. The label * marks results if p < .05, the label ** marks results if p < .01, the label *** marks results if p < .001*

Table S6. Correlation of STAI with measures of acquisition. Pearson Correlation between the State Trait Anxiety Inventory (STAI) (both trait and state scores) and changes in AEA, 2-AG and AA plasma concentration during acquisition training (T2-T1), behavioral measures of conditioned responses (CS+-CS- in fear ratings, US-expectancy ratings and SCR) and AEA, 2-AG and AA plasma concentration prior to fear acquisition training. Depicted p-values are not corrected for multiple comparisons.

Correlation of STAI with measures of acquisition						
			STAI T		STAI S	
timepoints	measure	N	*r*	*p*	*r*	*p*
Acquisition (T2-T1)	AEA	45	0.288	0.055	0.147	0.337
	2-AG	45	-0.028	0.853	-0.097	0.527
	AA	45	0.334	0.025*	0.193	0.203
	Fear Ratings	45	-0.109	0.477	-6.359e -4	0.997
	US-expectancy	41	-0.352	0.024 *	-0.069	0.669
	SCR	42	0.092	0.589	-0.085	0.615
Baseline (T1)	AEA	45	0.066	0.665	0.102	0.504
	2-AG	45	0.665	0.191	0.504	0.056
	AA	45	0.068	0.657	0.073	0.634

Note. The label * marks results if $p < .05$, the label ** marks results if $p < .01$

Table S7. Correlation of US-intensity and US-valence with measures of acquisition. Pearson correlation between US-intensity and US-valence with changes in AEA, 2-AG and AA plasma concentration (T2-T1) during acquisition training, behavioral measures of conditioned responses (CS+-CS- in fear ratings, US-expectancy ratings and SCR) and AEA, 2-AG and AA plasma concentration prior to fear acquisition training. Depicted p-values are not corrected for multiple comparisons.

Correlation of US-intensity and US-valence with measures of acquisition						
			US-intensity		US-valence	
timepoint	measure	N	*r*	*p*	*r*	*p*
Acquisition (T2-T1)	AEA	45	0.037	0.818	-0.195	0.227
	2-AG	45	-0.063	0.695	0.087	0.595
	AA	45	-0.139	0.387	-0.006	0.972
	Fear Ratings	45	-0.193	0.204	0.216	0.159
	US-expectancy	41	-0.039	0.607	-0.169	0.297
	SCR	42	-0.175	0.300	0.118	0.494
Baseline (T1)	AEA	45	-0.017	0.917	0.050	0.760
	2-AG	45	-0.047	0.772	0.132	0.415
	AA	45	-0.048	0.763	0.0472	0.774

Note. The label * marks results if $p < .05$, the label ** marks results if $p < .01$

Table S8. fMRI main effects of acquisition. Main effects of neural brain responses during acquisition training, reflecting the conditioned threat response (CS+>CS-), as well as the US. Results are solely depicted for calculated regions of interest analyses, namely amygdala, dorsal ACC, insula cortex and hippocampus.

fMRI main effects of acquisition						
main effects	ROIs	T	*p* FEW corrected	*P* uncorr	z	Coordinates [NMI]
CS+ > CS-	L Amygdala	4.26	0.009 **	<0.001	3.84	x=-26 y=-8 z=-12
	R Amygdala	4.95	0.002 **	<0.001	4.33	x=26 y=-12 z=-12
	L Insula	6.35	<0.001 ***	<0.001	5.23	x=-33 y=6 z=8
	R Insula	7.54	<0.001 ***	<0.001	5.89	x=34 y=18 z=4
	dACC	7.28	<0.001 ***	<0.001	5.75	x=-4 y=28 z=26
	R Hippocampus	4.38	0.015 *	<0.001	3.92	x=22 y=-38 z=0
US	L Amygdala	4.76	0.002 **	<0.001	4.20	x=-20 y=-3 z=-15
	R Amygdala	5.68	<0.001 ***	<0.001	4.82	x=22 y=0 z=-16
	L Insula	7.00	<0.001 ***	<0.001	5.60	x=-40 y=-6 z=-10
	R Insula	7.57	<0.001 ***	<0.001	5.90	x=39 y=4 z=-15
	dACC	7.06	<0.001 ***	<0.001	5.63	x=2 y=22 z=28
	L Hippocampus	4.38	0.015 *	<0.001	3.93	x=-21 y=-26 z=-12

Note. The label * marks results if $p < .05$, the label ** marks results if $p < .01$

Table S9. Regressor effects (AEA, 2-AG and AA – positive and negative) in fMRI regression model on association between neural brain responses and changes in eCBs and AA during acquisition training.

fMRI multiple regression: CS+ > CS- with eCBs and AA during acquisition						
measure	ROIs	T	*p* FEW corrected	*P* uncorr	z	coordinates
AEA positive	R Amygdala	3.91	0.028 *	<0.001***	3.54	x=27 y=-4 z=-16

Note. The label * marks results if $p < .05$, the label *** marks results if $p < 0.001$

Table S10. Regressor effects (AEA, 2-AG and AA – positive and negative) in fMRI regression model on association between modeled linear temporal dynamics of neural responses towards the US and changes in eCBs and AA during acquisition training.

fMRI modelled linear temporal dynamics of US with eCBs and AA during acquisition						
measure	ROIs	T	*p* FEW corrected	*P* uncorr	z	coordinates
AEA positive	dACC	4.92	0.008 **	<0.001	4.27	x= 0 y= 34 z= 27
2-AG positive	L Amygdala	3.62	0.048*	<0.001	3.32	x= -15 y= -6 z= -20
	L Insula	3.86	0.064	<0.001	3.51	x= -42 y= 6 z= -3
	L Hippocampus	4.69	0.008**	<0.001	4.12	x= -15 y= -9 z= -20
2-AG negative	R Amygdala	3.44	0.084	<0.001	3.18	x= 30 y=-2 z=-27

Note. The label * marks results if $p < .05$, the label ** marks results if $p < .01$

Table S11. Main effect of task during extinction. Effects of repeated measures ANOVA analysing the acquired conditioned response from fear ratings, trial-wise US-expectancy and skin conductance responses (SCR) during extinction training.

Main effects of tasks during extinction					
effect	**measure**	**N**	**F**	***p***	**η^2**
CS-Type	Fear Ratings	21	(1, 20) = 12.14	0.002**	0.38
	US-expectancy	21	(1, 20) = 30.53	<0.001***	0.60
	SCR	17	(1, 16) = 4.49	0.050	0.22
Time	Fear Ratings	21	(1, 20) = 15.66	<0.001***	0.44
	US-expectancy	21	(1.22, 24.42) = 16.95	<0.001***	0.46
	SCR	17	(1.28, 20.67) = 35.70	<0.001***	0.69
Time * CS	Fear Ratings	21	(1, 20) = 22.64	<0.001***	0.53
	US-expectancy	21	(1.26, 25.28) = 12.18	0.002**	0.34
	SCR	17	(0.49, 0.38 = 20.44	<0.001***	0.56

*Note. The label * marks results if p < .05, the label ** marks results if p < .01, the label *** marks results if p < .001*

Eidesstattliche Versicherung

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