NEURAL MECHANISMS AND PHARMACOLOGICAL MODULATION OF PAVLOVIAN LEARNING

DISSERTATION

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EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich,

- dass ich die vorliegende Arbeit selbstständig und ohne unerlaubte Hilfe verfasst habe,
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- dass mir die Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät II vom 17.01.2005, zuletzt geändert am 13.02.2006, veröffentlicht im Amtlichen Mitteilungsblatt Nr. 34/2006 bekannt ist.

Berlin, 29.08.2019 Claudia Ebrahimi

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TABLE OF CONTENTS

ABSTRACT

Pavlovian learning mechanisms are thought to play an important role in the development, maintenance, and relapse of psychiatric conditions like drug addiction and anxiety disorders. Although extinction learning can reduce conditioned responding towards drug- or fear-associated cues, animal research has convincingly characterized conditions that commonly result in return of fear or drug intake despite successful extinction. These Pavlovian relapse phenomena challenge the long-term success of extinction-based exposure treatments. As such, investigating pharmacological adjuncts that could help to improve extinction learning or long-term retention are of great clinical importance.

This dissertation comprises four studies applying translational human laboratory models of Pavlovian learning (i) to characterize the behavioral and neural mechanisms of appetitive Pavlovian relapse (*Studies I* and *II*), and (ii) to investigate D-cycloserine (DCS), a partial NMDA receptor agonist, as a pharmacological adjunct to augment Pavlovian extinction learning of appetitive and aversive stimuli (*Studies III* and *IV*).

In contrast to accumulating knowledge acquired in the domain of human fear conditioning, translational research on appetitive Pavlovian learning and relapse effects is still in its infancy. In *Study I*, we showed that appetitive Pavlovian relapse can be successfully modeled in the laboratory and provided evidence for opposing roles of amygdala and vmPFC in mediating the return of conditioned responding. As the scarcity of appetitive research has been partly attributed to a lack of established measures sensitive to quantify conditioned responding, *Study II* showed the usefulness of different and partly novel ocular response measures for appetitive conditioning research. Finally, *Studies III* and *IV* used a double-blind, placebo-controlled fMRI design to investigate the effect of DCS-augmented appetitive (*Study III*) and aversive extinction learning (*Study IV*). We found that DCS attenuated amygdala reactivity during appetitive extinction recall and enhanced amygdala-vmPFC coupling (*Study III*). Corroborating these results, *Study IV* showed DCS to reduce return of fear on behavioral arousal ratings and in brain areas associated with defense reactions like amygdala and posterior hippocampus.

Overall, the present work extends evidence on experimentally induced return of fear to the appetitive research domain and suggests an overarching regulatory role of the vmPFC during extinction recall. Finally, it supports the hypothesis that DCS can augment extinction learning, thereby reducing the risk of relapse phenomena.

ZUSAMMENFASSUNG

Einige psychische Störungen, darunter Angst- und Suchterkrankungen, zeichnen sich durch eine abnorme Beteiligung basaler assoziativer Lernprozesse aus. Obwohl Extinktionslernen konditionierte Reaktionen auf angst- oder suchtassoziierte Reize reduziert, existieren verschiedene Pawlow'sche Rückfallphänomene, die zum Wiederauftreten von Angst und Substanzkonsum trotz erfolgreicher Extinktion beitragen und damit den langfristigen Erfolg extinktionsbasierter Therapien gefährden. Damit kommt der Untersuchung pharmakologischer Interventionen zur Unterstützung des Extinktionslernens bzw. –abrufs eine zentrale Bedeutung zu.

Die vorliegende Dissertation umfasst vier Studien und bedient sich translationaler Pawlow'scher Lernmodelle, um (i) behaviorale und neuronale Mechanismen appetitiver Pawlow'scher Rückfallphänomene beim Menschen zu untersuchen (*Studien I* und *II*) sowie (ii) den Effekt des partiellen NMDA Rezeptor Agonisten D-Cycloserin (DCS) zur Unterstützung des Extinktionslernens appetitiver und aversiver Stimuli zu testen (*Studien III* und *IV*).

Die Untersuchung appetitiver Pawlow'scher Lern- und Rückfallprozesse beim Menschen steht noch am Anfang. *Studie I* demonstriert, dass appetitive Pawlow'sche Rückfalleffekte im Labor untersucht werden können und lieferte Evidenz für differenzielle Einflüsse der Amygdala und des vmPFC beim Wiederauftreten der konditionierten Reaktion. *Studie II* belegt die Sensitivität verschiedener, teilweise neuer okularer Reaktionsmaße für die appetitive Konditionierungsforschung. *Studie III* und *IV* nutzen ein doppelt-verblindetes, Placebo-kontrolliertes fMRT Design, um den Effekt des DCS-unterstützten Extinktionslernens zu untersuchen. *Studie III* zeigte, dass DCS mit einer attenuierten BOLD-Antwort in der Amygdala und einer gesteigerten funktionellen Amygdala-vmPFC Konnektivität während des appetitiven Extinktionsabrufs assoziiert war. *Studie IV* ergab, dass Probanden der DCS-Gruppe attenuierte Arousal Ratings wie auch neuronale Aktivierungen in der Amygdala und dem posterioren Hippocampus im Vergleich zur Placebo-Gruppe aufwiesen.

Die vorliegende Arbeit erweitert unser Verständnis appetitiver Pawlow'scher Rückfallphänomene und weist dem vmPFC eine bedeutsame Rolle beim Extinktionsabruf zu. Weiterhin unterstützt sie die Hypothese, dass DCS das Extinktionslernen unterstützt und damit Rückfallphänomene reduziert.

LIST OF ORIGINAL ARTICLES

This thesis is based on the following original research articles:

Study I

Ebrahimi, C., Koch, S. P., Pietrock, C., Fydrich, T., Heinz, A., & Schlagenhauf, F. (2019). Opposing roles for amygdala and vmPFC in the return of appetitive conditioned responses in humans. *Translational Psychiatry*, 9:148. https://doi.org/10.1038/s41398-019-0482-x.

Springer Nature kindly permits the use of the article for this thesis.

Study II

Pietrock, C, **Ebrahimi, C.**, Katthagen, T. M., Koch, S. P., Heinz, A., Rothkirch, M., & Schlagenhauf, F. (2019). Pupil dilation as an implicit measure of appetitive Pavlovian learning. *Psychophysiology,* 00:e13463. https://doi.org/10.1111/psyp.13463.

Wiley kindly permits the use of the article for this thesis.

Study III

Ebrahimi, C., Koch, S. P., Friedel, E., Crespo, I., Fydrich, T., Ströhle, A., Heinz, A., & Schlagenhauf, F. (2017). Combining D-cycloserine with appetitive extinction learning modulates amygdala activity during recall. *Neurobiology of Learning and Memory*, *142*, 209–217.

Elsevier kindly permits the use of the article for this thesis.

Study IV

Ebrahimi, C.*, Gechter, J.*, Lueken, U., Schlagenhauf, F., Wittchen, H.-U., Hamm, A. O., & Ströhle, A. (in revision). Augmenting extinction learning with D-cycloserine reduces return of fear: a randomized, placebo-controlled fMRI study. *Neuropsychopharmacology*.

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LIST OF ABBREVIATIONS

BOLD	blood oxygenation level dependent
CET	cue-exposure therapy
CS	conditioned stimulus
CR	conditioned response
DCS	D-cycloserine
fMRI	functional magnetic resonance imaging
HR	heart rate
IL	infralimbic cortex
NAcc	nucleus accumbens
NMDA	N-methyl-D-aspartate
PPI	psychophysiological interaction analysis
PAR	postauricular reflex
ROI	region of interest
SCR	skin conductance responses
US	unconditioned stimulus
vmPFC	ventral medial prefrontal cortex

LIST OF FIGURES

LIST OF TABLES

1 INTRODUCTION

"*When I go along the streets on a cold night and I see the warm lights shining from a bar and I hear the clinging of glasses – I am lost.*"

Anonymous patient

In order to survive in a constantly changing environment, individuals need to flexibly adapt their behavior. Environmental cues play an important role in guiding individuals to successfully approach desirable outcomes and avoiding danger via associative learning processes. Conversely, in the case of psychiatric conditions like addiction or anxiety disorders, these learning processes become maladaptive [1–4]. Instancing the case of addiction, environmental stimuli associated with drugs of abuse acquire motivational properties via Pavlovian conditioning and can act as powerful motivators for repeated drug use, thereby undermining the goal to stay abstinent, as exemplified by the above patients' quote (cited by A. Heinz, personal communication).

With that in mind, Pavlovian conditioning and extinction paradigms are thought to be valuable models to study the development, treatment, and relapse of these maladaptive learned associations in animals and humans [5,6]. One major challenge faced by extinction-based therapies for both anxiety and drug addiction is that conditioned responses (CRs) can easily recover despite successful extinction [7], which likely contributes to clinical relapse. Therefore it is of major clinical importance to understand the underlying behavioral and neural mechanisms of relapse behavior and to think of ways to counter it, for example by boosting extinction learning. However, in contrast to the accumulating evidence gained from learning models of fear [8,9], translational laboratory models of appetitive Pavlovian relapse phenomena are largely missing.

In the following, four original articles will be summarized that aim to shed light on the neural mechanisms and pharmacological modulation of Pavlovian learning processes. Specifically, in the first two studies we developed a translational laboratory model of appetitive Pavlovian learning and elucidated neural mechanisms mediating appetitive Pavlovian relapse effects, whereas in the last two studies we focused on the pharmacological modulation of extinction learning to attenuate these relapse effects across valence domains.

1.1 ROLE OF PAVLOVIAN LEARNING IN ADDICTION AND ANXIETY

Various lines of research have shown that basic associative learning mechanisms like Pavlovian and instrumental conditioning play an important role in the pathogenesis of anxiety and substance use disorders [2,3]. In Pavlovian conditioning, initially neutral environmental stimuli become conditioned stimuli (CS) through repeated pairing with a positive or negative reinforce (termed unconditioned stimulus, US), e.g. drugs of abuse or a threatening event. As a consequence, the CS is able to elicit a variety of CRs originally provoked by the US on its own. In posttraumatic stress disorder, for example, stimuli associated with the traumatic event can cause pathological conditioned fear responses and re-experiencing of the event [10]. Likewise in drug addiction, environmental stimuli present during drug intake become associated with the rewarding effects of the drug and elicit craving, which contributes to repeated drug use and relapse even after long phases of abstinence [11].

Exposure-based treatment approaches that target these maladaptive memories rely on mechanisms of extinction learning [5,6]. Extinction involves repeated CS presentations in the absence of the US, causing the CR to decline [12]. According to the inhibitory theory of extinction [7], extinction is an active learning process that results in a new, inhibitory memory that henceforth competes with the original excitatory memory for behavioral expression. As such, rodent work has demonstrated that CRs can recover under certain conditions, including the mere passage of time (spontaneous recovery), an unexpected encounter with the US (reinstatement), or a shift in context (renewal) [7,13].

Neural circuits of appetitive and aversive relapse

Preclinical work suggests overlapping neural circuits to be involved in appetitive and aversive Pavlovian relapse phenomena, including the amygdala, hippocampus, and prefrontal cortex [14-16]. The amygdala plays a central role during acquisition, extinction and expression of aversive and appetitive CRs [17–19]. Converging evidence implicated the amygdala in initial CS-US formation [20,21] and lesions of this structure have been shown to prevent both renewal and reinstatement of fear [22,23] and drug-seeking [24,25], supporting a wide-ranging role in relapse phenomena. In contrast, the infralimbic cortex (IL), assumed to constitute the rodent homologue of the human ventromedial prefrontal cortex (vmPFC) [8], is critical for successful extinction recall and regulates the return of both appetitive and aversive CRs after

extinction [14,16]. For example, pharmacological inactivation of the IL did not affect within-session extinction, but impaired long-term retrieval, suggesting it as a central site of extinction memory consolidation [26–30]. This regulatory role is thought to rely in part on projections to the amygdala, providing top-down control to inhibit CRs [15,16,31], although the role of IL-amygdala projections in return of drug-seeking behavior is less clear than in relapse of fear [14,32]. Furthermore, hippocampal engagement has been shown especially in context-sensitive relapse phenomena like renewal of fear, possibly mediated via ventral hippocampal projections to the prefrontal cortex and amygdala [33–35]. In addition, expression of conditioned approach behavior towards food or drug cues depends on an intact nucleus accumbens (NAcc) [36–38], a key structure for reward-related learning [39]. Animal models of drug-seeking have demonstrated amygdala projections to the NAcc to be involved in drug reinstatement [32], thereby extending the key neural structures supporting Pavlovian relapse in the appetitive domain.

1.2 TRANSLATIONAL HUMAN MODELS OF PAVLOVIAN RELAPSE

The neural circuits subserving Pavlovian conditioning, extinction, and relapse have been best described within the context of fear, and enormous progress has been made by translational research in this domain to extend these findings to humans [40]. In contrast, human research on appetitive Pavlovian learning processes is still in its infancy [41,42], especially the investigation of extinction learning and Pavlovian relapse effects [43–45]. This relative lack of research is remarkable, given the importance of Pavlovian processes in addiction and the limited efficacy of exposure-based treatments for substance use disorders [46,47]. Problems in finding universally rewarding USs comparable in intensity to the ones typically used in fear conditioning (i.e. electric shock), along with a lack of established measures sensitive to appetitive CRs [48–50] might account for this shortcoming.

Aversive human conditioning paradigms typically employ a differential conditioning procedure, whereby one stimulus (CS+) is repeatedly paired with an aversive US, e.g. loud noise or electric shock, while a second stimulus (CS-) is not, thereby controlling for initial orienting responses or overall habituation effects when contrasting both stimuli [51]. Using such paradigms, return of extinguished fear responses following a change in context (renewal) or unsignaled US presentations (reinstatement) has been demonstrated in humans on multiple response systems, including subjective ratings [52–54], SCRs [55–57], fear-potentiated startle [58–60]

and neuroimaging[61–63]. Of note, studies relying on more than one outcome measure to quantify the return of CRs often report diverging findings between different response measures[62,64]. Overall, the neural structures mediating return of fear in animal models have been widely confirmed in neuroimaging studies, suggesting them to be generally preserved across species. As such, enhanced amygdala activation has been observed in functional magnetic resonance imaging (fMRI) studies probing return of fear due to reinstatement or renewal[61,65–67]. Imaging studies further point to a specific role for the vmPFC in successful extinction recall[68] and inhibition of conditioned fear responses[66,69,70], which has been associated with increased amygdala-vmPFC functional connectivity[69,71,72] (but see[66]). In contrast, return of fear following reinstatement has been associated with decreased vmPFC involvement, while increasing blood oxygenation level dependent (BOLD) responses in structures like amygdala and hippocampus[56,61].

So far, the neural mechanisms guiding appetitive Pavlovian relapse in humans remain largely unknown. To our knowledge, experimentally induced return of appetitive CRs has been only demonstrated in US expectancy ratings[43,44], while more implicit, psychophysiological readout measures have not been evaluated. Therefore, it remains to be shown whether appetitive Pavlovian relapse effects can be modeled in a laboratory setting in order to investigate the conditions and neural structures mediating Pavlovian relapse effects.

1.3 PHARMACOLOGICAL MODULATION OF EXTINCTION LEARNING

Although cognitive-behavioral therapy incorporating exposure therapy is a first-line treatment in anxiety disorders[73,74], not all patients achieve complete symptom remission and relapse is frequently observed[75]. For addiction, the long-term success of currently available treatments remains poor, with relapse rates between 40 % and 60 % within one year post-treatment[76]. Evidence for the efficacy of cue-exposure therapy (CET) for substance use is limited and several methodological problems have been discussed that might prevent it from exploiting its full potential[46,47]. However, pharmacological adjuncts might be able to improve the efficacy of CET.

One pharmacological candidate to act as a cognitive enhancer is the partial N-methyl-D-aspartate (NMDA) receptor agonist D-cycloserine (DCS), which binds at the glycine site of the NMDA glutamate receptor, thereby increasing its activation probability[77]. Research has documented NMDA receptor involvement in synaptic plasticity, learning and memory[78,79].

Preclinical work on extinction of fear- and drug-paired cues has demonstrated that systemic administration as well as direct infusion of DCS into central structures of the extinction circuit – i.e. amygdala or hippocampus – enhanced extinction learning and prevented some Pavlovian relapse effects, such as spontaneous recovery[78,80,81]. DCS was also effective when administered shortly after extinction learning, suggesting it to primary support the consolidation of extinction memory[81].

Initial evidence from clinical trials on DCS-augmented exposure therapy for anxiety disorders revealed large effect sizes in favor of DCS[82,83]. However, meta-analytic investigations including more and larger RCTs have demonstrated smaller effect sizes[84–87], suggesting that the effect of DCS might depend on specific moderators. For example, Smits and colleagues[88,89] found that the effect of DCS depends on extinction success, as only patients with appropriate fear reduction at the end of the exposure sessions improved under DCS, while the opposite pattern emerged in those with high fear levels at the end of exposure. In the latter case, DCS might have promoted fear reconsolidation rather than extinction learning, suggesting that DCS could even have detrimental effects under certain conditions. It has also been suggested that DCS might primarily speed up treatment response, such that patients achieve symptom reduction earlier in treatment and that this benefit vanishes with more exposure sessions[90]. However, none of these potential moderators could be clearly confirmed in the latest individual patient data meta-analysis[87], which highlights the need for more research on how and under which conditions DCS exerts its therapeutic effect.

Compared to the field of anxiety, far less studies have investigated the effect of DCS as an adjunct to improve outcomes in CET for substance use disorders. Santa Ana and colleagues[91] were the first who reported that DCS-augmented CET for smoking cessation reduced subjective and physiological cue-reactivity compared to placebo, although effects on smoking behavior were not significant. Overall, more null than positive effects have been reported from this line of research[78,92]. Given the promising preclinical results and the clinical evidence from DCS-augmented exposure therapy in anxiety disorders, several methodological factors have been discussed that may account for some negative results.

In line with the notion that the efficacy of DCS might depend on extinction success[88,89], these critiques include insufficient reductions in craving at the end of CET sessions[93,94] and concerns about reconditioning experiences under the influence of DCS between CET-sessions in studies that did not control for between-session drug use[94,95]. More recent studies controlling for between-session sensitization experiences indeed found DCS-augmented CET

to reduce ventral striatal cue reactivity in alcohol dependent patients[96]. It further reduced self-reported craving and skin conductance reactivity to smoking cues in smokers, which was associated with a moderate-to-large, albeit non-significant effect, on follow-up abstinence rates (33 % DCS vs. 13 % placebo) [97].

Taken together, the inconsistent findings between preclinical and clinical work call for a deeper understanding of the precise effect of DCS on appetitive as well as fear extinction learning and recall and its underlying mechanism of action in humans. Moreover, the neural structures involved in DCS-augmented human extinction learning had not been investigated thus far. The few translational human studies using Pavlovian learning models to investigate the effect of DCS-augmented extinction learning in a controlled setting remained inconclusive. While DCS-augmented extinction learning had no effect on SCRs[98,99] or startle responses[98] during delayed fear extinction recall, SCRs were attenuated after a reactivation procedure (i.e. recall after a CS-US reactivation trial) [100] and only one study investigated the effect of DCS during appetitive extinction learning[101]. The authors administered DCS or placebo after context conditioning and extinction of sexual responses in females and found no group differences during simple recall, but attenuated subjective and physiological CRs when tested outside the extinction context. Although promising, concomitant conditioning and extinction learning in one session complicates the interpretation of these results.

2 OWN RESEARCH WORK

This chapter outlines the main research questions this thesis aims to shed light on, introduces core design and methodological aspects, and summarizes the main findings of each of the four studies.

2.1 RESEARCH OBJECTIVES

Based on the evidence outlined in Chapter 1, the following research questions arise:

1. Can appetitive Pavlovian relapse effects be observed in healthy participants in a laboratory setting? If so, which neural structures are involved in these effects and mediate individual relapse intensity? (*Study I*)
2. Which implicit response measures prove to be sensitive to evaluate appetitive CRs in human laboratory models of Pavlovian learning? (*Studies I* and *II*)
3. Can DCS enhance long term recall of extinguished appetitive and aversive CSs, i.e. reduce associated Pavlovian relapse phenomena in humans? If so, which neural structures are involved in DCS-augmented appetitive and aversive extinction learning? (*Studies III* and *IV*)

2.2 RESEARCH METHODS

Studies I - III were part of a DFG-funded research group investigating learning in alcohol dependence (FOR 1617). *Study III* originated from an additional collaboration with the multicenter national research network "Panic-Net" (2nd funding period), which further included *Study IV*. In order to investigate the above outlined research questions, the articles spanning the present thesis employed an appetitive and/or aversive conditioning paradigm in combination with a multimodal approach including fMRI as well as explicit and implicit conditioning[102]. Healthy participants were recruited from student mailing lists (Freie Universität Berlin, Humboldt Universität zu Berlin, Technische Universität Berlin, Charité – Universitätsmedizin Berlin; *Studies I* and *II*), as well as via local advertisement in Berlin (*Studies III* and *IV*) and Dresden (*Study IV*).

2.2.1 PAVLOVIAN LEARNING PARADIGMS

Except for *Study II*, which used a one-day design to investigate ocular response measures during appetitive conditioning, all studies employed a three-day design comprising a conditioning session (day 1), extinction learning (day 2), and an extinction recall/reinstatement test (day 3). These phases were spaced 24 hours apart to allow for memory consolidation between sessions, thereby representing a more ecologically valid model for Pavlovian learning and relapse [102], and necessary to evaluate DCS effects. Figure 1 shows the three-day design and conditioning paradigm of *Study I*. During conditioning (day 1), one stimulus (CS+) is repeatedly paired with a US, while another stimulus (CS-) is never followed by the US. The acquired CS–US association is extinguished on day 2, where only unreinforced CS+ and CS- trials are presented. Finally, the return of conditioned responding is tested during unreinforced CS+/CS- presentations, which in the case of *Study I* took place after a reinstatement procedure with unsignaled US administrations.

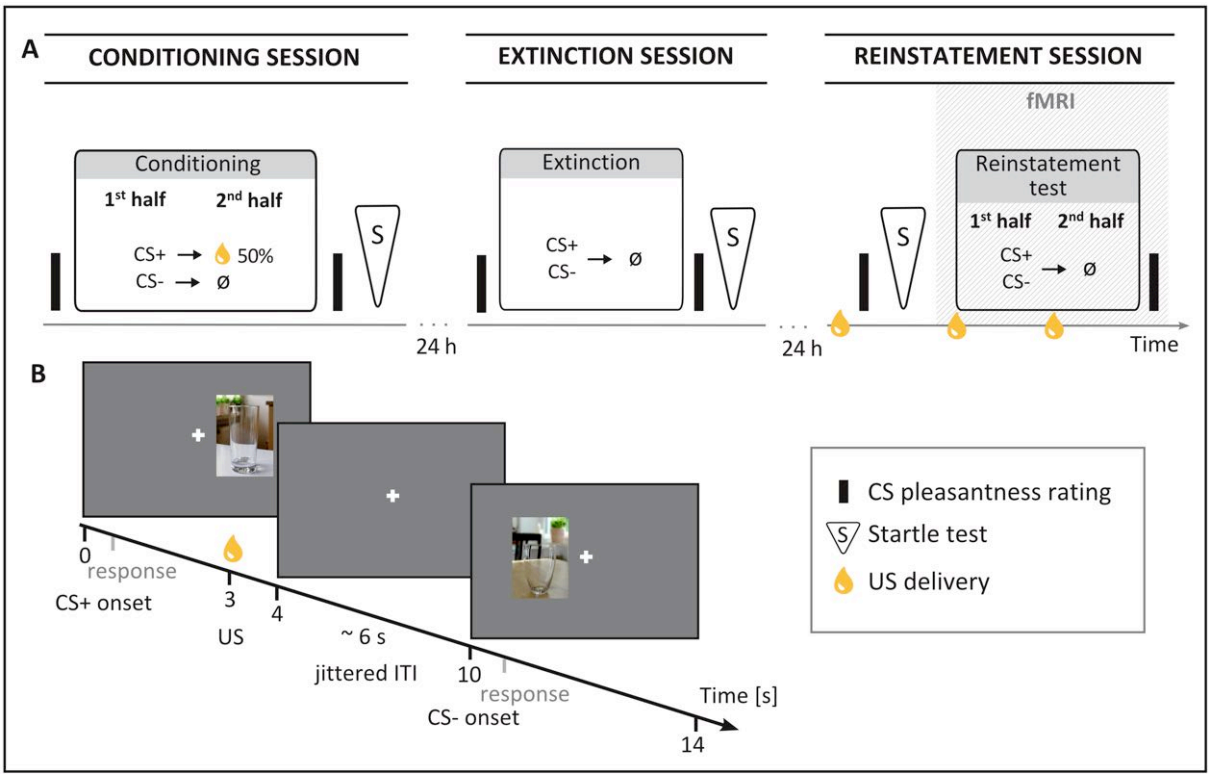

Figure 1 Pavlovian learning paradigm of *Study I*. A Three-day design with appetitive conditioning on day 1, extinction on day 2, and a reinstatement test on day 3. Conditioning comprised 60 CS+/CS- trials, extinction and the reinstatement test comprised 30 unreinforced CS+/CS- each, presented in pseudorandom order. Return of appetitive CRs on day 3 was probed after a reinstatement procedure (3 unsignaled US administrations) occurring once before the startle test and twice during the fMRI reinstatement test. SCRs, HR, and RTs were acquired continuously during each session. Acoustic startle tests and CS pleasantness ratings were conducted separately at different time points. **B** Exemplary trial sequence during conditioning (day 1): In each trial, one out of two different cues was

presented either on the left or right side of a fixation cross for 4 s. In half of the CS+ trials, 1 ml of subjects' preferred liquid food (US) was delivered 3 s after cue onset (50 % reinforcement schedule).

Table 1 summarizes important characteristics of the paradigms used in each study. *Studies I*, *II* and *IV* employed a delay conditioning design on day 1, where CS+ and US co-terminate with each other, whereas *Study III* used a trace conditioning design, in which CS+ and US are separated by a temporal delay (in this case 3 seconds). Moreover, *Study III* aimed to investigate appetitive and aversive conditioning in one paradigm, using monetary wins and losses as appetitive and aversive USs, respectively. Importantly, while *Study III* used a secondary reinforcer (money) as US, *Studies I* and *II* used a primary reinforcer, namely liquid food (fruit juice), delivered directly into the subject's mouth via a programmable syringe pump.

2.2.1 MULTIMODAL ASSESSMENT OF CONDITIONED RESPONDING

Human Pavlovian learning can be described on different response levels, including subjective reports, psychophysiological responses, behavioral reactions (i.e. approach or avoidance) or neurobiological changes[51]. Since different response measures represent different dimensions of Pavlovian learning and hence do not necessarily converge, recent methodological recommendations advocate a multimodal approach to assess CRs[49,51,102]. This is of special importance in appetitive conditioning research, where CRs are comparably weak and there is (yet) no established gold standard measure to assess conditioned responding[42,49].

The outcome measures used in each study are summarized in Table 1. The most common psychophysiological measure in human fear conditioning is the skin conductance response (SCR) [51], a phasic increase in electrodermal activity elicited by salient stimuli that reflects sympathetic arousal[103], which was acquired continuously in all studies of this thesis. In addition, *Study I* further assessed heart rate (HR) changes as well as two acoustic startle reflexes, which are modulated by stimulus valence, namely the eyelid reflex[41] and the postauricular reflex (PAR) [48] in separate post-session startle tests (see also Figure 1). *Study II* then investigated the sensitivity of ocular response measures (pupil dilation, gaze dwelling time, blink count and duration) as outcome measures of appetitive conditioned responding in addition to the abovementioned psychophysiological conditioning indices. All studies acquired subjective ratings, like CS valence (*Studies I–IV*), arousal (*Studies II* and *IV*), or attractiveness (*Study II*), and assessed contingency knowledge after the conditioning session.

As a behavioral measure of conditioning, *Studies I* and *III* acquired trialwise reaction times (RTs) obtained via cue (*Study I*) or outcome (*Study III*) discrimination.

2.2.2 Functional Magnetic Resonance Imaging

Studies I, III and *IV* used fMRI to infer neuronal activation in cortical and subcortical brain areas during Pavlovian conditioning, extinction, and extinction recall in three event-related paradigms. This method is based on the BOLD response, representing an indirect measure of neuronal activation [104].

2.2.3 DCS ADMINISTRATION IN STUDIES III AND IV

To test the hypothesis that DCS can enhance extinction learning by supporting post-learning memory consolidation, investigated in *Studies III* and *IV*, participants received 50 mg of DCS or placebo one hour before extinction training under double-blind conditions, as 50 mg of DCS have been shown to enhance fear exposure-therapy in anxiety disorders [86] and plasma concentration peaks approximately 1-2 hours after ingestion [105].

2.2.4 STATISTICAL ANALYSES

fMRI Analyses

All imaging analyses were performed within the general linear model approach of SPM (www.fil.ion.ucl.ac.uk/spm/) using region of interest (ROI) analyses at $p < .05$ family wise error (FWE) correction, complemented with exploratory whole-brain analyses. *Studies I* and *III* further investigated the cue-dependent functional connectivity between the amygdala and the vmPFC during appetitive extinction recall using psychophysiological interaction (PPI) analysis [106,107], a measure of change in the functional association between both regions depending on the experimental condition (CS+ vs. CS-).

Psychophysiological Modeling of Skin Conductance Data

Analysis of skin conductance data was performed using a model-based approach that explicitly formalizes how sudomotor nerve activity elicited by sympathetic arousal generates measured SCRs, thereby allowing for separation of SCRs in fast event-related designs and increasing the signal-to-noise ratio [108–110].

Table 1: Characteristics of Pavlovian learning paradigms used in *Studies I-IV*

Study	Design	CS-US contingency	CS+/CS-	US	Sessions	Outcome measures
Study I	Delay	50 %	pictures of empty glasses	participant's preferred juice	conditioning (day 1) extinction (day 2) extinction recall after reinstatement (day 3)	valence & contingency ratings, SCRs, HR, RTs, startle reflexes, BOLD responses (day 3 only)
Study II	Delay	50 %	female faces with neutral expression	participant's preferred juice	conditioning (day 1)	valence, arousal, attractiveness & contingency ratings, forced-choice preference ratings, SCRs, HR, startle reflexes, pupillary responses (pupil dilation, gaze dwelling time, blink count, blink duration), US expectancy ratings
Study III	Trace	100 %	geometric figures	+2€ coin image (appetitive US) -2€ coin image (aversive US) blurred coin image ('noUS')	conditioning (day 1) extinction (day 2) extinction recall after reactivation trials (day 3)	valence (day 1 only) & contingency ratings, RTs, SCRs, BOLD responses
Study IV	Delay	100 %	male faces with neutral expression	auditory panic scream	conditioning (day 1) extinction (day 2) extinction recall (day 3)	valence, arousal & contingency ratings, SCRs, BOLD responses

SCR: skin conductance response; HR: heart rate; RT: reaction time

2.3 SUMMARY OF RELATED ARTICLES

2.3.1 STUDY I: OPPOSING ROLES FOR AMYGDALA AND VMPFC IN THE RETURN OF APPETITIVE CONDITIONED RESPONSES IN HUMANS

Ebrahimi *et al.* (2019), *Translational Psychiatry*

Objective: Animal and human research on fear conditioning, extinction learning, and return of fear phenomena has greatly informed our understanding of the development and treatment of anxiety disorders and fostered novel interventions to optimize exposure therapy[5]. In contrast, there is a paucity of comparable translational human research on Pavlovian learning of appetitive or drug cue associations[45], and so far, human reinstatement effects have been exclusively investigated in the fear domain. In *Study I* we aimed to establish a human laboratory model of appetitive Pavlovian relapse, with special emphasis on the neural structures involved in the return of appetitive CRs after a reinstatement procedure in healthy participants.

Method: We used a three-day design comprising differential delay conditioning with liquid foods as primary reinforcer (day 1; n=63), extinction (day 2; n=33) and reinstatement test (day 3; n=33) in combination with a multimodal approach to evaluate CRs on a behavioral (i.e. valence ratings), psychophysiological (i.e. skin conductance and startle responses), and neural level using fMRI (day 3 only).

Main findings: Conditioning was associated with increased valence ratings, enhanced SCR and differential startle modulation (attenuation of the eyelid reflex and enhancement of the PAR) towards the CS+ compared to the CS-, which were successfully extinguished on day 2, demonstrating the validity of our paradigm to investigate appetitive Pavlovian learning. Of most interest, we observed a return of conditioned responding in terms of enhanced SCRs following unsignaled US presentations (reinstatement procedure) on day 3, along with significant BOLD activation within the amygdala and, more transient, within the NAcc. On an individual level, psychophysiological reinstatement intensity (SCRs) was anticorrelated with vmPFC activation and further marginally with enhanced amygdala-vmPFC functional connectivity during CS+ compared to CS- presentations (gPPI), which emerged during the second phase of the reinstatement test.

Conclusions: In this study, we demonstrate for the first time that appetitive Pavlovian relapse can be modeled in a laboratory setting in healthy participants using an implicit response

measure (SCR) and provide evidence for opposing roles of the amygdala and vmPFC in regulating appetitive Pavlovian relapse. Our results therefore extend evidence from return of fear phenomena to the appetitive research domain and suggest that the vmPFC might be a promising target for novel interventions that aim to counteract Pavlovian relapse phenomena.

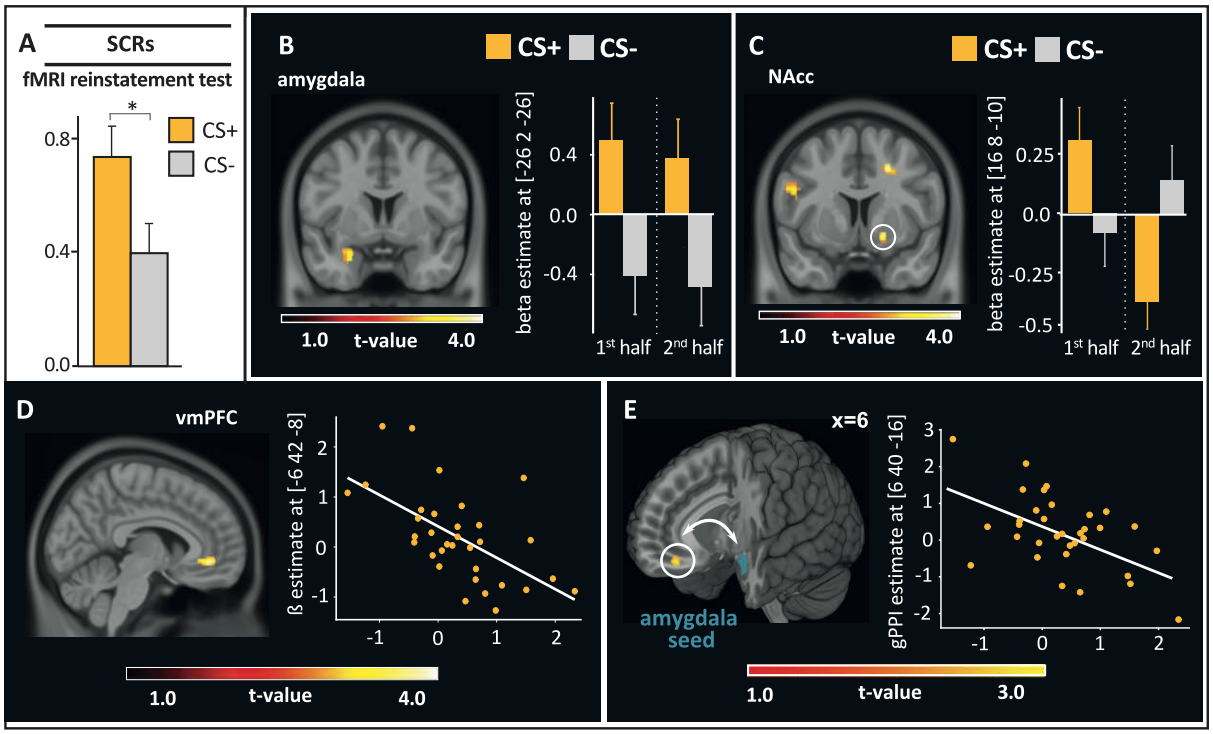

Figure 2. Psychophysiological and neural responses during reinstatement test. A Significant differential SCRs during reinstatement test ($t(32) = 2.25$, $p = .031$). Error bars represent SEM[111,112]. **B** Elevated BOLD response in the contrast CS+ > CS− in the left amygdala over phases (MNI peak at [x: −26, y: 2, z: −26], $p_{FWE\ ROI} = .01$). **C** Interaction of differential BOLD responses with test phase in the right NAcc (MNI peak at [x: 16, y: 8, z: −10], $p_{FWE\ ROI} = .016$). **D** Inverse correlation between differential SCRs and vmPFC activation (MNI peak at [x: −6, y: 42, z: −8], $p_{FWE\ ROI} = .022$). **E** Differential SCRs were further marginally inversely correlated with functional amygdala-vmPFC connectivity (gPPI) observed in the second test phase (MNI peak at [x: 6, y: 40, z: −16], $p_{FWE\ ROI} = .061$). Error bars represent SEM. All t-maps are displayed on a visualization threshold of $p < .005$ uc with $k \geq 20$ cluster extend.

2.3.2 STUDY II: PUPIL DILATION AS AN IMPLICIT INDEX OF APPETITIVE PAVLOVIAN LEARNING

Pietrock *et al.* (2019), *Psychophysiology*

Objective: Although Pavlovian learning processes are assumed to play an important role in the development of drug addiction [11], translational human research in the domain of appetitive Pavlovian learning is widely lacking. One proposed reason for this shortcoming has been the absence of an established sensitive measure of appetitive CRs [42,49]. In *Study II*, we evaluated the suitability of the pupil diameter and other ocular response measures to quantify appetitive Pavlovian learning. Furthermore, we examined how different conditioning indices were related intra-individually.

Method: Differential delay conditioning was investigated in 29 healthy participants using a slightly modified version of the conditioning paradigm from *Study I*. Eye-tracking was used to simultaneously acquire pupil diameter, gaze dwelling time, blink duration and blink count, along with additional behavioral (i.e. valence and attractiveness ratings) and psychophysiological (SCRs, startle responses, HR) measures of conditioning. Moreover, we applied different Rescorla-Wagner learning models to participants' pupil diameter data to infer learning on a trial-by-trial basis.

Main findings: Our appetitive conditioning procedure induced robust CRs in all but one ocular response measure. Specifically, conditioning resulted in increased pupil dilation, longer gaze duration and shorter blink duration towards the CS+ compared to the CS-, while blink count was marginally attenuated. Model comparisons revealed that a Pearce-Hall attention model predicting pupil diameter with dynamic attention weights explained the data best. Conditioning was further associated with increased (forced-choice) CS preference ratings and HR decelerations towards the CS+. Interestingly, there were no notable intra-individual associations between these different conditioning indices.

Conclusions: In this study we showed that pupil dilation represents a sensitive index to study human appetitive CRs and that trial-by-trial pupil diameter changes were consistent with a reinforcement learning mechanism incorporating attentional processes. By providing first evidence that gaze dwelling time and blink duration represent additional indices of appetitive learning, we argue that ocular response measures represent a promising and powerful tool that may help advance translational research in the domain of human appetitive Pavlovian learning.

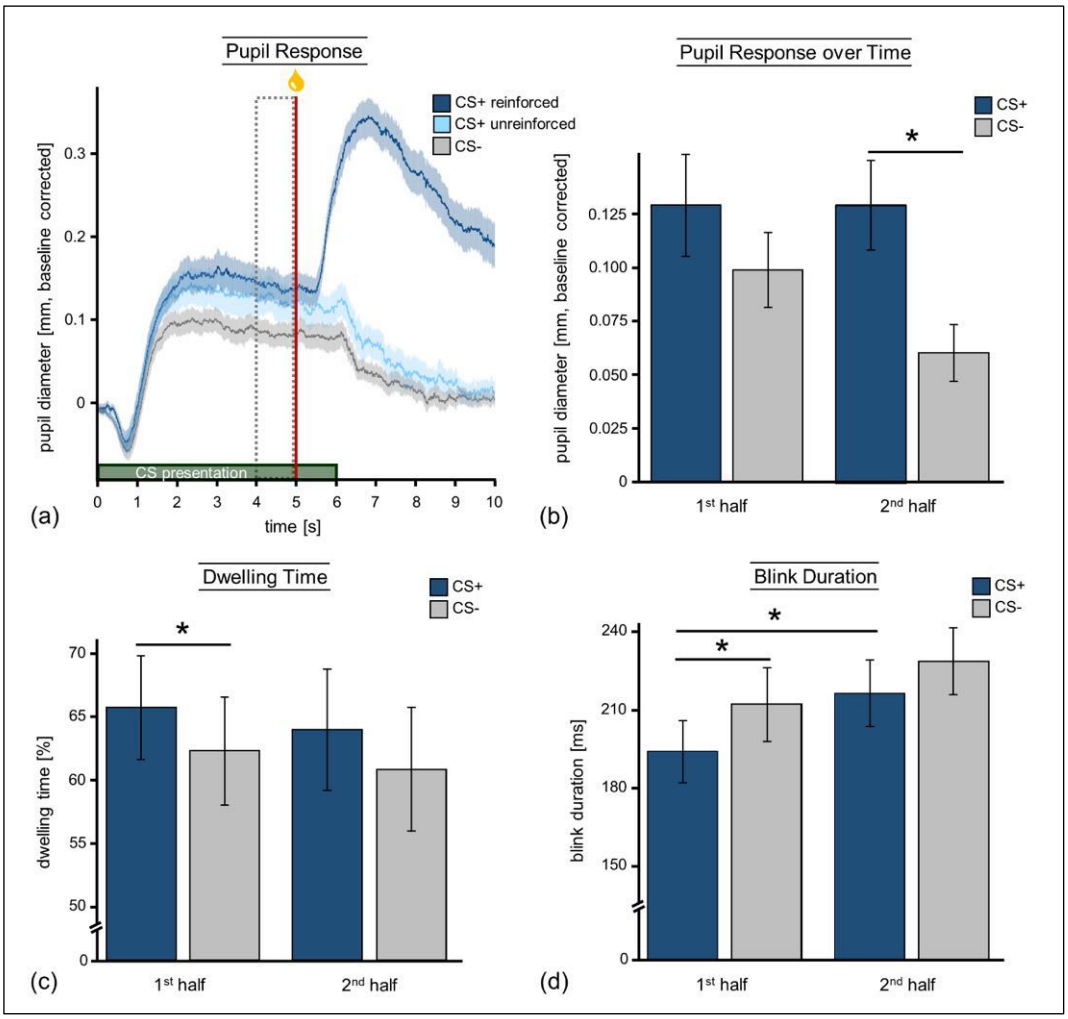

Figure 3. Ocular responses during appetitive Pavlovian learning. A Mean pupil diameter time course in CS+ (reinforced/unreinforced) and CS− trials over participants. The dotted area corresponds to the predefined analysis time window (second 4–5 after CS onset). **B** Stronger pupil dilation during the reward-predicting cue in the first and second half of the experiment (main effect condition: $F(1,24) = 9.64$, $p = .005$). **C** Longer gaze ‐ dwelling time on the reward ‐ predicting cue than on the control cue (main effect of condition: $F(1,26) = 7.74$, $p = .010$). **D** Blink duration was significantly shorter in CS+ compared to trials trials (main effect of condition: $F(1,28) = 10.99$, $p = .003$). Error bars represent SEM. *$p \leq .05$

2.3.3 STUDY III: COMBINING D-CYCLOSERINE WITH APPETITIVE EXTINCTION LEARNING MODULATES AMYGDALA ACTIVITY DURING RECALL

Ebrahimi *et al.* (2017), *Neurobiology of Learning and Memory*

Objective: As demonstrated in *Study I*, although extinction learning can reduce conditioned responding, return of CRs is a frequently observed phenomenon. Such Pavlovian relapse phenomena could challenge the long-term success of exposure-based treatments for anxiety and addiction-related disorders, which rely on extinction processes. In animal studies, the glutamate partial NMDA receptor agonist DCS has been shown to improve extinction learning of appetitive and aversive Pavlovian associations [80,113], while clinical trials of DCS-augmented exposure therapy remain inconclusive [92]. To help close this gab and explore the precise working mechanism of DCS in humans, *Study III* aimed to investigate the behavioral and neuronal effect of DCS during recall of appetitive and aversive Pavlovian associations.

Method: We used a three-day differential appetitive and aversive trace conditioning paradigm comprising conditioning, extinction, and extinction recall after a reactivation procedure (three initial CS-US pairings) in a placebo-controlled, double-blind fMRI design. Monetary wins and losses served as USs. Thirty-three healthy participants underwent conditioning (day 1); the next day they were randomly allocated to receive either an oral dose of 50 mg of DCS or placebo one hour before extinction training (n = 15 DCS / n = 15 placebo; day 2). DCS was hypothesized to attenuate conditioned responding on a behavioral (RTs), psychophysiological (SCRs), and neuronal level following a reactivation procedure similar to Kuriyama et al. [100] (three initial CS-US pairings) to trigger the return of conditioned responding on day 3.

Main findings: The reactivation procedure successfully induced a return of differential RTs in the cued outcome discrimination task during the first extinction recall trial on day 3 in both groups, suggesting no effect of DCS on cognitive measures related to US expectancy. On a neural level, participants receiving DCS compared to placebo before extinction learning showed attenuated amygdala activation during appetitive extinction recall. Exploratory functional connectivity analysis (PPI) further revealed increased amygdala-vmPFC coupling in the DCS compared to the placebo group. As conditioning on day 1 did not result in differential SCRs, DCS effects could not be evaluated on a psychophysiological level. Furthermore, the aversive contrast '$CS+_{avers}$ vs. CS-' did not reveal significant differential BOLD responses in any session, which might be due to different methodological aspects.

Conclusions: Our finding of attenuated amygdala activation and increased amygdala-vmPFC coupling after DCS-augmented extinction learning is in line with the hypothesis that DCS facilitates human appetitive extinction learning by enhancing memory consolidation. While the absence of an additional psychophysiological measure precludes the evaluation of a behavioral DCS effect, these findings should encourage future research regarding the usefulness of DCS as a cognitive enhancer during appetitive extinction learning.

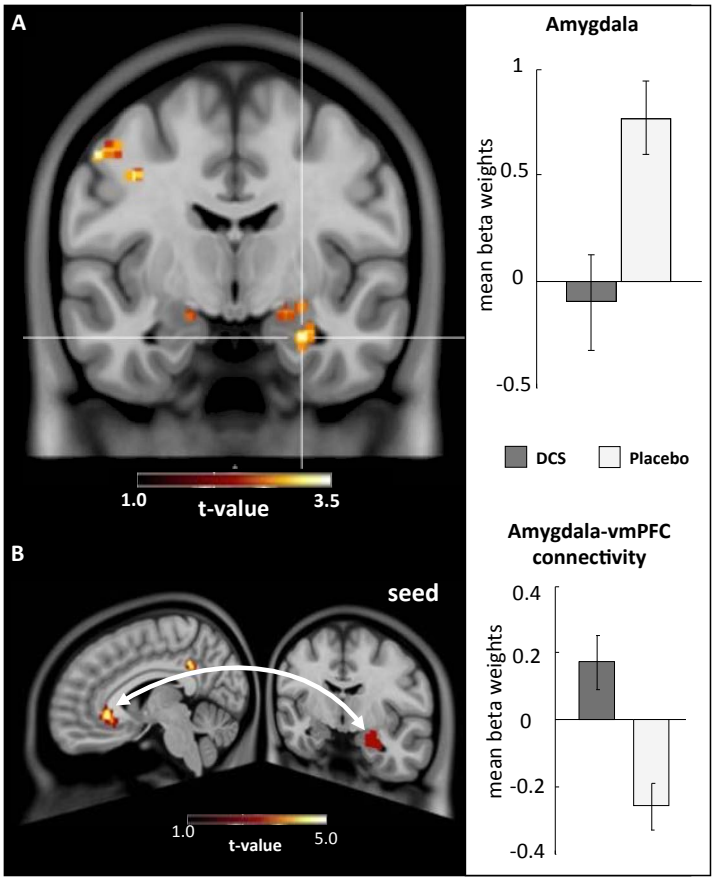

Figure 4. DCS effects during appetitive extinction recall. A Significant amygdala activation during appetitive extinction recall (CS+app >CS-) in the placebo compared to the DCS group (MNI peak at: [x:26, y:-8, z:-24], Z = 3.30, $p_{FWE\ ROI}$ = .021). **B** An exploratory PPI analysis revealed stronger amygdala-vmPFC connectivity in the DCS compared to the placebo group (MNI peak at: [x:6, y:34, z:-2], Z = 4.32, cluster size = 56 at p_{uc} < 0.001). Displayed in red is the seed region (anatomical amygdala mask) used for time course. Activations displayed at $t \geq 2.35$, cluster extent $k > 50$.

2.3.4 STUDY IV: AUGMENTING EXTINCTION LEARNING WITH D-CYCLOSERINE REDUCES RETURN OF FEAR: A RANDOMIZED, PLACEBO-CONTROLLED FMRI STUDY

Ebrahimi*, Gechter* *et al.* (in revision), *Neuropsychopharmacology*

Objective: In *Study IV*, we built upon *Study III,* solely investigating the effect of DCS on return of fear using an established differential fear conditioning and delayed extinction paradigm [114].

Method: The three-day design, administration and dosage of DCS were identical to *Study III*. Thirty-seven healthy participants completed differential fear conditioning using an auditory aversive panic scream as US, extinction following random allocation to either DCS or placebo group (n=20 DCS/n=17 placebo), and extinction recall. Return of fear, i.e. increased conditioned responding from extinction learning (day 2) to extinction recall (day 3), was assessed on a behavioral (CS ratings), psychophysiological (SCRs), and neural level (fMRI).

Main findings: The fear conditioning procedure resulted in increased arousal and decreased valence ratings of the fear-associated cue, along with enhanced SCRs and neural activation patterns in fear-related brain areas comprising bilateral insula, dACC, SMA, and midbrain. Evaluation of group differences in return of fear measures revealed that only participants receiving placebo but not DCS experienced a rather generalized return of fear in arousal ratings, and showed comparably increased BOLD responses in left amygdala and posterior hippocampus, suggesting stronger fear memory recall in placebo-treated participants. In line with this, an exploratory analysis revealed higher differential BOLD responses in right dACC and left insula in the placebo compared to the DCS group during the early recall phase. We found no evidence for return of fear in SCRs across or within groups, impeding the evaluation of a potential DCS effect in this measure.

Conclusions: We found that DCS prevented the return of fear in subjective arousal ratings and attenuated differential BOLD responses in brain areas involved in fear acquisition and expression, like amygdala and posterior hippocampus. This adds further support to the hypothesis that DCS enhances human extinction learning, thereby reducing return of fear.

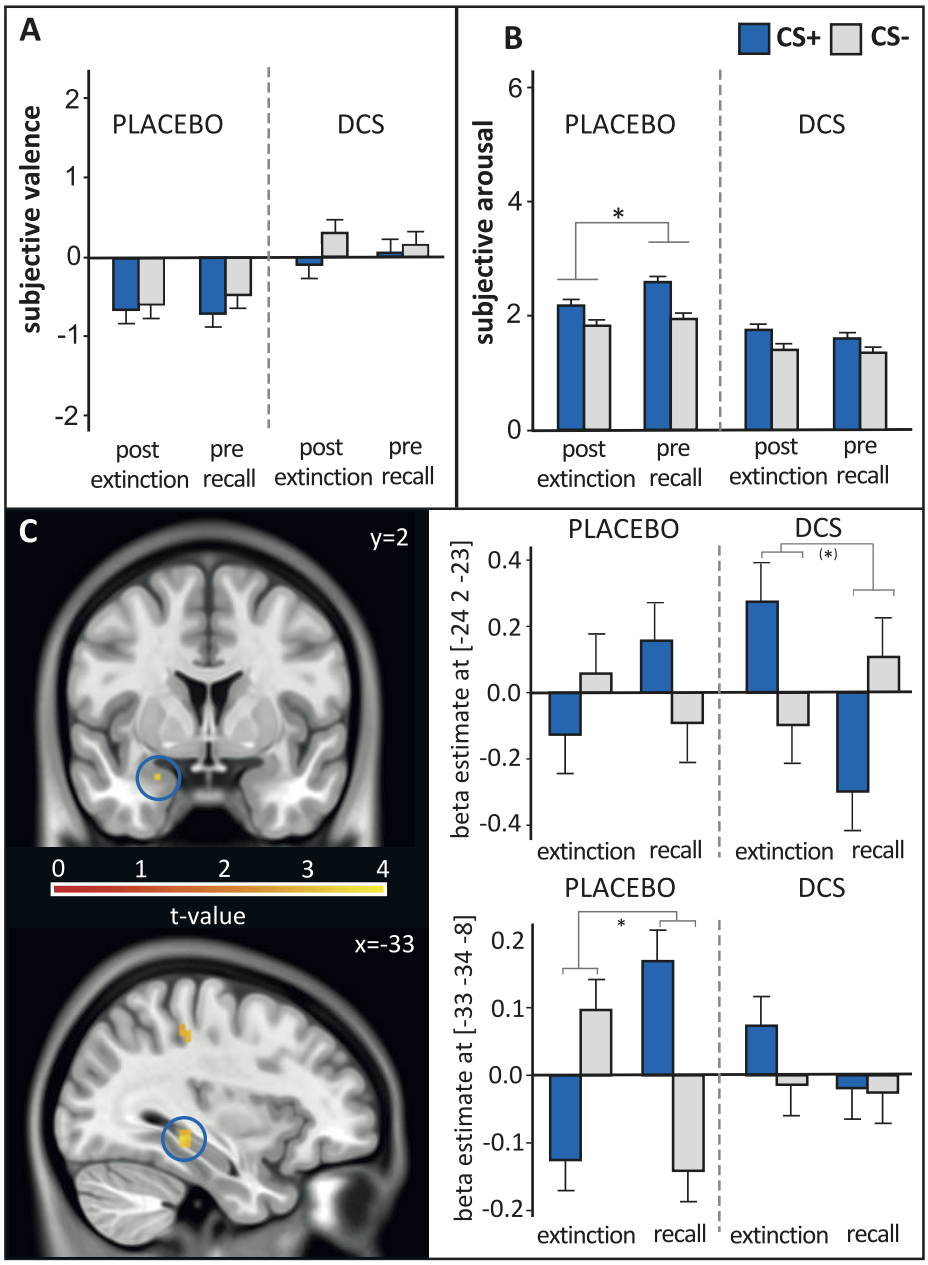

Figure 5 DCS effects on behavioral and neural measures of return of fear. A+B While no significant differential return of fear was observed across or between groups in rating measures of CS valence or arousal, subjects in the placebo but not DCS group showed non-differential return of fear with increased arousal ratings from extinction training to extinction recall. Bar graphs represent the mean ± SEM [111,112]. **C** Participants in the placebo compared to the DCS group showed stronger return of fear on a neural level, that is increased differential BOLD responses from extinction learning to extinction recall in left amygdala and posterior hippocampus. Bar graphs represent mean parameter estimates from a 6-mm sphere surrounding peak voxel activation ± SEM [111,112]. T-maps are displayed on a visualization threshold of $p<.005$ uc. with $k\geq5$ cluster extent.

3 GENERAL DISCUSSION

This thesis was devoted to the study of behavioral and neural correlates of human Pavlovian learning processes and its pharmacological enhancement via DCS, with special emphasis on learned appetitive associations due to its clinical relevance for addiction. We established a human laboratory model of appetitive Pavlovian relapse and characterized its involved neural structures, and provide first evidence that DCS-augmented extinction learning may promote successful extinction recall by attenuating BOLD responses in structures essential for acquisition and expression of CRs, such as the amygdala.

Differential Involvement of Amygdala and vmPFC in Appetitive Pavlovian Relapse

According to the inhibitory model of extinction learning[7], extinction does not erase the original CS-US association but establishes a new, inhibitory and context sensitive CS-noUS association that henceforth competes with the original association for behavioral expression. This duality explains several Pavlovian relapse phenomena like spontaneous recovery, reinstatement, or renewal, which have been extensively studied in animal models[7,13]. Anxiety research has already started to experimentally investigate these return of fear phenomena in humans[9,64,115], while comparable research in the appetitive domain is scarce (for exceptions see[43,44]).

In *Study I*, we established a human laboratory model of appetitive Pavlovian relapse and demonstrated – for the first time – a return of differential SCRs following unsignaled US presentations (reinstatement procedure). This is in line with results on experimentally reinstated fear in humans[55,62]. By using neuroimaging, we were able to identify heightened amygdala and NAcc activation during the reinstatement test, the latter decreased from the early to the late reinstatement phase. Neuroimaging studies revealed amygdala and ventral striatum activation, including the NAcc, during appetitive conditioning with primary rewards[116,117]. NAcc activation during appetitive Pavlovian reinstatement therefore involves anticipation of reward stimuli[118] and initiation of cue-induced approach behavior, which has been recently shown to be predictive of later relapse in detoxified alcohol dependent patients using a Pavlovian-to-instrument transfer task[119]. Heightened amygdala activity has been observed in return of fear following unsignaled US presentations[56,61,67] or a context change[65,66]. Given its central role for the acquisition of CS-US associations in appetitive and aversive Pavlovian learning[17,18], amygdala activation seems to be a neural correlate of

recalling the acquisition memory. In contrast, we observed an inverse association between differential BOLD responses within the vmPFC and reinstated SCRs, and a median split revealed significantly higher vmPFC activity in participants in the low compared to the high reinstatement group. This result extends evidence from fear extinction recall to the appetitive domain, where vmPFC activation has been linked to successful extinction recall[56,66,68,69], being anticorrelated with differential SCRs[69,71,120] and reduced during reinstated fear[61,62]. Our finding of increased functional connectivity between amygdala and vmPFC during CS+ relative to CS- presentations in the late reinstatement phase, along with a marginal significant anticorrelation with reinstated SCRs, is well in line with an inhibitory top-down signal from vmPFC to the amygdala. In line with results reporting amygdala-vmPFC coupling during fear extinction recall[69,71,72], functional amygdala-vmPFC coupling seems to be an important feature for successful appetitive extinction recall as well.

Knowing that Pavlovian responses can easily recover despite successful extinction learning raises the question of whether we can – at least to some degree – overcome this weakness of extinction learning, which constitutes an important element in treatment approaches for anxiety and could also benefit addiction interventions[47].

D-Cycloserine as a Pharmacological Intervention to Enhance Extinction Learning

Various lines of experimental research in the fear domain aim to improve extinction learning using behavioral or pharmacological interventions[5]. One such pharmacological agent is DCS, which is assumed to primarily target the consolidation process required for long-term extinction memory formation[121]. However, the large effect sizes observed in animal models combining DCS with fear or drug-cue extinction have not been replicated in clinical trials[84,87,122]. In humans, it therefore remains unclear if DCS-augmented extinction training can improve later extinction recall, or which neural mechanisms might accompany such effects. In *Study III* we investigated this issue using a combined appetitive and aversive conditioning paradigm, while in *Study IV* we specifically focused on DCS effects on return of fear.

Study III showed that 50 mg of DCS administered one hour prior to extinction learning attenuated BOLD responses in the amygdala during later appetitive extinction recall, an effect that might be mediated via top-down influence from vmPFC as suggested by functional connectivity analysis. While *Study III* failed to provide an additional psychophysiological indicator for the effectiveness of DCS, we interpret this neural pattern to reflect improved extinction memory retrieval in participants receiving DCS compared to placebo. This

interpretation is well in line with our findings from *Study I*, where we showed significant amygdala activation during an appetitive reinstatement test and a marginally significant correlation between heightened amygdala-vmPFC coupling and reduced psychophysiological reinstatement intensity. As such, these results provide first evidence that DCS may augment appetitive extinction learning, resulting in reduced neural signatures associated with the return of appetitive CRs and increased inhibitory functional connectivity between amygdala and vmPFC.

Corroborating our findings from *Study III*, *Study IV* found attenuated BOLD responses in the amygdala as well as within the posterior hippocampus in DCS- compared to placebo-treated participants when probing return of fear on the neural level. Previous work suggests a functional segregation along the longitudinal axis of the hippocampus, associating the posterior hippocampus with return of fear[63,66,123], while the anterior hippocampus, in concert with the vmPFC, has been found to be involved in extinction recall[66,68,69]. Of special interest, Kalisch et al.[123] observed enhanced fear expression in terms of increased SCRs following DCS-augmented fear acquisition, which was accompanied by increased BOLD responses in posterior hippocampus/collateral sulcus, ACC, and trendwise in the amygdala, suggesting DCS to enhance fear memory consolidation. In *Study IV*, posterior hippocampal activation under placebo therefore likely reflects increased processing of the initial threat association, which was attenuated under DCS. In the same vein, DCS abolished the rather generalized return of fear in subjective arousal ratings observed in the placebo group.

In contrast to these neural results, evidence on behavioral and psychophysiological DCS effects is more heterogeneous between laboratory studies. While *Study III* only acquired subjective valence ratings post-conditioning to confirm initial CS-US acquisition and not during extinction or extinction recall, Brom et al.[101] found post-learning DCS administration to attenuate valence and arousal ratings, as well as physiological CRs but not US expectancy during a renewal test following appetitive sexual conditioning and extinction in healthy women. This dovetails with our results in *Study III*, were RTs from a cued outcome discrimination task during extinction recall did not differ between groups, jointly indicating that DCS affects subjective ratings but did not alter explicit outcome expectancies. Further human laboratory studies from the fear domain only relied on psychophysiological outcome measures: in two studies, DCS administration before extinction learning failed to attenuate SCRs[98,99] or fear potentiated startle[98] during simple extinction recall, whereas one study found DCS to attenuate SCRs after a reactivation procedure similar to the one used in *Study III*[100]. In both *Studies III* and *Study IV*, it was not possible to evaluate DCS effects on return

of SCRs; either because of initial missing conditioning effects (*Study III*) or because of floor effects during extinction recall (*Study IV*). Floor effects, i.e. insufficient recovery of extinguished responses during test in the placebo group, also precluded the evaluation of DCS effects on fear extinction consolidation in the study by Kalisch and colleagues [123] and might have contributed to the reported null findings from studies probing simple extinction recall.

Taken together, *Studies III* and *IV* corroborate the hypothesis that DCS facilitates consolidation of reward- and fear-associated extinction memories, thereby improving subsequent extinction recall. Relating to the dual-model theory of conditioning [124], which assumes human conditioning depends on a reflexive, lower-order defense system and a cognitive, higher-order system, our results suggest DCS exerts its facilitating effect primarily on the lower-order mechanisms, attenuating BOLD responses in subcortical brain areas associated with Pavlovian relapse effects like the amygdala, while not affecting cognitive measures of US expectancy.

Is There a 'Gold Standard' to Assess Human Conditioned Responses?

As outlined earlier, human CRs can occur on various response dimensions, including explicit and implicit ones. From a methodological viewpoint, this raises the question of which measures to use in a specific study. In the field of translational research in anxiety disorders, SCRs and fear potentiated startle responses have been established as the most common outcome measures to quantify threat responses [51]. In contrast, lack of research on appetitive Pavlovian learning has partly been attributed to difficulties in finding universally rewarding reinforcers comparable in intensity to the ones typically used in fear conditioning and a lack of established measures sensitive to appetitive CRs [48–50]. Especially in *Study I* and *II* we therefore adopted a multimodal approach to evaluate appetitive conditioned responses, not only taking on measures proven reliable in fear conditioning like SCRs, but extending the repertoire to test new and perhaps more sensitive measures for appetitive Pavlovian learning. For example, *Study I* showed that the PAR, a vestigial microreflex that serves to pull the ear backwards, is attenuated after a conditioning procedure with liquid food rewards. The PAR has been previously found to be enhanced towards positive compared to aversive or neutral pictures [125,126], especially towards appetitive food pictures [127]. In *Study I*, we were able to confirm the sensitivity of the PAR to quantify appetitive Pavlovian learning with liquid food USs in humans. Similarly, this has been recently demonstrated in appetitive odor conditioning [48]. However, applying acoustic startle probes during learning sessions to assess startle responses could interfere with learning itself, which could be especially problematic during

appetitive Pavlovian learning. *Studies I* and *II* therefore only assessed startle responses post-learning during separate startle tests, which on the flip side precludes the assessment of changes in this measure during learning.

Emphasizing the need for careful selection of readout measures, our studies pointed out that different response measures do not necessarily converge, i.e. although we observed a significant return of conditioned SCRs, valence ratings and startle responses showed no reinstatement effect in *Study I*. Furthermore, only one out of three appetitive conditioning studies (*Study I*) revealed significant differential SCRs during acquisition, emphasizing the need for alternative, perhaps more sensitive measures in appetitive conditioning research. *Study II* followed this line of research and investigated different pupillary response measures during appetitive Pavlovian conditioning via eye-tracking. In line with our result of increased pupil diameter towards the CS+, pupil dilation has been previously observed towards cues predicting liquid food rewards [128–130], as well as threat-signaling cues in fear conditioning [131–134]. We further propose gaze dwelling time and blink duration as novel, complementary conditioning indices. Longer gaze and decreased blink duration observed towards the CS+ compared to the CS- might reflect higher attentional capture or alertness towards the reward-associated cue [135–138]. Moreover, the computational modeling results in *Study II* revealed that a Pearce-Hall attention-weighted learning model best captured the observed trial-by-trial pupil diameter changes. According to Pearce and Hall [139], learning is driven by modulating the attention devoted towards stimuli, which is highest when the associated outcome is unpredictable, and declines as the predictive value of a stimulus increases. This dovetails with to the partial reinforcement schedule in our paradigm, where the outcome associated with the CS+ remains unpredictable and hence increasing attention towards the CS+ in contrast to the CS-. As such, our results suggest that conditioning-related pupil diameter changes may prominently capture attentional processes compared to stimulus value.

Taken together, our results highlight the importance of a multimodal approach when investigating Pavlovian learning mechanisms, as different response measures do not necessarily converge and cannot be treated interchangeably. Careful selection of appropriate response measures applies in particular to the appetitive research domain, where CRs might be smaller compared to the ones in fear conditioning [42]. Here, *Study II* suggests that pupillary response measures provide sensitive psychophysiological readout measures that were also unaffected by habituation. The suitability of pupillometry with simultaneous fMRI acquisition should particularly encourage future translational neuroimaging research interested in Pavlovian learning processes.

3.1 LIMITATIONS

The studies presented here bear some limitations. First, heterogeneity in design and methods (see Table 1) limits the comparability of results between studies. Especially *Study III* differentiates from the appetitive conditioning procedure employed in *Studies I* and *II*. *Study III* used a trace conditioning paradigm, were CS and US are separated by a temporal delay, and secondary (monetary) instead of primary (food) reinforcers as US. Furthermore, this paradigm used a more stringent control condition, where the CS- was associated with an image of a blurred coin, while in the other studies the CS- was not followed by an outcome. These factors might have contributed to the missing differentiation in SCRs during appetitive conditioning in *Study III*, i.e. secondary reinforcers might induce only weak psychophysiological responses, and trace conditioning has been associated with slowed learning[140] and weaker CRs compared to delay conditioning[141]. Moreover, associating the CS- with a blurred coin image could have positively biased this condition, hence reducing differential effects.

Second, the samples from *Studies III* and *IV* partly overlap (i.e. 40 % of participants in *Study IV* also participated in *Study III*) and sample sizes were small, raising the need for replicating our results.

Third, while *Studies I, III*, and *IV* concurrently point to the amygdala as a central structure in the return of appetitive CRs and return of fear, interestingly, we did not observe significant amygdala involvement during initial appetitive (*Study III*) or fear conditioning (*Study IV*). While this finding is in line with a meta-analysis on human fear conditioning, which found no evidence for consistent amygdala activity across studies[142], it appears in contradiction with the key role the amygdala plays in fear- and drug-cue conditioning, as evident from preclinical research[17–19]. Such heterogeneous findings might be partly explained by rapid habituation processes in this structure, as amygdala activation has been observed especially early in acquisition[143,144]. As such, the 100 % reinforcement schedule implemented in both *Studies III* and *IV* could have reduced the BOLD signal by increasing US expectancy and promoting fast learning. Robust differential BOLD responses in dACC and insula, as observed in *Study IV* and confirmed in the meta-analysis by Fullana et al.[142], could further suggest that especially structures subserving conscious experience of threat rather than the classical amygdala circuit might be engaged across fear conditioning paradigms. Since *Study I* focused on the neural mechanisms involved in Pavlovian relapse phenomena and conducted the conditioning session in the laboratory, one could only speculate whether using primary

instead of secondary reinforcers[42] in combination with a partial reinforcement schedule, which increases outcome uncertainty and slows learning, would induce amygdala activation during appetitive conditioning.

Fourth, although *Study I* used a reinstatement procedure, the observed return of conditioned responding cannot solely be interpreted as reinstatement effect, as spontaneous recovery (due to the 24 hour delayed test) and renewal (due to the context change from laboratory to fMRI environment) likely also contributed to the observed results. While this represents a more ecologically valid design, future studies should also start to investigate similarities and differences of specific relapse phenomena in more detail in order to better understand appetitive Pavlovian relapse effects in humans. Relatedly, *Study III* used a similar reactivation procedure to Kuriyama et al.[100], where initial pairings of CS+ and US precede extinction recall. As such, DCS might not only attenuate spontaneous recovery, as tested in *Study IV*, but possibly also deters rapid reacquisition.

As an important methodological aspect, *Study II* highlighted that different appetitive conditioning indices were not significantly related on an individual level. This is in line with a recent study by Wardle and colleagues[49], who assessed various subjective, behavioral, and psychophysiological response measures in different tasks after a conditioning procedure with primary food rewards and reported, if any, only weak correlations among different measures. Importantly, *Study II* showed that this even holds for different response measures acquired simultaneously during the conditioning procedure. This finding is highly relevant for future research, suggesting different response measures to reflect different response levels and/or intra-individual variability in responsiveness (i.e., more idiosyncratic response patterns).

3.2 CLINICAL IMPLICATIONS AND FUTURE DIRECTIONS

The results presented in this thesis have important clinical implications and offer several starting points for future research. The observed negative correlation between reinstated SCRs and vmPFC activation during the appetitive reinstatement test in *Study I,* as well as the enhanced functional amygdala-vmPFC connectivity in DCS-treated participants during appetitive extinction recall in *Study III* suggest that the vmPFC and its functional connections with the amygdala mediate appetitive Pavlovian relapse and might be a promising target for novel intervention approaches that aim to improve extinction memory retrieval. Future studies should investigate a causal link between vmPFC involvement and extinction learning and retrieval, e.g. using transcranial direct current stimulation (tDCS) in order to directly

modulate vmPFC activity (for preliminary results in fear extinction, see[145]). Our result is of particular clinical relevance, as drug addiction has been associated with grey matter volume decreases and activation impairments in prefrontal areas including vmPFC[35,146–148], potentially increasing an individual's susceptibility to cue-induced craving during the course of the disorder, initiating a vicious circle.

The individual variation in reinstatement effects observed in *Study I* further raises the question about individual vulnerability or protective factors that moderate Pavlovian relapse risk, which should be addressed in future studies. In this respect, it is surprising that, to our knowledge, Pavlovian learning processes in addiction have so far only been investigated using aversive conditioning protocols[149–151]. While these studies suggested impaired acquisition of CRs that has been interpreted as reduced risk aversion[4], it is unknown if patients suffering from substance use disorders might be characterized by altered Pavlovian reward learning and extinction retrieval processes that either represent a susceptibility factor or develops during the addiction cycle. However, characterizing such deficits could inform novel treatment approaches.

Our findings from *Studies III* and *IV* are of special clinical importance, as they concurrently provide initial experimental evidence that DCS-augmented extinction learning supports delayed extinction memory recall by modulating neural structures subserving the acquisition and expression of appetitive as well as aversive CRs. These promising findings should encourage further investigations on the precise mechanisms of action of DCS in patient populations. As some anxiety disorders have been associated with abnormal fear generalization and extinction impairments[152,153], DCS might be even more effective in this population[98]. Furthermore, studies should systematically investigate boundary conditions and potential moderating factors of DCS efficacy like dosage and timing of DCS administration[78], success of within-session extinction[88,89,97], or concurrent use of antidepressants[154]. For example, the majority of studies investigating DCS-augmented exposure therapy for anxiety disorders have administered 50 mg of DCS one hour before extinction learning[87], as we did in *Studies III* and *IV*. However, so far it remains unknown whether this is in fact the ideal dose.

Translational laboratory models of appetitive Pavlovian learning could also be used to systematically investigate alternative augmentation techniques that could inform actual cue-exposure protocols for substance use disorders, like 'deepened extinction', use of retrieval cues to reduce renewal[155], or virtual reality exposure in order to simulate extinction in various and individual meaningful contexts, possibly in combination with pharmacological adjuncts

(for review of such techniques in translational anxiety research, see[5]). Besides cue exposure relying on Pavlovian extinction processes, cognitive bias modification trainings targeting automated behavioral approach tendencies towards drug cues have been proposed and implemented with some success[156], although more evidence is needed on this topic[157].

Lastly, the use of model-based analyses of psychophysiological data (*Studies I-IV*), computational modeling (*Study II*) and new imaging approaches like multi-voxel-pattern analysis[158] hold promise to increase the comparability of studies through more standardized analysis procedures and to enable more fine-grained analyses of Pavlovian learning processes.

Final Conclusion

In summary, the studies constituting this thesis successfully established a human laboratory model for appetitive Pavlovian learning and relapse effects and showed for the first time that amygdala and vmPFC modulate the return of appetitive CRs. In doing so, they also highlight the need for a multimodal approach to cover various response systems that may be differentially involved in human appetitive Pavlovian learning. Furthermore, we provide novel evidence that DCS can augment aversive as well as appetitive extinction learning in healthy individuals resulting in reduced activation of limbic brain structures during extinction recall, which should inspire future research to investigate the mechanisms of action of DCS along with efficacy moderating factors in more detail. This line of research may constitute an important building block to bridge the gap between animal models of drug use and anxiety on the one hand and clinical studies on the other. Eventually, this may guide the way towards more effective, individually tailored intervention techniques able to counteract maladaptive learned associations.

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Opposing roles for amygdala and vmPFC in the return of appetitive conditioned responses in humans

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Abstract

Learning accounts of addiction and obesity emphasize the persistent power of Pavlovian reward cues to trigger craving and increase relapse risk. While extinction can reduce conditioned responding, Pavlovian relapse phenomena—the return of conditioned responding following successful extinction—challenge the long-term success of extinction-based treatments. Translational laboratory models of Pavlovian relapse could therefore represent a valuable tool to investigate the mechanisms mediating relapse, although so far human research has mostly focused on return of fear phenomena. To this end we developed an appetitive conditioning paradigm with liquid food rewards in combination with a 3-day design to investigate the return of appetitive Pavlovian responses and the involved neural structures in healthy subjects. Pavlovian conditioning (day 1) was assessed in 62 participants, and a subsample ($n = 33$) further completed extinction (day 2) and a reinstatement test (day 3). Conditioned responding was assessed on explicit (pleasantness ratings) and implicit measures (reaction time, skin conductance, heart rate, startle response) and reinstatement effects were further evaluated using fMRI. We observed a return of conditioned responding during the reinstatement test, evident by enhanced skin conductance responses, accompanied by enhanced BOLD responses in the amygdala. On an individual level, psychophysiological reinstatement intensity was significantly anticorrelated with ventromedial prefrontal cortex (vmPFC) activation, and marginally anticorrelated with enhanced amygdala-vmPFC connectivity during late reinstatement. Our results extend evidence from return of fear phenomena to the appetitive domain, and highlight the role of the vmPFC and its functional connection with the amygdala in regulating appetitive Pavlovian relapse.

Introduction

Learning about environmental cues that signal desirable outcomes constitutes an important mechanism to flexibly adapt behavior and foster survival. However, learning theories of addiction and obesity emphasize the persistent power of Pavlovian reward cues (conditioned stimuli, CS+)—a beer brand label in the super market or the smell of a freshly-baked cake—to trigger the desire for the associated drug/food (unconditioned stimulus, US), drive habits, and increase the risk of relapse long after abstinence[1–4].

Although extinction—repeatedly presenting a CS+ without the US—reduces conditioned responding[5], it does not "erase" the original cue-reward association, but induces new, highly context-dependent inhibitory learning[6]. Several Pavlovian relapse phenomena—a return of conditioned responding towards the extinguished CS+—originate from these properties of extinction, including the mere passage of time (spontaneous recovery), an unpredicted encounter with the US (reinstatement), or a change in context (renewal)[6,7]. Reinstatement, the return of conditioned responding towards an extinguished CS

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after an unpredicted encounter with the US, is well documented in rodents[8,9].

From a clinical perspective, this challenges the efficacy of cue-exposure therapy to prevent relapse despite reducing cue reactivity in the clinic[10,11]. Translational laboratory models of human Pavlovian relapse could therefore represent a valuable tool to investigate the mechanisms that mediate relapse and develop new techniques to counteract it[12]. Anxiety research experimentally investigates return of fear following extinction on multiple response systems, including psychophysiological measures (skin conductance, fear-potentiated startle) and neuroimaging[13–15]. Conversely, experimental research on appetitive Pavlovian conditioning and relapse in humans is still in its infancy[16–18]. This research gap has been explained by difficulties to find universally-rewarding USs and a lack of established measures sensitive to appetitive responses[19–21].

To this end we developed a differential delay conditioning paradigm with liquid food as natural unconditioned stimulus (US) in combination with a 3-day design to evaluate conditioning (day 1), extinction (day 2) and return of conditioned responding following a reinstatement procedure (3 day) while allowing consolidation of learning between sessions. A multimethod approach was used to assess conditioned responses, including explicit (CS pleasantness and US contingency ratings), behavioral (reaction times), and implicit measures (SCRs, heart rate, startle responses). Moreover, return of appetitive conditioned responses was investigated on a neuronal level using fMRI.

Preclinical work points toward an important role of the ventromedial prefrontal cortex (vmPFC) in mediating appetitive Pavlovian relapse after extinction[22,23], suggesting it as a central site of extinction memory storage[24]. This regulatory role is accomplished via projections to key structures involved in reward-related learning, particularly basolateral amygdala and nucleus accumbens (NAcc)[25,26]. Corroborating animal findings, human neuroimaging confirmed the involvement of the amygdala as well as the ventral striatum, including the NAcc, during appetitive Pavlovian learning with primary rewards[27,28]. Furthermore, vmPFC activation was related to inhibiting previously learned appetitive responses[29], possibly through enhanced functional connectivity with the amygdala[28,30]. In the fear domain, neuroimaging highlighted an important role for the vmPFC in extinction recall[31], and amygdala, hippocampus and vmPFC have been shown to be differentially involved in reinstated fear[32,33].

The aims of this study were twofold: (1) to test the hypothesis that appetitive conditioned responses in healthy subjects recover after a reinstatement procedure 24 h after extinction learning; (2) to characterize the neural structures involved in appetitive reinstatement and their relation to individual differences in reinstatement intensity. Based on the outlined findings, we hypothesized that amygdala, NAcc and vmPFC mediate reinstatement and that inhibition of conditioned responses depends on functional amygdala-vmPFC connectivity[30].

Materials and methods

Subjects

Seventy-one healthy, right-handed volunteers recruited from student mailing lists gave written informed consent to participate in the appetitive conditioning session (day 1); the last 36 were followed up for extinction (day 2) and a reinstatement test (day 3). Participants were excluded in case of current or past medical, psychiatric or neurological disorders, drug or alcohol abuse, pregnancy, color blindness or weakness, being on a diet, and allergies or food intolerances to the delivered liquid foods (self-report). All subjects were right-handed and had normal or corrected-to-normal vision. Subjects were required to fast for at least four hours before each session[34]. A priori defined inclusion criteria (see '*Behavioral data acquisition*') resulted in a final sample of 62 subjects during conditioning (M (SD)$_{age}$ = 24.42(3.28) years; 35 women) and 33 subjects during extinction and reinstatement test (M (SD)$_{age}$ = 24.06(3.81) years; 18 women). The study was approved by the Ethics Committee of the Charité – Universitätsmedizin Berlin and conducted in accordance with the Declaration of Helsinki.

Experimental procedure

We used a differential delay conditioning paradigm with liquid food as natural unconditioned stimulus (US) with a multimethod approach over three sessions (conditioning, extinction, and reinstatement; Fig. 1a), 24 h apart to allow consolidation between sessions. Return of conditioned responding on day 3 was assessed after confronting subjects with unsignaled US presentations (reinstatement procedure). We provide publically available source code of the paradigm via Bitbucket (https://bitbucket.org/LearningAndCognition/appreinstatement).

Stimuli

One of four possible juices/smoothies (apple, orange, orange-passionfruit, strawberry-banana) served as US, depending on subject's preference. US administration consisted of 1 ml of liquid delivered directly into the subjects' mouth via clear PVC tubes and a programmable syringe pump (World Precision Instruments, Inc., Sarasota, USA). Two different pictures combined with one of two possible tones (400 or 500 Hz; 100-ms duration) served as cues (CS+/CS−, counterbalanced across subjects; see Fig. 1b).

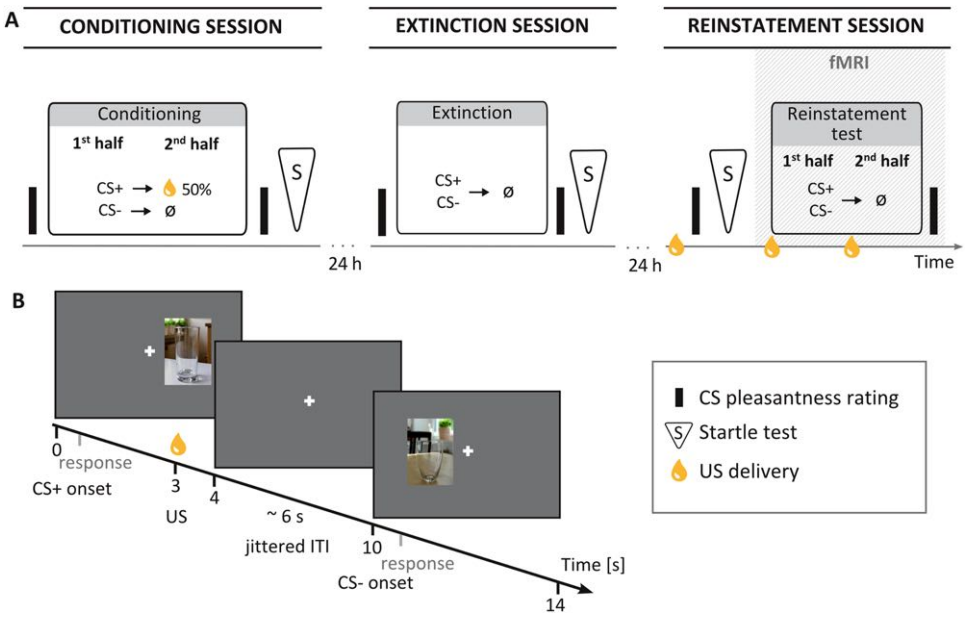

Fig. 1 Experimental design and paradigm. a Subjects underwent appetitive differential delay conditioning on day 1, extinction on day 2, and a reinstatement test on day 3. Acoustic startle tests were conducted separately in each session. Return of conditioned appetitive responses on day 3 was probed after a reinstatement procedure occurring once before the startle test and twice during fMRI reinstatement test. **b** Exemplary trial sequence during conditioning on day 1. In each trial, one out of two different cues was presented either on the left or right side of a fixation cross for 4 s and subjects were asked to indicate the stimulus presentation side as fast as possible via button press. In case of a reinforced CS+ trial, 1 ml of subjects' chosen liquid food (US) was delivered 3 s after cue onset

Instructions

Subjects underwent uninstructed conditioning, extinction, and a reinstatement test, i.e., no information about CS-US contingencies or changes across sessions was provided. Until a final debriefing, subjects were told they were adult controls in an experiment investigating hand-eye coordination and attention in small children, where juice served to keep children's motivation during the experiment. Subjects were asked to indicate the side of cue appearance via button press with their right index and middle fingers as quickly and accurately as possible.

Design

Conditioning (day 1) consisted of two phases, each with 30 CS+ and 30 CS− trials (total, 120 trials). In each trial, a cue was presented for 4 s to the left or right side of a white fixation cross. The CS+ was paired with US delivery 1 s before cue offset in 50% of the trials; the CS− was never followed by liquid food. To extinguish conditioned responses acquired on day 1, extinction (day 2) comprised one phase with 30 unreinforced CS+ and 30 CS− trials. The reinstatement test (day 3, in MRI) consisted of two identical phases, each with 15 unreinforced CS+/CS− trials. To trigger return of conditioned responding, subjects received three unsignaled, jittered US deliveries within 30 s prior to each phase (reinstatement procedure). Of note, due to the temporal spacing between sessions (24 h) and context changes from laboratory to scanner, spontaneous recovery and renewal might contribute to return of conditioned responding following reinstatement, providing a more ecologically valid model of appetitive Pavlovian relapse, where reinstatement effects inevitably follow some time after treatment (spontaneous recovery) and likely occur in a context different from initial acquisition (renewal). Trial sequences were pseudo-randomized across subjects (see Supplementary Material for further details). The inter-trial interval (ITI) ranged 3.5–12 s ($M = 6$ s).

Behavioral data acquisition and preprocessing

Thirst and hunger ratings

Thirst and hunger ratings were collected prior to each session on separate 100-mm visual analog scales (VAS) ranging from 0 = 'not thirsty/hungry at all' to 100 = 'very thirsty/hungry'.

Pleasantness of US and CS

US pleasantness was evaluated pre- and post-conditioning, and before the startle test on day 3 by applying a single US delivery, followed by a computerized VAS ranging from −50 = 'very unpleasant' to 50 = 'very pleasant'. Only subjects with mean positive ratings pre-/post-conditioning were included in the study, ensuring US appetence during learning (subjects excluded: $n = 6$).

On-screen pleasantness ratings for CS+ and CS− were acquired on an identical VAS before and after each session (Fig. 1a).

Contingency awareness

After conditioning, subjects rated the reward probability of each CS on a 100-point VAS ('Immediately after this picture, I received a sip of juice…') ranging from 'never' to 'always'. Difference scores [CS+ *minus* CS−] were calculated as a dimensional awareness indicator, with large positive values implying contingency awareness and values around zero unawareness[35]. Subjects with notable negative difference scores (≤−20) were excluded from the study ($n = 3$), as these indicate (explicit) conditioning towards the CS− rather than unawareness. Following a worthwhile reviewer comment, we further explored associations between contingency awareness and conditioning indices on day 1 (see Supplementary Material).

Psychophysiological data acquisition and preprocessing

Psychophysiological data were acquired at 250 Hz using an MR-compatible amplifier (BrainAmp ExG, Brain Products, Munich, Germany).

Skin conductance

Skin conductance responses (SCRs) were recorded during all sessions from the participant's middle phalanges of the left index and middle finger using MR-compatible Ag/AgCl electrodes. Two datasets were excluded because of technical failures during recording or low data quality. Preprocessing and analysis of single-subject data was performed within the PsPM toolbox (version 3.1.1; http://pspm.sourceforge.net/), using the general linear convolution model (GLM) approach. Preprocessing comprised linear interpolation of movement-related artefacts, band-pass filtering (first-order Butterworth, 0.05–5 Hz), downsampling (10 Hz), and normalization to remove between-subject variance in response amplitudes[36]. Event onsets (CS+/CS−/US) were modeled as stick functions and convolved with a canonical SCR function. GLMs for conditioning and extinction included CS+ and CS− onsets per phase as regressors of interest, and an additional regressor to model the US effect on day 1. To test for return of conditioned responses on day 3, cue onsets of the first five CS+ and CS− trials following unsignaled US deliveries in each phase were modeled as two regressors of interest. US onsets and the remaining cues were modeled as three regressors of no interest. Within each GLM, regressors were fitted to the SC time series, yielding an SCR-amplitude estimate per regressor.

Startle responses

Cue-related modulation of eyeblink and postauricular (PAR) reflexes was assessed by auditory startle tests in each session (Fig. 1a). During the reinstatement session, three unsignaled US deliveries (reinstatement procedure) preceded the startle test. Startle probes consisted of a 50-ms, 90-dB burst of white noise presented binaurally via headphones. The test was initiated by four jittered startle probes while viewing the fixation cross (habituation startles; mean inter-probe-interval 2 s). Thereafter, startle probes were presented in each of four unreinforced CS+/CS− trials with varying stimulus-onset asynchronies (0.5, 1, 1.5, or 2 s). Four additional startle probes were presented during the ITI to reduce startle predictability and avoid a cue-related association. The ITI ranged 9–13 s ($M = 10.9$ s). Trial order and stimulus side were fully counterbalanced across subjects by assigning one of four fixed trial sequences. Startle responses were recorded with four Ag/AgCl electrodes on left orbicularis oculi and auricularis muscles[37,38]. Due to technical reasons, startle data from the first 12 participants are missing, and two datasets from the conditioning sample were lost because of technical failures. Electromyography (EMG) signal was notch-filtered at 50 Hz and band-pass filtered (2nd order Butterworth, 28–110 Hz), after which data were rectified and smoothed (3rd order Savitzky-Golay filter) using a moving average of 15 and 20 consecutive data points for PAR and eyeblink reflex, respectively. Startle responses were defined as the difference between the maximum amplitude within 20–115 ms and 10–40 ms after probe onset for eyeblink reflex and PAR, respectively, and startle baseline, i.e., mean EMG activity 50 ms before probe onset (ranges comparable to[37,38]), and averaged over cue type for group analyses. Eyeblink data from two subjects on day 1 and from one subject on day 3 had to be excluded for low quality.

Acquisition and preprocessing of heart rate (HR) and reaction times (RTs) is provided in the Supplementary Material.

Imaging data acquisition and preprocessing

On day 3, MR data were acquired on a 3 Tesla scanner (Trio, Siemens AG, Erlangen, Germany) using a 32-channel head coil with a standard EPI sequence (40 slices, $3 \times 3\ mm^2$ in-plane voxel resolution, TR = 2.09 s, TE = 22 ms, 90° flip angle, 64 × 64 matrix; 192 × 192 mm FOV). Preprocessing and statistical analyses were performed within SPM12 (www.fil.ion.ucl.ac.uk/spm/) implemented in Matlab R2015b (The MathWorks, Inc., Natick, Massachusetts, United States). Preprocessing included slice time correction, realignment to the mean EPI volume, unwarping using the acquired field map, segmentation of the structural T1 image, coregistration of the segmented structural image to the mean EPI, spatial normalization to MNI space based on normalization parameters derived from each subjects' structural image (2-mm isotropic voxel resolution), and

smoothing using a 6-mm full-width at half maximum Gaussian Kernel.

Statistical analysis of behavioral and physiological parameters

Effects of conditioning and extinction

CS pleasantness ratings during conditioning and extinction were analyzed separately with repeated measures ANOVA (rmANOVA) with within-subject factors cue (CS+/CS−) and time (pre/post). SCR, HR and RT during conditioning were analyzed analogously, with the factor time referring to early/late phase of conditioning. Paired *t*-tests were used to analyze cue differences during the extinction phase and all startle tests.

Reinstatement effects

Differential responding on day 3 was only probed for measures showing a significant conditioning effect on day 1 and successful extinction on day 2. Differential valence ratings and startle responses were evaluated using paired *t*-tests. For the fMRI reinstatement test, SCRs were estimated for the first five trials per cue after each reinstatement (10 trials/cue) and analyzed using a paired *t*-test.

Statistical analyses were performed within R version 3.4.3[39] and rmANOVAs were Greenhouse-Geisser-corrected when necessary. Significant interaction effects were followed by *post-hoc t*-tests. The alpha level was set at .05 for all analyses and effect sizes were estimated using partial Eta^2 (η^2p) and Cohen's *d*.

Statistical analysis of imaging data (day 3)

An event-related analysis was applied using SPMs GLM approach on two levels. On the subject level, onsets for CS+ and CS− were included for each phase after convolution with the canonical HRF. US onsets and movement parameters were entered as regressors of no interest. Baseline contrasts for CS+ and CS− were computed for each phase and entered into a flexible factorial model on the group level. Reinstatements effects were analyzed by contrasting CS+ vs. CS− across phases. Possible time effects during the reinstatement test were investigated by assessing the cue × phase interaction. Based on evidence showing amygdala and NAcc involvement in appetitive Pavlovian conditioning[27,28,40] and the role of the vmPFC in successful extinction recall[41,42] and reinstated fear[32,33], we applied small volume correction for amygdala, NAcc, and vmPFC at $p \leq .05$ FWE-corrected. In our sample ($n = 33$) we had a power of 0.88 to detect medium effects ($d = 0.5$) at this threshold (G*Power 3[43]). Bilateral amygdala and NAcc masks were derived from WFU PickAtlas (http://www.fmri.wfubmc.edu/download.htm). For the vmPFC mask, a 10-mm sphere centered on [x = 0, y = 40, $z = -12$] was used based on previous studies on reinstated fear[32,33].

For completeness, exploratory whole-brain analyses at $p < .001$ uncorrected are provided in the Supplementary Material.

gPPI analysis. Based on previous findings[30], we investigated the interplay between amygdala and vmPFC during the reinstatement test and analyzed their cue-dependent functional connectivity using generalized psychophysiological interaction (gPPI) analysis (gPPI toolbox, http://www.nitrc.org/projects/gppi)[44]. For each participant, the first eigenvariate time series was extracted from the left amygdala seed and deconvolved to generate the neuronal signal[45]. For each cue type and phase, a PPI term was created by multiplying the respective cue onsets with the neural time series and convolving it with the HRF. The four PPI terms and the seed region time course then entered as regressors into first-level models otherwise similar to the primary analysis first-level GLM. Estimated connectivity parameters for each cue type and phase were analyzed in SPM's full factorial design on the second level. Cue-specific connectivity was analyzed by contrasting PPI terms for CS+ vs. CS- across phases, and time-dependent changes by interacting cue differences with phase. A region-of-interest (ROI) analysis for the vmPFC was applied at $p \leq .05$ FWE-corrected, following the proposed modulatory influence from vmPFC on amygdala activity during aversive[41,42] and appetitive[30] extinction recall.

Associations with psychophysiological reinstatement effects. In order to link the observed psychophysiological reinstatement effect (SCRs) to brain activation, simple regression analyses were performed within SPM introducing differential SCRs during reinstatement test as a covariate using the contrast images 'CS+ vs. CS−', as well as cue-specific connectivity differences (CS+ vs. CS−) from the gPPI analysis in separate SPMs. These analyses were complemented by subgroup analyses based on a median split of differential SCRs, thereby contrasting a low reinstatement ($n = 17$) with a high reinstatement group ($n = 16$).

Following a reviewer's suggestion, an exploratory gPPI analysis using the right NAcc as seed region was applied (see Supplementary Material).

Results

Manipulation checks

Thirst and hunger

Ratings confirmed thirst and moderate hunger before conditioning (thirst: *M* (SD) = 63.7(20.6); hunger: *M* (SD) = 43.3(27.1)), extinction (thirst: *M* (SD) = 68.7 (21.8); hunger: *M* (SD) = 51.8(29.0)), and reinstatement test (thirst: *M* (SD) = 65.0(18.6); hunger: *M* (SD) = 48.4 (26.6)).

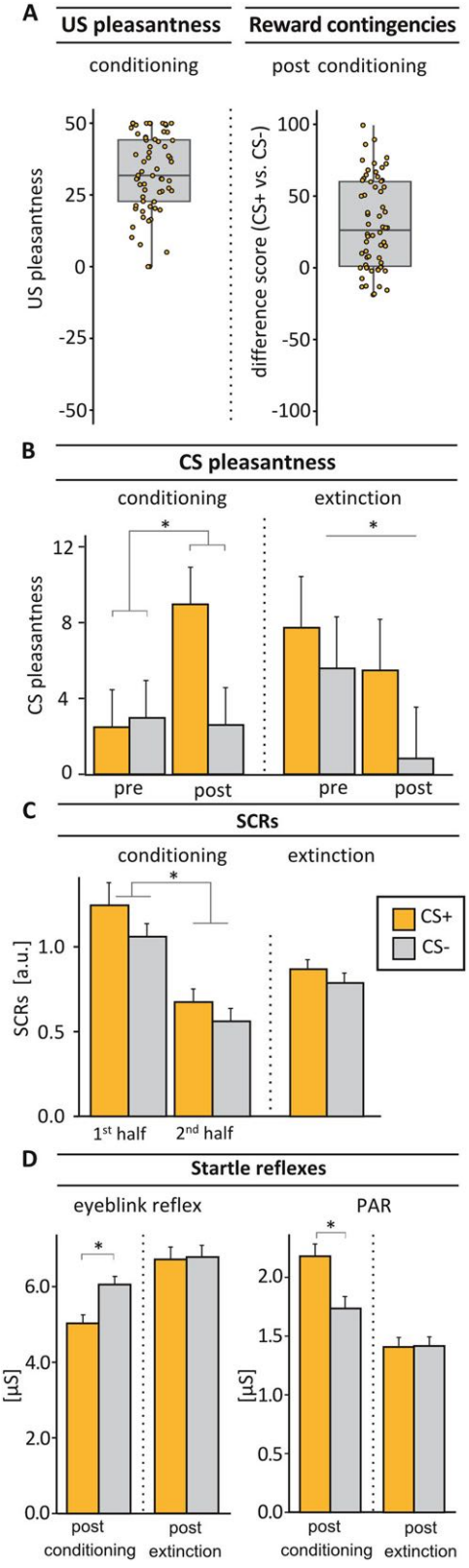

Fig. 2 Indices of conditioning and extinction. a Study inclusion criteria of mean US pleasantness ratings (US pleasantness rating ≥ 0; left panel) and difference scores of rated reward contingencies (CS+*minus* CS− <−20; right panel) on day 1. **b** CS pleasantness ratings increased selectively for CS+ from pre to post conditioning, resulting in a significant cue × time interaction (F (1,61) = 4.32, p = .042). During extinction, a general decline in CS pleasantness was observed (main effect of time: F (1,32) = 4.93, p = .034). **c** Larger SCRs towards the CS + compared to the CS− across both acquisition phases were observed during conditioning (main effect of cue: F (1,59) = 7.08, p = .010). This differentiation was successfully extinguished on day 2 (t(32) = 0.99, p = .329). **d** Conditioning resulted in marked differences between startle responses during CS+ compared to CS− presentations in a subsequent acoustic startle test. While the eyeblink reflex was attenuated, the PAR was enhanced (p ≤ .005). Differential modulation of startle responses disappeared completely after extinction (p ≥ .894). Note that only a subsample of subjects participating on day 1 (conditioning sample) was further investigated during extinction and reinstatement test. For sample sizes in each measure, please see methods section. Error bars represent within-subject SEM[81,82]; a.u., arbitrary units; *p ≤ .05

US pleasantness

The perceived pleasantness of the chosen juice/smoothie was high throughout conditioning (M (SD) = 32.32(13.60); Fig. 2a) and before the reinstatement test (M (SD) = 32.61 (15.68)), and remained unchanged over sessions (day 1 vs. day 3: Z = −0.08, p = .935; Wilcoxon signed-rank test).

Contingency awareness

Reward contingencies for CS+ were rated significantly higher than for CS− after conditioning, indicating overall contingency knowledge across subjects that varied to different degrees (M (SD)$_{diff}$ = 30.40(31.58), t(61) = 7.58, p < .001, range: −18.84 to 99.34; Fig. 2a, see Supplementary Material for further details), suggesting that unin-structed conditioning in addition to our cover story allowed for variability regarding explicit learning.

Conditioning and extinction

CS pleasantness

Analysis of CS pleasantness during conditioning revealed a significant cue × time interaction (F (1,61) = 4.32, p = .042, η^2p = 0.07; Fig. 2b). Closer inspection showed that subjective pleasantness of the CS+ increased significantly from pre- to post-conditioning (t(61) = −2.96, p = .004), while CS− pleasantness remained unchanged (t(61) = 0.13, p = .900), resulting in a trendwise differentiation between CS+ and CS− after acquisition (t(61) = 1.98, p = .052) but not at baseline (t(61) = −0.21, p = .840). During extinction, a significant main effect of time (F (1,32) = 4.93, p = .034, η^2p = .13) indicated an overall decrease in CS pleasantness, but no cue × time interaction or main effect of cue (all p ≥ .413).

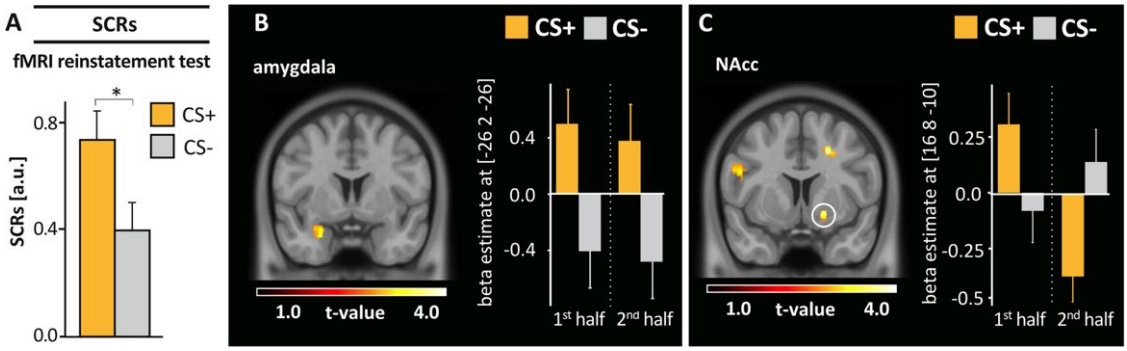

Fig. 3 Psychophysiological and neural responses during reinstatement test. a Return of appetitive conditioned responses was observed during fMRI reinstatement test with significant larger SCRs towards the CS+ compared to the CS− during the first 5 CS+ and CS− presentations in both phases ($t(32) = 2.25$, $p = .031$). Error bars represent within-subject SEM[81,82]. **b** Elevated BOLD response in the contrast CS+ > CS− in the left amygdala over phases (MNI peak at [x: −26, y: 2, z: −26], $p_{FWE\ ROI} = .01$). **c** Interaction of differential BOLD responses with test phase in the right NAcc due to CS+ related activation declines from early to late reinstatement test (MNI peak at [x: 16, y: 8, z: −10], $p_{FWE\ ROI} = 016$). All t-maps are displayed on a visualization threshold of $p < .005$ uc with $k \geq 20$ cluster extend

Skin conductance responses (SCRs)

During conditioning, SCRs towards the CS+ were significantly larger compared to the CS− across phases (main effect cue: $F\ (1,59) = 7.08$, $p = .010$, $\eta^2p = .11$; Fig. 2c). We also observed a significant time effect ($F\ (1,59) = 22.16$, $p < .001$, $\eta^2p = .27$) due to declining SCRs towards both cue types, but no cue × time interaction ($F(1,59) = 0.53$, $p = .468$). As expected, differential SCRs were no longer observed during extinction on day 2 ($t(32) = 0.99$, $p = .329$).

Startle reflexes

Successful conditioning was confirmed by a significantly attenuated eyeblink reflex ($t(45) = -3.19$, $p = .003$, $d = -.47$) as well as a significantly enhanced PAR ($t(47) = 2.98$, $p = .005$, $d = .46$; Fig. 2d) when contrasting startle reflexes during CS+ compared to CS− presentations post-conditioning. After extinction, neither eyeblink reflex nor PAR were differentially modulated by cue type, indicating complete extinction (all $p \geq .894$).

Heart rate (HR)

Analysis of HR during conditioning revealed HR increases towards both cue types (main effect time: $F\ (1,58) = 8.03$, $p = .006$, $\eta^2p = .12$) but no main effect of cue or cue × time interaction ($F\ (1,58) \leq 1.95$, $p \geq .168$). HR responses did not differ during extinction ($t(32) = -0.82$, $p = .418$).

Reaction times (RTs)

RTs obtained from the stimulus side detection task revealed no significant main or interaction effects during conditioning ($F\ (1,60) \leq 1.80$, $p \geq .185$) nor extinction ($t(32) = -0.31$, $p = .760$).

The reported conditioning effects remained unchanged when controlling for possible sample effects (conditioning sample vs. 3-day sample).

Behavioral and psychophysiological reinstatement effects

Return of appetitive conditioned responding was investigated for measures showing successful conditioning and extinction, i.e., CS pleasantness ratings, startle responses, and SCRs. Extinguished differences of eyeblink reflex or PAR did not recover at test ($p \geq .500$). In contrast, following reinstatement in the fMRI, a significant return of conditioned responding was observed in SCRs, with larger SCRs towards CS+ compared to CS− ($t(32) = 2.25$, $p = .031$, $d = .39$; Fig. 3a). CS pleasantness ratings did not differ significantly at either time point ($p \geq .170$), although CS+ pleasantness significantly increased from laboratory to fMRI reinstatement test ($t(32) = -2.88$, $p = .007$) while CS− pleasantness remained unchanged ($t(32) = -0.83$, $p = .414$).

Neural responses during fMRI reinstatement test

The fMRI reinstatement test was accompanied by a significant differential BOLD response in the left amygdala with stronger activation towards the CS+ compared to the CS− ([x:−26, y:2, z:−26]; $Z = 3.82$; $p_{FWE\ ROI} = .010$, Fig. 3b). The inverse contrast (CS−> CS+) revealed no significant activation differences. We also looked for time dependent effects, as return of conditioned responding may decline with repeated unreinforced CS presentations despite the second reinstatement between phases. We observed a significant decline in differential BOLD response over time (cue × time interaction) in the right NAcc ([x:16, y:8, z:−10]; $Z = 3.35$, $p_{FWE\ ROI} = .016$; Fig. 3c) and trendwise also in the left amygdala ([x:−20, y:−6, z:−18]; $Z = 2.23$; p_{FWE}

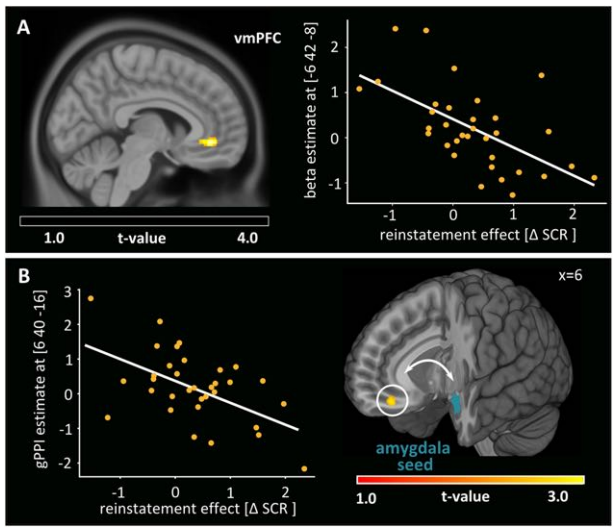

Fig. 4 Neural responses associated with psychophysiological reinstatement effects. a Differential BOLD responses in the vmPFC during reinstatement test were inversely correlated with Pavlovian reinstatement intensity, indexed by differential SCRs (MNI peak at [x: −6, y: 42, z: −8], $p_{FWE\ ROI} = .022$). **b** Differential SCRs were further marginally inversely correlated with functional amygdala-vmPFC connectivity (gPPI) observed in the second test phase (MNI peak at [x: 6, y: 40, z: −16], $p_{FWE\ ROI} = .061$). Overlayed in cyan is the unilateral anatomical ROI mask of the left amygdala used to extract the time course of the seed region for the gPPI analysis. All t-maps are displayed on a visualization threshold of $p < .005$ uc with $k \geq 20$ cluster extend

$_{ROI} = .064$), but no differential BOLD response increase over phases.

Neural responses associated with psychophysiological reinstatement effects

We investigated brain regions associated with the psychophysiological reinstatement effect by performing regression and subgroup analyses based on differential SCRs during the reinstatement test. Regression analysis revealed a significant negative correlation between differential SCRs and activation within the vmPFC ([x:−6, y:42, z:−8]; $Z = 3.63$; $p_{FWE\ ROI} = .022$; Fig. 4a), indicating stronger vmPFC activity towards CS+ compared to CS− in subjects showing attenuated differential SCRs. Corroborating this association, directly contrasting participants with high vs. low differential SCRs confirmed significant higher vmPFC activation in the low reinstatement compared to the high reinstatement group ([x:−4, y:42, z:−8], $Z = 3.83$, $p_{FWE\ ROI} = .011$; Fig. S1A).

Functional connectivity between amygdala and vmPFC

Based on the proposed inhibitory role of the vmPFC over amygdala to support successful extinction recall[30,41,42], we further investigated cue-dependent functional connectivity between the amygdala and the vmPFC during the reinstatement test. The gPPI analysis showed that, while no significant amygdala-vmPFC connectivity was evident across phases, connectivity during CS+ compared to CS− presentation was significantly enhanced in the second phase of the reinstatement test ([x:8, y:44, z:−16]; $Z = 3.49$; $p_{FWE\ ROI} = .032$). Interestingly, this connectivity was further marginally anticorrelated with the psychophysiological reinstatement effect ([x:6, y:40, z:−16]; $Z = 3.25$; $p_{FWE\ ROI} = .061$; Fig. 4b), i.e., tended to be enhanced in participants with low compared to high differential SCRs during reinstatement test ([x:6, y:40, z:−16], $Z = 3.08$, $p_{FWE\ ROI} = .077$; Fig. S1B).

Discussion

This study investigated the return of experimentally conditioned appetitive responses in healthy subjects as a translational laboratory model of appetitive Pavlovian relapse. We showed that SCRs recover after a reinstatement procedure 24 h after extinction and provide evidence for opposing roles of amygdala and vmPFC in mediating Pavlovian relapse. During the reinstatement test, amygdala activation towards the CS+ was enhanced, while psychophysiological reinstatement intensity was significantly anticorrelated with vmPFC activation and marginally with enhanced amygdala-vmPFC connectivity observed during late reinstatement.

Amygdala and NAcc activity during appetitive Pavlovian relapse

The reinstatement test showed increased BOLD responses in the left amygdala towards CS+ compared to CS− presentations. Amygdala activity was present over both test phases, declining trendwise over time. Preclinical studies have demonstrated the central role of the amygdala in appetitive Pavlovian learning[25,46] and cue-induced relapse in animal models of drug addiction[47]. Corroborating animal findings showing the relevance of the amygdala in the formation of CS-US associations[48], human neuroimaging studies have repeatedly observed amygdala activation during appetitive Pavlovian learning with primary rewards[27,28,40]. The increased amygdala activation present in our study therefore likely reflects retrieval of the original CS−US association. In line with our results, enhanced amygdala activation towards a previously extinguished fear cue has also been observed following unsignaled aversive US presentations[32,33,49] or context changes[50,51]. Enhanced amygdala activation has further been observed towards an extinguished monetary CS+ following a reactivation procedure 24 h after extinction in healthy controls[30]. We further observed time-dependent differential NAcc activity due to CS+ related declines in BOLD responses from early to late reinstatement, suggesting a more transient involvement of

this structure during the reinstatement test. Animal and human work have identified the NAcc within the ventral striatum as a key structure in the brain's reward circuit, being involved in reward processing and reward-related learning[52,53]. In rodents, expression of conditioned approach behavior towards food or drug cues depend on an intact NAcc[54–56] and human neuroimaging has shown increased BOLD responses in the ventral striatum towards cues predicting primary rewards[27,28,40,57].

vmPFC mediated inhibition of appetitive conditioned responses

In contrast to enhanced amygdala and NAcc activity, we did not observe significant vmPFC activation towards CS+ compared to CS− presentations during the reinstatement test. Differential BOLD responses in this region were instead inversely related to psychophysiological reinstatement intensity (i.e., differential SCRs), whereby increased vmPFC involvement was only present in subjects experiencing weak and not in those showing strong reinstatement effects. This finding directly adds to animal evidence supporting a role for the vmPFC in inhibiting maladaptive learned associations[25,26]. Rodent studies have demonstrated that lesions of the infralimbic (IL) cortex as the homolog region of the human vmPFC do not impair acquisition or within-session extinction of appetitive Pavlovian responses, but impair retrieval of extinction the following day, resulting in increased spontaneous recovery, reinstatement, and renewal[22,23]. Conversely, optogenetic activation of IL neurons has been shown to suppress the return of appetitive conditioned responses[58]. Animal models of cue-induced reward seeking after extinction further revealed IL involvement in both drug and natural reward seeking responses[26,59,60] and specific CS-responsive neuronal ensembles within the IL have been shown to exert inhibitory control over alcohol seeking[61].

In line with our result, human neuroimaging has implicated the vmPFC in successful aversive extinction learning and recall[31–33,42,50,62], and vmPFC activity[41,42,62] and cortical thickness[63] scaled inversely with conditioned SCRs. Our results further extend recent evidence linking reduced vmPFC involvement to return of fear following reinstatement[32,64]. In these studies, differential vmPFC activity was present during simple extinction recall but not after a reinstatement procedure[32], and reduced CS− related vmPFC activity during reinstatement test compared to extinction recall was associated with increased SCRs, indicating a "release from inhibition"[64]. Our results further suggest an important regulatory role for the vmPFC in appetitive Pavlovian relapse. Adding to this, increased ventral vmPFC activity has been shown towards a cue no longer paired with a monetary reward, consistent with an inhibitory signal[29].

The rodent vmPFC is widely connected[65]. Given its strong projections to the amygdala[66,67] and previous findings on functional amygdala-vmPFC connectivity during appetitive extinction recall[30], we investigated cue-dependent functional connectivity between amygdala and vmPFC during the reinstatement test. While there was no evidence of enhanced connectivity across phases, amygdala-vmPFC coupling during CS+ relative to CS− presentations was significantly enhanced during late reinstatement. On an individual level, amygdala-vmPFC connectivity might be further inversely related to the psychophysiological reinstatement effect, indicated by a marginally significant anticorrelation. These findings add to imaging studies reporting functional amygdala-vmPFC connectivity during fear extinction recall[41,42] (but see[50]) and indicate that amygdala-vmPFC coupling constitutes an important neural correlate of successful extinction recall despite adverse circumstances. In line with this, enhanced cue-dependent amygdala-vmPFC coupling has been observed during appetitive extinction recall in subjects receiving the NMDA receptor agonist D-cycloserine hypothesized to enhance extinction consolidation compared to placebo[30]. Moreover, increased amygdala-vmPFC connectivity during initial appetitive conditioning seems to attenuate amygdala activity and acquisition of SCRs[28]. Our finding that amygdala-vmPFC connectivity only emerged during the late reinstatement test is consistent with the proposed disinhibition due to decreased vmPFC activity observed in reinstated fear[64]. Amygdala-vmPFC connectivity might therefore increase as reinstatement effects decline, in line with declining differential BOLD responses observed over test phases for NAcc and trendwise for amygdala.

Psychophysiological and behavioral measures of conditioned responding

We observed a differential return of conditioned responding during the reinstatement test in an implicit measure (SCRs), providing evidence that human appetitive Pavlovian relapse can be modeled in the laboratory. Most of what is known about Pavlovian relapse effects in humans stems from investigations on return of fear phenomena[13,15,68]. The relative lack of translational research in the appetitive domain has been explained by difficulties in finding suitable USs and measures sensitive to appetitive conditioned responses[19–21]. In line with a previous study using food US[16], we observed successful conditioning and extinction in pleasantness ratings, SCRs and eyelid startle, clearly indicating the validity of our design. Conditioning also resulted in a significantly enhanced PAR, thereby replicating recent evidence demonstrating the sensitivity of this microreflex as an appetitive conditioning index[20]. By contrast, HR and RTs did not provide sensitive indices of conditioning in our paradigm. Unlike SCRs, neither

pleasantness ratings nor startle responses showed significant reinstatement effects. Diverging findings across multiple response measures are also commonly reported in fear reinstatement studies[13,69], and multimodal investigation of appetitive conditioning suggests that different conditioning indices are only weakly related on an individual level[21]. We observed a rather weak conditioning effect in pleasantness ratings with only trendwise differentiation after conditioning. Explicit ratings might primarily reflect cognitive learning components[70] as indicated by the influence of contingency awareness in our study (see Supplementary Material), while our uninstructed learning paradigm and cover story was intended to reduce cognitive demands[71]. Moreover, pleasantness ratings have been shown to be rather insensitive to extinction[70,72,73] and, in contrast to SCRs or BOLD responses, might not distinguish experimental groups well[28,57]. While we observed a robust startle modulation after conditioning, participants may have learned to distinguish the non-reinforced and aversive startle context from appetitive acquisition in this session, potentially impeding assessment of startle modulation on subsequent days. Apart from that, the scanner environment possibly enhanced the return of conditioned responses observed during the fMRI reinstatement test, i.e., a stronger renewal effect might have added to the return of conditioned responding.

Conclusions

Our finding that appetitive conditioned responses returned after unsignaled US presentations 24 h after extinction extends existing evidence on return of fear phenomena in humans to the appetitive research domain. Moreover, our results suggest opposing roles for amygdala and vmPFC in mediating appetitive Pavlovian relapse effects. This is of particular clinical relevance, as drug addiction is associated with heightened amygdala responses towards disease-related cues[74], while, at the same time, addicted patients exhibit grey matter volume decreases and activation impairments in vmPFC[75–79]. Future studies could investigate appetitive Pavlovian conditioning and relapse phenomena in patients with addiction and explore inter-individual differences in these processes as well as its interaction with instrumental responding (e.g., Pavlovian-instrumental transfer (PIT)[80]). Although correlational, our findings suggest that the vmPFC could be a promising target for novel intervention techniques that aim to counteract appetitive Pavlovian relapse.

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Conflict of interest
The authors declare that they have no conflict of interest.

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Supplementary Material to:

Opposing roles for amygdala and vmPFC in the return of appetitive conditioned responses in humans

Claudia Ebrahimi, Stefan P. Koch, Charlotte Pietrock, Thomas Fydrich, Andreas Heinz, Florian Schlagenhauf

Paradigm and trial structure

Trial sequences were pseudo-randomized across subjects and sessions, but restricting the occurrence of identical cue type or stimulus side to a maximum of three successive trials. *Additional restriction conditioning*: Within subjects, both phases followed the same trial sequence except that the very first four trials were fixed to initiate learning equivalently across subjects (reinforced CS+, CS-, reinforced CS+, CS-).
Additional restriction reinstatement test: As reinstatement effects are assumed to be transient, each phase started with two alternating CS+/CS- presentations (either [CS+ CS- CS+ CS-] or [CS- CS+ CS- CS+]), counterbalanced across subjects.

The paradigm was programmed in Matlab (R2011a; The Mathworks, Natick, United States) using Cogent (Wellcome Department of Imaging Neuroscience, Institute of Neurology, London, UK) and presented on a 19" computer screen except during fMRI, when stimuli were presented on an MR-compatible LCD screen (32″, NNL LCD Monitor®, NordicNeuroLab, Bergen, Norway).

Data Acquisition and Preprocessing

Heart Rate. Heart rate (HR) during conditioning and extinction was measured using electrocardiography (ECG) with bipolar leads. Adhesive electrodes were placed in right parasternal costoclavicular space and left mid-clavicular line in the fifth intercostal space. The ground electrode was placed on the costal margin in the right mid-clavicular line. During the reinstatement test, photoplethysmography (PPG) attached to the left index finger was used as a measure of cardiac activity, recorded at 50 Hz via the Siemens Physiological Monitoring Unit. The QRS detection algorithm proposed by Pan & Tompkins[1] was used to extract QRS complexes from ECG data. For the PPG, online pulse period (PP) detection within the PMU was used. Subsequently, the complete time sequence with detected RR

intervals (PP intervals on day 3, respectively) was visually inspected and manually corrected, if necessary. Sequences with artefacts or low signal in the data preventing reliable heart beat detection were treated as missing data points. The time series of non-uniform inter-beat

intervals was converted to HR and interpolated to the sample rate of acquisition. Trials with missing data in a window from -1 to 4 s with respect to CS onset were excluded from further analyses. Mean HR was calculated for the time window 1-3 s after cue onset. Trialwise HR data were normalized and aggregated over each phase and cue type. In the conditioning sample technical failures during recording caused data loss in three subjects, while for day 3 another five subjects were excluded due to failed PP detection (low signal-to-noise ratio/ PPG dislocation). Because swallowing causes prolonged HR changes after US delivery[2], only trials without preceding reinforcement were considered for analysis of HR during conditioning (day 1).

Reaction Time. RTs from the cue side detection task were collected in the laboratory and fMRI with a 2-button keypad and MR-compatible response buttons, respectively. RTs between 200-2400 ms were considered valid responses. Data were log transformed to reduce skewness, and averaged over each phase and cue type. Data from one subject are missing due to technical malfunction.

Neural responses during fMRI reinstatement test

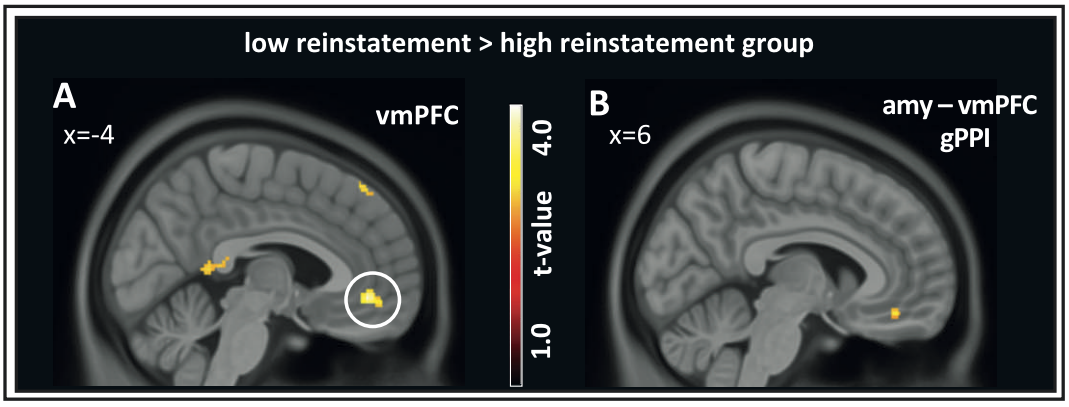

Figure S1. Subgroup analyses based on median split on differential SCRs during reinstatement test. A Higher differential BOLD responses (CS+ > CS-) in vmPFC in participants experiencing low opposed to high psychophysiological reinstatement ([x:-4, y:42, z:-8], Z=3.83, p_{FWE_ROI}=.011). **B** Cue-dependent functional amygdala–vmPFC connectivity (gPPI) during late reinstatement test was trendwise enhanced in the low compared to the high reinstatement group ([x:6, y:40, z:-16], Z=3.08, p_{FWE_ROI}=.077).

Supplementary Table S1: Exploratory whole-brain results during reinstatement test, displayed at p<.001 uncorrected using a cluster-forming threshold of k=10 contiguous voxels

Contrast	Region	Side	Voxel	Peak voxel MNI			Z_{max}	P_{uc}
				x	y	z		
Full reinstatement test [all CS+ > CS-]	Amygdala	L	22	-26	2	-26	3.82	<.001
	Cerebellum	L	14	-16	-38	-30	3.59	<.001
	Middle temporal gyrus	R	11	46	-62	16	3.55	<.001
	Precuneus	R	10	6	-58	22	3.49	<.001
Early reinstatement $_{[CS+ > CS-]}$ > late reinstatement $_{[CS+>CS-]}$	Middle frontal gyrus	R	23	22	6	42	4.21	<.001
	Putamen	R	16	18	8	-10	3.82	<.001
	Middle cingulum	L	14	-16	-34	34	3.71	<.001
	Middle temporal gyrus	L	15	46	-48	-6	3.70	<.001
	Supramarginal gyrus	R	16	64	-40	34	3.69	<.001
	Precuneus	R	29	10	-58	62	3.67	<.001
	Medial orbitofrontal gyrus	L	13	-8	60	-14	3.58	<.001
	Precentral gyrus	L	26	-50	4	26	3.44	<.001

Exploratory connectivity analysis using the right NAcc as seed region

Following a reviewer's suggestion, we further explored functional cue-dependent connectivity between right NAcc, which showed a time-dependent effect during the reinstatement test with stronger BOLD response towards CS+ compared to CS- during the early compared to the late reinstatement phase, and the vmPFC by applying a similar gPPI analysis as for the amygdala but using the right NAcc as seed region. This analysis revealed no significant cue-dependent NAcc-vmPFC involvement across or within phases of the reinstatement test ($p_{FWE\ ROI}$≥.648). Interestingly, we instead observed heightened functional connectivity between right NAcc and amygdala ([x:23, y:0, z:-26]; *Z*=3.40; $p_{FWE\ ROI}$=.035) during the early reinstatement phase, suggesting these two structures to closely interact upon CS+ compared to CS- presentation.

Effects of contingency awareness on conditioning measures

Following a worthwhile reviewer comment, we explored possible associations between contingency awareness and measured indices of conditioning on day 1. As our study was not designed to unambiguously classify participants as contingency aware or unaware, we based our analyses on a median split on difference scores of rated reward probabilities (CS+ *minus* CS-) acquired after conditioning (see Figure S2A). We then re-evaluated each conditioning measure by introducing a between subject group factor (aware vs. unaware), i.e. CS pleasantness ratings, SCRs, RTs, and HR were analyzed in separate mixed ANOVAs with within subject factors cue type (CS+ vs. CS-) and time (pre/early vs. post/late) and between subject factor group (aware vs. unaware), and startle responses were analyzed in a mixed

ANOVA with within subject factor cue type and between subject factor group. Significant interactions with contingency awareness were followed up by groupwise post-hoc analyses.

CS pleasantness ratings. Including awareness as an additional predictor revealed a significant cue x time interaction ($F(1,60)=4.60$, $p=.036$, $\eta^2p=.07$), as well as a significant three-way interaction cue x time x group ($F(1,60)=4.97$, $p=.030$, $\eta^2p=.08$; Figure S2B). No further main or interactions effects were observed ($F(1,60)\leq2.48$, $p\geq.120$). Post-hoc ANOVAs showed a significant cue x time interaction only in aware subjects ($F(1,30)=5.62$, $p=.024$, $\eta^2p=.16$), while no significant main or interaction effects were present in the unaware group ($F(1,30)\leq0.72$, $p\geq.402$), indicating that CS pleasantness ratings were mediated by contingency awareness.

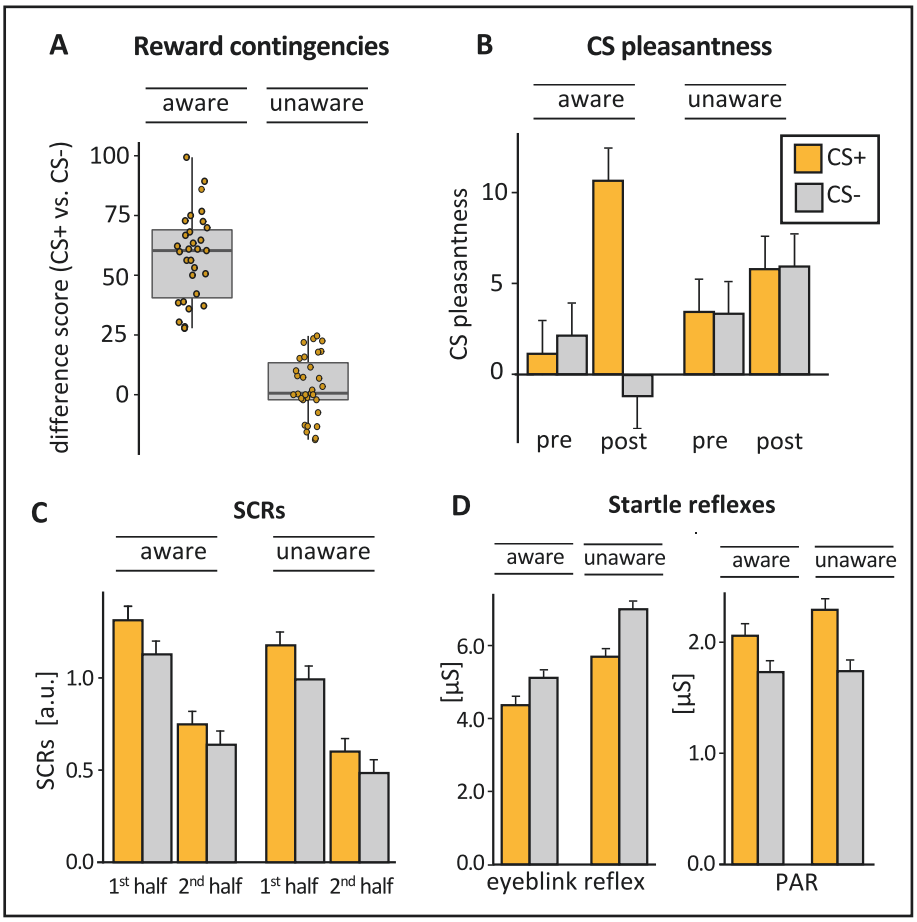

Figure S2. Effects of contingency awareness on conditioning measures on day 1. A. Classification of participants into contingency aware vs. unaware participants was based on a median split on difference scores of rated reward probabilities for each cue (CS+ *minus* CS-) obtained after conditioning. **B-D.** CS pleasantness, SCRs, and startle responses for contingency aware and unaware participants. Error bars represent within-subject SEM[3,4]; a.u., arbitrary units; PAR, postauricular reflex.

SCRs. Validating our main analysis, we observed a significant main effect of cue ($F(1,58)=6.96$, $p=.011$, $\eta^2p=.11$) due to increased SCRs towards the CS+ compared to the

CS- and a main effect of time (F(1,58)=21.78, p<.001, η^2p =.27) due to overall decreasing SCRs over the conditioning session (Figure S2C). No further main or interaction effects were significant (F(1,58)≤0.63, p≥.430), suggesting that SCRs were unaffected by contingency awareness.

Startle responses. A significant main effect of cue confirmed differential modulation of both startle measures after conditioning (eyeblink reflex: F(1,44)=10.13, p=.003, η^2p=.19; PAR: F(1,46), p=.005, η^2p=.16), while no main or interaction effects with awareness were observed for both measures (eyeblink reflex: F(1,44)≤1.32, p≥.256; PAR: F(1,46)≤0.56, p≥.457; Figure S2D).

RTs. In line with our main analysis, no significant main or interaction effects were observed in RTs towards cues (all F(1,59) ≤3.93, p≥.052).

HR. Analysis of HR revealed only a main effect of time due to general HR increases over phases (F(1,57)=8.08, p=.006, η^2p=.12), but no significant conditioning effects or interactions with contingency awareness (all F(1,57) ≤3.37, p≥.072).

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ORIGINAL ARTICLE

PSYCHOPHYSIOLOGY SPR WILEY

Pupil dilation as an implicit measure of appetitive Pavlovian learning

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Abstract

Appetitive Pavlovian conditioning is a learning mechanism of fundamental biological and pathophysiological significance. Nonetheless, its exploration in humans remains sparse, which is partly attributed to the lack of an established psychophysiological parameter that aptly represents conditioned responding. This study evaluated pupil diameter and other ocular response measures (gaze dwelling time, blink duration and count) as indices of conditioning. Additionally, a learning model was used to infer participants' learning progress on the basis of their pupil dilation. Twenty-nine healthy volunteers completed an appetitive differential delay conditioning paradigm with a primary reward, while the ocular response measures along with other psychophysiological (heart rate, electrodermal activity, postauricular and eyeblink reflex) and behavioral (ratings, contingency awareness) parameters were obtained to examine the relation among different measures. A significantly stronger increase in pupil diameter, longer gaze duration and shorter eyeblink duration was observed in response to the reward-predicting cue compared to the control cue. The Pearce-Hall attention model best predicted the trial-by-trial pupil diameter. This conditioned response was corroborated by a pronounced heart rate deceleration to the reward-predicting cue, while no conditioning effect was observed in the electrodermal activity or startle responses. There was no discernible correlation between the psychophysiological response measures. These results highlight the potential value of ocular response measures as sensitive indices for representing appetitive conditioning.

KEYWORDS

associative learning, attention, eye-tracking, pupil dilation, reward

1 | INTRODUCTION

Appetitive Pavlovian conditioning is the learning process by which an initially neutral stimulus (CS, conditioned stimulus), after repeated pairings with a salient pleasant experience (US, unconditioned stimulus), is able to elicit the innate physiological response that was originally confined to the US (Pavlov, 1927; Rescorla, 1988). This constitutes a central learning mechanism that enables organisms to survive and thrive in dynamic environments; however, if maladaptive, it can also contribute to pathological states including addiction, depression, and eating disorders (Grosshans, Loeber,

& Kiefer, 2011; Kiefer & Dinter, 2013; Martin-Soelch, Linthicum, & Ernst, 2007; Robinson & Berridge, 2000; van den Akker, Jansen, Frentz, & Havermans, 2013).

In contrast to its aversive counterpart (Delgado, Jou, & Phelps, 2011; Fullana et al., 2016; Li & McNally, 2014), appetitive conditioning is only rarely explored in humans (Andreatta & Pauli, 2015; Konova & Goldstein, 2018). This is predominantly ascribed to two challenges: the identification of suitable reinforcement as well as clear criteria for established conditioning. Regarding the first, it is difficult to determine a US whose rewarding properties or subjective pleasantness is inter-individually equivalent. So far, a variety of both primary and secondary stimuli have been used for appetitive reinforcement, for example, food (Andreatta & Pauli, 2015; Blechert, Testa, Georgii, Klimesch, & Wilhelm, 2016; van den Akker et al., 2017a; Wardle, Lopez-Gamundi, & Flagel, 2018), drink (Ebrahimi et al., 2019; O'Doherty, Buchanan, Seymour, & Dolan, 2006; O'Doherty, Dayan, Friston, Critchley, & Dolan, 2003; Pauli et al., 2015; Prévost, McNamee, Jessup, Bossaerts, & O'Doherty, 2013), odor (Gottfried, O'Doherty, & Dolan, 2002; Hermann, Ziegler, Birbaumer, & Flor, 2000; Stussi, Delplanque, Corai, Pourtois, & Sander, 2018), attractive faces (Bray & O'Doherty, 2007), erotic images (Klucken et al., 2009, 2013, 2015; Klucken, Wehrum-Osinsky, Schweckendiek, Kruse, & Stark, 2016), and money (Austin & Duka, 2010; Delgado, Gillis, & Phelps, 2008; Ebrahimi et al., 2017; Tapia León, Kruse, Stalder, Stark, & Klucken, 2018). Although there exists a certain overlap, primary and secondary rewards are processed in distinct neural systems (Sescousse, Caldú, Segura, & Dreher, 2013). Both primary and secondary appetitive reinforcers rarely result in physiological responses comparable to those evoked by reinforcers in aversive conditioning research (e.g., pain and noise), and the appetitive value of the US is difficult to standardize (Martin-Soelch et al., 2007; Stussi et al., 2018). Furthermore, the physiological responses toward secondary reinforcers may be weaker compared to those elicited by primary reinforcers (Andreatta & Pauli, 2015; Ebrahimi et al., 2017).

Associated with this is the second challenge facing human appetitive conditioning research, namely, the lack of an established gold standard measurement to assess conditioned responding. A frequently implemented method to confirm successful conditioning are ratings, for example, CS valence (Andreatta & Pauli, 2015; Ebrahimi et al., 2017, 2019; Klucken et al., 2015, 2009, 2013, 2016; Prévost et al., 2013), CS dichotomous preference (Bray & O'Doherty, 2007; Kahnt, Heinzle, Park, & Haynes, 2011; Metereau & Dreher, 2013; Prévost et al., 2013), US expectancy (van den Akker, Havermans, & Jansen, 2015), and contingency awareness (Bray & O'Doherty, 2007; Ebrahimi et al., 2017, 2019; Klucken et al., 2015, 2009, 2013, 2016; Stussi et al., 2018; Tapia León et al., 2018). A shortcoming of ratings is that they only reflect the explicit component of learning and are prone to influences of social desirability when the learning task is simple. Therefore, a thorough investigation of appetitive associative learning should incorporate both explicit and implicit conditioning indices. Unfortunately, due to the scarcity of multi-methodological studies that compare implicit learning parameters, along with nonstandardized approaches of analysis, it is still unclear which measure is most suited in appetitive conditioning experiments (Stussi et al., 2018; Wardle et al., 2018).

Implicit behavioral indices of appetitive conditioning, like reaction time, are hitherto inconclusive, with results showing both conditioned increases (O'Doherty et al., 2006), decreases (Ebrahimi et al., 2017; Gottfried et al., 2002), or no differentiation (Ebrahimi et al., 2019; Metereau & Dreher, 2013) in response times. Psychophysiological measures similarly often present inconsistent results. Electrodermal activity, which is a common learning index used in aversive conditioning paradigms (Lonsdorf et al., 2017; Ney et al., 2018), has shown both an enhanced skin conductance response (SCR; Andreatta & Pauli, 2015; Ebrahimi et al., 2019; Klucken et al., 2013, 2015, 2016; Tapia León et al., 2018), as well as no differential response (Klucken et al., 2009; Stussi et al., 2018; van den Akker et al., 2017a) to the reward-associated stimulus and appears to be dependent on task context (van den Akker et al., 2017b). Heart period response (HPR) has seldom been examined in an appetitive context and has not yielded a conclusive differential effect (Hermann, Ziegler, Birbaumer, & Flor, 2000; Wardle et al., 2018). Interestingly, fear-conditioned cardiac deceleration (bradycardia) has been observed in experiments using aversive US (Castagnetti et al., 2016; Prévost et al., 2013). Both SCR and HPR are characterized by long response latencies and durations, which unfortunately prolong the experiment's duration (Lonsdorf et al., 2017; Sjouwerman & Lonsdorf, 2018). In contrast, acoustic startle responses (eyeblink reflex, EBR; Andreatta & Pauli, 2015; Ebrahimi et al., 2019; Hermann et al., 2000; Stussi et al., 2018; Wardle et al., 2018) and the vestigial postauricular microreflex (PAR; Aaron & Benning, 2016; Ebrahimi et al., 2019; Sandt, Sloan, & Johnson, 2009; Stussi et al., 2018) have short reaction latencies; however, their inherent aversive quality limits their utility in the appetitive conditioning domain, where they are confined to being post-hoc measures.

In the current study, we decided to explore the ocular response as a potential measure of appetitive conditioning. Eye-tracking is an accurate, non-invasive tool and specifically pupil diameter constitutes a powerful implicit measure in cognitive tasks with short response latency (van der Wel & van Steenbergen, 2018). Non-luminance-mediated pupil dilation is generally associated with a broad range of cognitive processes causing sympathetic nervous activation (Sirois & Brisson, 2014; van der Wel & van Steenbergen, 2018), including, but not limited to, mental processing load (Just, Carpenter, & Miyake, 2003; Kahneman & Beatty, 1966), emotional processing (Granholm & Steinhauer, 2004;

Kinner et al., 2017), arousal (Bradley, Miccoli, Escrig, & Lang, 2008; Leuchs, Schneider, Czisch, & Spoormaker, 2017; Prévost et al., 2013; Seymour, Daw, Dayan, Singer, & Dolan, 2007), attention (Eldar, Cohen, & Niv, 2013; Laeng, Sirois, & Gredebäck, 2012; Lasaponara et al., 2019), surprise (Kloosterman et al., 2015), exerted effort (Varazzani, San-Galli, Gilardeau, & Bouret, 2015), learning and memory (Aston-Jones & Cohen, 2005; Brocher & Graf, 2016; Eldar et al., 2013; Goldinger & Papesh, 2012; Nassar et al., 2012; Silvetti, Vassena, Abrahamse, & Verguts, 2018; Tzovara, Korn, & Bach, 2018). Prior research in the context of appetitive conditioning is scarce and has focused only peripherally on pupil diameter (Bray, Rangel, Shimojo, Balleine, & O'Doherty, 2008; O'Doherty et al., 2003, 2006; Seymour et al., 2007; Pauli et al., 2015; Prévost et al., 2013), showing pupil dilation toward both primary (Pauli et al., 2015; Prévost et al., 2013; O'Doherty et al., 2003, 2006) and secondary (Seymour et al., 2007) conditioned stimuli. Moreover, we decided to explore gaze dwelling time, that is, the amount of time gaze lingers on a stimulus, as a measure of visual attention (Isaac, Vrijsen, Rinck, Speckens, & Becker, 2014) in conditioned learning. We further investigated blink responding (blink frequency and duration). Analyses of blink frequency have thus far been isolated to the aversive conditioning domain, where a greater frequency to the aversive conditioned stimulus has been observed (Pauli et al., 2015; Prévost et al., 2013). Blink duration is commonly used as an indicator of alertness, as long blinks are found to signal drowsiness and fatigue (Caffier, Erdmann, & Ullsperger, 2003; Stern, Boyer, & Schroeder, 1994). Both gaze dwelling time and blink responses have, to our knowledge, never been systematically examined in an appetitive conditioning paradigm. With the purpose of contributing to the quest for a sensitive psychophysiological parameter, we tested whether the ocular response measures (pupil diameter, gaze dwelling time, blink duration, blink count) are suitable measures for representing appetitive conditioning.

To address the elaborated challenges in appetitive conditioning, we designed a conditioning paradigm using a primary reinforcer to test the hypothesis that pupil dilation is a sensitive marker for appetitive conditioning. Furthermore, we, to our knowledge, for the first time assess additional ocular response measures such as gaze dwelling time and blink responding in the appetitive conditioning context.

In line with budding research (Koenig, Uengoer, & Lachnit, 2018; Leuchs et al., 2017), we investigated whether latent and dynamic learning mechanisms could be inferred from the trial-by-trial pupil response by means of computational modeling techniques. Using learning models based on a Rescorla-Wagner framework, we explored whether this trial-by-trial measure depicted the expected stimulus value or its associated Pearce-Hall attention weight. To corroborate our data and search for possible relations between ocular and other psychophysiological measures (Wardle et al., 2018), we assessed additional psychophysiological parameters (SCR, HPR, EBR, and PAR) previously used in appetitive conditioning research.

2 | METHOD

2.1 | Participants

A total of 32 right-handed, healthy volunteers participated in the present study. Participants were recruited via the student mailing lists of the Humboldt-University of Berlin and Charité–Universitätsmedizin Berlin. All participants were free of current or past neurological, psychiatric, and metabolic disorders, had normal or corrected-to-normal vision, intact color vision, and consumed no therapeutic or recreational drugs. Inclusion criteria were regular daily food intake and no allergies or dietary limitations. Students of psychology were not permitted to take part in the experiment. Three participants were excluded from the analysis (two as a cause of technical difficulties during data acquisition and one due to an average negative US rating (≤50 %), see Section 2.3.1., Ratings, for further details). This left 29 participants (16 female) ranging in age from 18–30 years, $M(SD)_{age} = 24.49(3.45)$ years and ranging in body mass index (BMI) from 18–27 kg/m^2, $M(SD)_{BMI} = 22.12(2.26)$ kg/m^2. All participants provided written informed consent and received 20€ for their participation. The study was performed in accordance with the Declaration of Helsinki and was approved by the local ethics committee of the Charité.

2.2 | Experimental procedure

Participants completed an appetitive Pavlovian learning task, where they learned to associate sequentially presented audiovisual stimuli (two female faces coupled with a distinct bell chime) with a rewarding outcome (juice delivery) or no reward, respectively. Throughout the task, we acquired a variety of psychophysiological measures (ocular response measures, heart period, electrodermal activity). Directly before and after the learning task, participants rated the CS and US and indicated their awareness for CS-US contingency. As a further parameter of conditioning, an auditory startle task was performed following the conditioning task.

To enforce the craving of the US, participants were asked to abstain from eating and drinking in the respective 6 and 4 hrs preceding the experiment (Ebrahimi et al., 2019; Metereau & Dreher, 2013). The mean reported fasting time was 9.6 hr for food and 4.4 hr for drink. Participants selected and rated their preferred US from four fruit juices (apple, orange, mango-passion fruit, berry) and, after viewing a 4-min priming presentation showing various appetizing dishes and drinks, rated their current state of hunger and thirst on a visual analogue scale (VAS) ranging from 0–100%.

2.2.1 | Design

The differential delay conditioning procedure consisted of 96 trials (48 trials with CS+ condition, 48 with CS− condition). An additional habituation phase of 8 CS presentations (4 per condition) with no reinforcement, but analogous timing, preceded the experiment. During the conditioning phase, each trial began with the presentation of the CS for 6 s to the left or right side of a central fixation cross. In half of the CS+ trials (24 trials), the CS+ was followed by the US 5 s after CS onset (50% reinforcement schedule). In reinforced trials, the phrase "Please swallow!" appeared on screen during the intertrial interval (ITI) with a jittered interval of 3–6 s after US delivery in order to mitigate swallowing artifacts (Pauli et al., 2015). The CS− was never reinforced. Trials were separated by a variable ITI starting at CS offset with a mean duration of 11 s (min. 9 s, max. 16 s; see Figure 1a).

2.2.2 | Trial order

The experiment was divided into two halves. Before the start of the first and second half of the experiment, a standardized 9-point eye-tracking calibration was carried out. Stimuli were presented in a pseudorandomized order: The first and second half consisted of quasi-identical trial sequences, where the first two appearances of the CS+ in the experiment were always reinforced. The three possible pairings of CS and outcome (CS+ reinforced, CS+ unreinforced, and CS− trials) appeared equally often in the first and second half of the experiment (Klucken et al., 2016). Additionally, the following criteria were applied to the trial sequences: There were never more than three consecutive trials of the same condition, cues were never displayed for more than three successive trials on the same side of the fixation cross, and there was a balanced succession of CS+ and CS− trials following a trial with US delivery (Ebrahimi et al., 2017, 2019).

2.2.3 | Stimuli

Visual stimuli were presented on a 36.5 cm × 27.4 cm computer monitor with a spatial resolution of 1,280 × 960 pixels. The monitor was placed 60 cm in front of the participant, whose head was stabilized on a chin rest. Two high-resolution images of young, female faces with a neutral facial expression from the FACES database (Max Planck Institute for Human Development, Berlin; image ID 132, 182; Ebner, Riediger, & Lindenberger, 2010) served as CS. The images presented resembled each other regarding relevant perceptual and social parameters comprising perceived attractiveness, competence, dominance, familiarity, trustworthiness, and distinctiveness, based on empirical ratings provided in an aesthetic preference study (Kiiski, Cullen, Clavin, & Newell, 2016). Both stimuli had equal mean luminance and were presented on a gray background. The stimuli were cropped onto an 82.1 mm × 70.0 mm ellipsoid template and covered 5.65% of the whole screen each. The CS+ and CS− were each coupled with a distinct bell sound (50 dB, duration: 100 ms, 2,349.32 Hz = D_7 and 2,637.02 Hz = E_7) that coincided with CS onset and was presented binaurally via headphones. We employed compound CS as this permits two different sensory modalities to be associated with the US and therefore facilitates the conditioning procedure (Talmi, Seymour, Dayan, & Dolan, 2009). The assignment of the visual-auditory stimuli to the CS+ and CS− condition was counterbalanced across participants. The experiment was coded in MATLAB R2016a (The Mathworks, Natick, MA) using Psychtoolbox-3 (http://psychtoolbox.org; Brainard, 1997; Kleiner et al., 2007). The individually selected appetitive liquid was delivered by a programmable syringe pump (World Precision Instruments, Inc., Sarasota, FL). The pump administered 3 ml of the juice through a 3-m long polyvinyl tube (Oldoplast GmbH, Marl, Germany; outside diameter: 6 mm, inside diameter: 4 mm) with an attached exchangeable straw continuously held between the individual's lips.

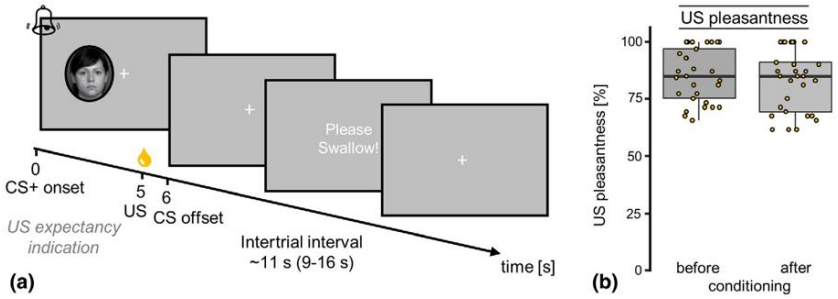

FIGURE 1 Appetitive conditioning procedure. (a) Sequence and timing of an example reinforced Pavlovian learning trial. Participants learned to associate two neutral audiovisual stimuli with a reward or no reward. At the beginning of a trial, one of two female faces was displayed to the left or right of a central fixation cross for 6 s. Upon display of cue, participants had to indicate their binary juice expectancy via button press. In reinforced CS+ trials, the US was delivered 5 s after CS onset. The intertrial interval ranged from 9–16 s (mean: 11 s) after cue offset. In reinforced CS+ trials, the signal to swallow appeared 2–5 s following cue offset. (b) US pleasantness rating before and after conditioning. All error bars represent *SEM*. *$p \leq .05$

2.2.4 | US expectancy

In each trial, participants were instructed to indicate their binary expectancy of US delivery as quickly as possible via button press using their dominant hand. The button indicating a positive or negative expectation was counterbalanced across participants. Due to a technical error, no responses exceeding a reaction latency of 1.5 s after trial onset were recorded causing a loss of 55.3% of the data. Analyses of US expectancy and reaction time are therefore restricted to the online supporting information, Appendix S1, and to be treated with caution.

2.3 | Ratings

2.3.1 | Dimensional ratings

Immediately before and after the conditioning experiment, participants rated the CS attractiveness, pleasantness, and arousal each on a dimensional 100-point VAS ranging from *very unattractive* to *very attractive* for attractiveness and correspondingly, *very unpleasant* to *very pleasant* for pleasantness, and *not at all arousing* to *very arousing* for arousal rating. US pleasantness was also rated on a 100-point VAS ranging from *very unpleasant* to *very pleasant* before and after conditioning. To ensure that the US fulfilled its appetitive potency, participants with an average negative rating (<50%) were excluded from the analysis ($n = 1$).

2.3.2 | Dichotomous preference rating

In addition, participants performed a dichotomous preference rating: Both cues and four further images of young female faces from the FACES database (image ID 63, 22, 150, 171) most similar in rated attractiveness were used (Kiiski et al., 2016). During each choice, two stimuli were presented simultaneously, and participants were asked to promptly indicate via button press which image they preferred based on their current judgment (Bray & O'Doherty, 2007; Kahnt et al., 2011; Metereau & Dreher, 2013; Prévost et al., 2013). Each image was paired with every other image exactly once, resulting in 15 choices.

2.3.3 | Contingency awareness

After the learning session, participants' explicit contingency awareness regarding the pairing of visual stimuli with reward outcomes was assessed on a categorical four-level Likert-type scale. To this end, each CS was presented individually and participants had to indicate how often they received the juice after the respective image was presented. The response options were *always*, *sometimes*, *never*, and *I am unsure*. Participants were considered contingency aware when they chose the always or sometimes options for the CS+ and the never option for the CS−. Using this awareness criterion, 28 participants reached awareness and 1 participant was unsure. An additional dimensional awareness measure on a VAS from 0–100% also confirmed CS+ versus CS− differentiation ($t = 8.71$, $p < .001$; paired t test; Tapia León et al., 2018).

2.4 | Ocular response measures: data acquisition and pre-processing

We tracked participants' eye movement and pupil diameter using a high-speed video-based eye-tracker (Cambridge Research Systems Ltd., UK; sampling rate: 250 Hz, spatial accuracy: 0.05°). For each participant, the activity of the right eye was measured. Preprocessing of eye-tracking data comprised a visual inspection of the raw data. Untracked data points were treated as missing data points. Subsequently, data were smoothed using a second-order Savitzky-Golay filter over seven consecutive data points. The data were segmented from CS onset until potential US onset (0–5 s after CS onset within each trial).

2.4.1 | Pupil diameter

The pupil diameter data were baseline corrected using the mean pupil diameter in a time window of 2 s prior to CS onset for correction. Due to the temporal proximity between the calibration of the eye-tracker and the start of the second half of the conditioning experiment, baseline correction was not possible for the first trial of the second half, resulting in the elimination of this trial from all further eye-tracker analyses. We performed statistical analyses on the pre-outcome pupil size (4–5 s after CS onset) as this is considered the interval of strongest CS differentiation (Koenig et al., 2018; Leuchs et al., 2017). All participants with ≥35% missing data were excluded from analyses (Korn, Staib, Tzovara, Castegnetti, & Bach, 2017). In addition to the latter analysis, we also performed a model-based approach (Korn et al., 2017; Korn & Bach, 2016) on pupil size response (PSR) using the PsPM toolbox (version 4.0, http://pspm.sourceforge.net/, details of analysis below).

2.4.2 | Dwelling time

Dwelling time was computed by averaging the percentage of time participants' gaze fell on the displayed stimulus in the segmented time window of 0–5 s after CS onset (Rothkirch, Stein, Sekutowicz, & Sterzer, 2012). Within this window of analysis, participants had the opportunity of gazing at the displayed CS, the fixation cross, or anywhere else on the gray background. Presenting the CS on the left or right side of the central fixation cross allowed us to assess the relative gaze proportion of the participants on the stimulus. All trials with ≥25% untracked data points were excluded from further analysis.

2.4.3 | Blink duration and frequency

Blink duration was assessed by calculating the mean blink length per condition in the segmented time window. A blink was defined as a series of continuous missing data points with a duration of 50–750 ms (Holmqvist et al., 2011; Stern, Walrath, & Goldstein, 1984). All blinks that coincided with the start or end of the designated time window were removed from the analysis. For the eyeblink rate, the number of eyeblinks in the identical time frame was counted.

2.5 | Further psychophysiological measures: data acquisition and pre-processing

Heart period, electrodermal activity, breathing, and startle responses were recorded using a BrainAmp MR amplifier (Brain Products GmbH, Munich, Germany; sampling frequency: 250 Hz). Due to a technical malfunction, data from one participant in these parameters were lost. All data were preprocessed using MATLAB R2016a. For HPR, SCR, and PSR, we used psychophysiological modeling techniques by means of the PsPM toolbox (Bach et al., 2018).

2.5.1 | HPR

Heart rate was measured using electrocardiography (ECG) with bipolar leads. Pre-gelled adhesive electrodes (45 mm) were placed in the right parasternal second intercostal space and fifth intercostal space in the left midclavicular line. All raw data underwent a visual inspection. Two participants were removed from further analysis due to data loss. The data were band-pass filtered using the PsPM default second-order Butterworth filter with desired cutoff frequencies of 5–15 Hz. QRS detection was performed semiautomatically using PsPM's modified version of the Pan & Tompkins algorithm (Pan & Tompkins, 1985). All deviating detected or undetected QRS complexes were manually corrected if necessary. The ECG signal was linearly interpolated at a 10 Hz sampling rate, converted to heart period, and normalized (Castegnetti et al., 2016; Paulus, Castegnetti, & Bach, 2016).

2.5.2 | SCR

A pair of 11-mm Ag/AgCl-electrodes placed on the medial phalanx of the second and third digit of the nondominant hand and secured with eudermic tape was used to detect SCR. An initial visual inspection was performed on the raw SCR data, resulting in the exclusion of 8 data sets due to poor signal quality (i.e., flatline due to disconnection of electrodes). The remaining data were filtered using PsPM's default 0.05–5 Hz unidirectional first-order Butterworth filter and downsampled to 10 Hz (Bach et al., 2013).

2.5.3 | PsPM first-level general linear model for HPR, SCR, and PSR

For HPR, SCR, and PSR separately, we executed a first-level analysis using PsPM's general linear convolution model (Bach, Flandin, Friston, & Dolan, 2010; Bach, Friston, & Dolan, 2013; Castegnetti et al., 2016; Korn et al., 2017). Psychophysiological modeling of HPR, SCR, and PSR has been shown to discriminate conditioned CS+ from CS− responses more precisely than corresponding model-free alternatives (Bach, 2014; Castegnetti et al., 2016; Korn et al., 2017). Each general linear model (GLM) included six regressors of interest, modeling cue onsets for CS+ unreinforced, CS+ reinforced, and CS−, for both halves of the experiment separately. Cue onsets of the habituation phase and US onsets were included as regressors of no interest. Regressors were convolved with the modality-specific (i.e., canonical HPR, SCR, and PSR) response function, yielding a beta estimate of each regressor. For primary group analysis, CS+ (mean of unreinforced and reinforced) and CS− estimates for each phase entered the second level. To assess the influence of conditioning on the responses uncontaminated by US, these analyses were complemented by an analysis of only unreinforced CS+ versus CS− responses.

2.5.4 | Startle task

Auditory startle reflexes were assessed subsequently to the learning session as a further index of appetitive conditioning. The startle session consisted of eight trials (four per condition, with no reinforcement) in which the cues were presented individually at the center of the screen. Participants did not have to indicate US expectancy. At asynchronous onset latencies (0.3, 0.6, 0.9, 1.2 s after stimulus onset), a white noise startle probe (90 dB, duration: 50 ms) was presented binaurally via headphones. Additionally, four startle probes occurred 0.1 s after ITI onset, in order to prevent a CS-startle association. Four initial habituation startle probes with analogous timing, but no cue display preceded the startle session. The ITI had a mean duration of 3.5 s after CS offset (min. 1.4 s, max. 5.8 s). Trial order and timing were randomized within and counterbalanced across participants.

2.5.5 | Startle response

The startle-induced EBR was measured using electromyography (EMG) of the left musculus orbicularis oculi. Two 5-mm Ag/AgCl electrodes were used and, adhering to human EMG eyeblink startle guidelines (Blumenthal et al., 2005), placed 1 cm below the eye's central vertical axis and 1 cm temporal of the lateral canthus. The PAR was measured using EMG of the left musculus auricularis posterior by positioning two 5-mm Ag/AgCl electrodes 1 cm posterior of the auricular auris directly above and below

the height of the meatus acusticus externus. Due to high electrical impedance noise detected in the primary visual examination of the data, only $n = 13$ and $n = 17$ data sets remained in the EBR and PAR analysis, respectively. The remaining data were fourth-order high-pass Butterworth filtered with a cutoff frequency of 40 Hz (EBR) and 28 Hz (PAR). Mains hum was removed using a 50 Hz notch filter. The EMG signal was rectified, and the orbicularis oculi data were further smoothed with a fourth-order low-pass Butterworth filter using a time constant of 3 ms (equivalent to 53.05 Hz; Khemka, Tzovara, Gerster, Quednow, & Bach, 2017). The peak startle magnitude was defined as the maximum value in the time interval of 20–120 ms for EBR (Blumenthal et al., 2005; Schumacher et al., 2018) and 5–35 ms for PAR (Aaron & Benning, 2016; Gable & Harmon-Jones, 2009; Sandt et al., 2009; Stussi et al., 2018) after startle onset subtracted by the mean EMG amplitude in a time window of 10 ms before startle onset for baseline correction. All negative peak values were transformed to zero. We applied the following quality criteria: (a) all trials with a baseline shift ≥5 μV were rejected from further analysis (EBR, PAR); (b) peak startle magnitudes ≤5 μV in the window of analysis were converted to zero (EBR; Genheimer, Andreatta, Asan, & Pauli, 2017; Glotzbach-Schoon, Andreatta, Mühlberger, & Pauli, 2015). Lastly, all data were *t* scored (*z* scored × 10 + 50).

2.6 | Self-report questionnaires

Prior to the learning task, participants completed the following self-report questionnaires: NEO-FFI (Neo Five-Factor Inventory; Costa & McCrae, 1992; German version: Borkenau & Ostendorf, 1993), BIS/BAS (Behavioral Inhibition System/ Behavioral Activation System Scale; Carver & White, 1994), and STAI (State-Trait Anxiety Inventory; Laux, Glanzmann, Schaffner, & Spielberger, 1981). For sample characteristics, see supporting information, Table S1.

2.7 | Statistical analysis

We conducted all statistical analyses using the R software environment (version 3.4.3, R Core Team, 2017) with an alpha level set at 0.05. Partial eta squared (η_p^2) or Cohen's *d* were used to estimate effect size. Ratings were analyzed using separate 2 × 2 repeated measures analyses of variance (rmANOVAs) with within-subject factors condition (CS+ vs. CS−) and time (pre- vs. postconditioning). Ocular response measures, HPR, SCR, reaction time, and US expectancy, were analyzed analogously, with time referring to the first versus second half of the experiment. Habituation trials were excluded from analyses to reduce orienting response confounds (Kruse, Tapia León, Stark, & Klucken, 2017). In addition, for the physiological measures, only unreinforced CS+ responses were initially contrasted with CS− responses. As these analyses did not change our results substantially (see Appendix S1), the differentiation between both CS+ types (reinforced/unreinforced) was henceforth discontinued. The reported results contrast all CS+ with all CS− responses. The startle data were analyzed using a paired *t* test, contrasting CS+ versus CS− conditions. To investigate intraindividual associations between conditioning indices, bivariate correlations between measures showing a significant differential conditioning effect were computed (Pearson's product-moment correlation or Spearman's rank correlation for associations with CS preference rating scores). As the personality traits neuroticism and extraversion potentially modulate the responsiveness to appetitive conditioning (Depue & Fu, 2013; Hooker, Verosky, Miyakawa, Knight, & D'Esposito, 2008; Schweckendiek, Stark, & Klucken, 2016), we correlated these subscales from the NEO-FFI with CS-related pupil size, HPR, and dichotomous CS ranking.

2.8 | Computational modeling of pupil data

As pupil diameter was strongly affected by appetitive conditioning, we investigated whether latent and dynamic learning mechanisms could be inferred from trial-by-trial pupil responses (individual trial-by-trial means, determined for the pre-outcome pupil size time window). By using computational modeling techniques, individual pupil responses were predicted by either (a) expected values of the displayed CS, or (b) the dynamic attention weight associated with the displayed CS. All learning models were based on a Rescorla-Wagner framework, where trial-wise prediction errors (reflecting the discrepancy between the received reward and the expected value; see Equation 1: *k* denotes trial number; $\delta_{v'}^{(k)}$ denotes the prediction error on trial *k*; *r* is the received reward, and $v'^{(k)}$ is the expected value) are used to update the expected value of the displayed CS (Equation 2).

$$\delta_{v'}^{(k)} = r^{(k)} - v'^{(k)} \tag{1}$$

$$v'^{(k+1)} = v'^{(k)} + \alpha^{(k)}\delta_{v'}^{(k)} \tag{2}$$

In our model space, the influence of the prediction errors on the value update was varied via (a) fixed learning rates (one free parameter α for both stimuli vs. two separate parameters per outcome), or (b) dynamic attention weights. The latter was determined via a Pearce-Hall update rule (as used by Diederen et al., 2016) that takes into account a general decay across accumulating trials, as well as the absolute prediction error from the previous trial (Equation 3: γ denotes the decay constant; $\left|\delta_{v'}^{(k-1)}\right|$ denotes the absolute prediction error from the preceding trial). Transferred to our paradigm,

the latter operationalization would model relatively steady attention weights for the values attributed to the CS+ because prediction errors remain high due to the 0.5 reinforcement rate. In contrast, the attention weights for the CS− would slowly decrease when participants have learned that this stimulus is not followed by the reward, reflected by expected values and prediction errors approximating 0 (figure 4b; Pearce & Hall, 1980).

$$\alpha_{v'}^{(k)} = \gamma \left| \delta_{v'}^{(k-1)} \right| + (1-\gamma)\, \alpha_{v'}^{(k-1)} \quad (3)$$

In a trial where the respective CS was not shown, the attention weight as well as the expected value remained constant. Further, the prediction error of the last trial where the respective CS was shown was used in the Pearce-Hall update rule. Learning trajectories ($learning^k$; reflecting either expected values or dynamic attention weights) were defined to linearly predict individual trial-by-trial pupil responses (Equation 4: ζ = Gaussian noise).

$$pupil\ diameter = \beta_0 + \beta_1 learning^k + \zeta \quad (4)$$

In sum, there were six Rescorla-Wagner learning models: (1) one fixed learning rate and expected value as predictor (RW-1α), (2) two fixed learning rates and expected value as predictor (RW-2α), (3) Rescorla-Wagner Pearce-Hall hybrid model with value as predictor with the same parameters for both conditioned stimuli (RW-PH-value-same), and (4) with distinct parameters (RW-PH-value-distinct), as well as these hybrid models with the attention weights predicting the pupil response—(5) RW-PH-attention-same, (6) RW-PH-attention-distinct. In order to compare whether pupil responses reflected such dynamic learning effects or stationary reactions to two cues, a null model was added that only predicted pupil responses via the displayed CS. Models were fitted using the HGF toolbox 4.15 (http://www.translationalneuromodeling.org/tapas/; Mathys, Daunizeau, Friston, & Stephan, 2011; Mathys et al., 2014) applying a quasi-Newton algorithm for optimization. For prior means and variances of parameters, see supporting information, Table S2.

2.8.1 | Model selection

A random-effects Bayesian model selection (Stephan, Penny, Daunizeau, Moran, & Friston, 2009) was used to compare the negative variational free energy of the following model families: null model, Rescorla-Wagner (RW-1α, RW-2α; value predicting pupil responses), Pearce-Hall models with values predicting responses (RW-PH-value-same, RW-PH-value-distinct), and Pearce-Hall attention weight (RW-PH-attention-same, RW-PH-attention-distinct). The exceedance probability (XP) of each model family, which reflects the certainty about the probability that the data from a randomly chosen participant are best explained by this respective model (i.e., this model family is more likely than any of the others considered) was reported. In addition to the family-wise comparison, all models were compared directly, which was quantified using the protected exceedance probability (PXP) that is protected against the null hypothesis that there are no differences across models (Rigoux, Stephan, Friston, & Daunizeau, 2014).

2.8.2 | Recovery of raw data effects

As a sanity check of our modeling data, the same analyses performed on the raw pupil data were repeated on the simulated data based on the best fitting model (rmANOVA with condition and time as within-subject factors (see Section 2.7, Statistical analysis).

2.8.3 | Confusion matrix

We calculated a confusion matrix in order to probe the specificity of our models (Tzovara et al., 2018; Wilson & Collins, 2019; Wilson & Niv, 2012). We simulated 200 data sets for each of the seven models from our model space, for which we drew parameter values from distributions based on our empirical data. We then fitted the seven models to these simulated data sets. For the confusion matrix, we compared the Bayes information criterion (BIC) scores from these 7 × 7 model fits within every individual subject. For every simulated model (columns), we summed up in how many subjects (percentage) the fitted model (rows) explained the data best. Thus, the diagonal in the created matrix shows how often the true model explained these simulated data best compared to the other candidate models in the model space.

3 | RESULTS

3.1 | Motivational state and perceived US valence

Ratings confirmed that participants were in a hungry and thirsty state before conditioning took place (hunger: $M(SD)$ = 64.5% (22.0); thirst: $M(SD)$ = 63.3% (20.5). The final study population evaluated the US as very pleasant, $M(SD)$ = 84.2% (12.5); this was also consistent over the course of the experiment (before conditioning: $M(SD)$ = 85.4% (11.6); after conditioning: $M(SD)$ = 83.0% (13.4); Figure 1b).

3.2 | CS ratings and US expectancy

Ratings of CS+ and CS− face stimuli before and after the experiment showed no significant conditioning effects regarding pleasantness, arousal, and attractiveness (all Fs(1, 28) ≤ 2.88, $p \geq .101$, $\eta_p^2 \leq .09$), except for a trend-wise time

effect for CS attractiveness, $F(1, 28) = 3.47$, $p = .073$, $\eta_p^2 = .11$.

However, in a dichotomous preference rating, participants chose the CS+ more often after conditioning when selecting between two out of six face stimuli including the CS+ and CS−. We observed a significant Condition × Time interaction, $F(1, 28) = 7.34$, $p = .011$, $\eta_p^2 = .21$, and main effect of time, $F(1, 28) = 5.40$, $p = .028$, $\eta_p^2 = .16$ (Figure S1a). Post-hoc analyses (with Bonferroni correction) showed that the CS+ was preferred more often after conditioning had taken place ($t = -4.54$, $p < .001$; paired t test) and also became the most preferred stimulus from all six stimuli (Figure S1b). CS preference did not change significantly over time ($t = 0.53$, $p = .602$; paired t test) and the difference between CS+ and CS− postconditioning did not reach statistical significance ($t = 1.81$, $p = .081$; paired t test).

Trial-by-trial US expectancy ratings indicated that learning was successful; due to the amount of missing data, these results are to be interpreted with caution (Appendix S1).

3.3 | Ocular response measures

3.3.1 | Pupil diameter

A significant main effect of condition, $F(1, 24) = 9.64$, $p = .005$, $\eta_p^2 = .29$, along with a trend in Condition × Time interaction, $F(1, 24) = 3.23$, $p = .085$, $\eta_p^2 = .12$, was found for the pupil diameter response (Figure 2a,b). The rmANOVA of the model-based PSR showed a significant main effect of condition, $F(1, 24) = 15.15$, $p = .001$, $\eta_p^2 = .39$, and time, $F(1, 24) = 4.71$, $p = .04$, $\eta_p^2 = .16$, along with a trend in Condition × Time interaction, $F(1, 24) = 4.23$, $p = .051$, $\eta_p^2 = .15$. In both analysis approaches, the CS+ elicited a stronger pupil dilation in comparison to the CS−, and this difference was more pronounced in the second half of the experiment.

3.3.2 | Dwelling time

The rmANOVA showed a significant main effect of condition, $F(1, 26) = 7.74$, $p = .010$, $\eta_p^2 = .23$ (Figure 2c) with a longer gaze-dwelling time on CS+ stimuli than on CS− stimuli, yet no main effect of time or Condition × Time interaction (all Fs$(1, 26) \leq 0.62$, $p \geq .437$, $\eta_p^2 \leq .02$).

3.3.3 | Blink duration

A significant main effect of condition, $F(1, 28) = 10.99$, $p = .003$, $\eta_p^2 = .28$, and time, $F(1, 28) = 9.69$, $p = .004$, $\eta_p^2 = .26$, but no Condition × Time interaction between these two variables, $F(1, 28) = 0.39$, $p = .537$, $\eta_p^2 = .01$, was found in the rmANOVA examining mean blink duration. The blink duration was generally increased in CS− trials and in the latter part of the experiment (Figure 2d).

3.3.4 | Blink count

The mean amount of blinks quantified after CS onset showed a trend for the condition, $F(1, 28) = 3.17$, $p = .086$, $\eta_p^2 = .10$, with a higher frequency of blinks in CS− trials. There was no main effect of time or Condition × Time interaction (all Fs$(1, 28) \leq 0.34$, $p \geq .567$, $\eta_p^2 \leq .01$).

3.4 | Modeling results

To further elucidate the mechanism of the observed conditioning effect on pupil dilation, we used different computational models to explain the individual trial-by-trial pupil response in combination with Bayesian model comparison. This revealed that the Pearce-Hall models that predicted the pupil response via the dynamic attention weights associated with the displayed stimulus explained the data best ($XP_{PearceHallAttention} = .6433$, $XP_{RescorlaWagnerValue} = .2361$, $XP_{NullModel} = .0881$, $XP_{PearceHallValue} = .0325$; Figure 3a). The pattern was more ambiguous in the direct comparison of all single models, but in line with the family comparison, the Pearce-Hall attention model with distinct learning parameters per stimulus (RW-PH-attention-distinct) displayed the best model fit (PXP = .1529).

Next, we simulated pupil response data using the best fitting model (RW-PH-attention-distinct). When performing the same analyses as for the raw data, we were able to recover the raw pupil data effects from the simulated pupil responses. The Condition × Time ANOVA across the two halves of the experiment revealed a significant Condition × Time interaction, $F(1, 24) = 30.58$, $p < .001$, $\eta_p^2 = .56$, as well as a significant main effect of condition, $F(1, 24) = 35.25$, $p < .001$, $\eta_p^2 = .59$, and time, $F(1, 24) = 28.09$, $p < .001$, $\eta_p^2 = .54$ (Figure 4a).

Our confusion matrix discerning the specificity of the candidate models showed that, apart from the null model that does not use any dynamic learning trajectories (23%), the other true models clearly predominate the model fits (≥70% of subjects' data are best explained by their true model) with our best fitting model also showing the highest specificity (82%; Figure 3b).

3.5 | Additional psychophysiological measures

The rmANOVA of the HPR showed a significant main effect of condition, $F(1, 25) = 98.85$, $p < .001$, $\eta_p^2 = .80$, with no main effect of time or Condition × Time interaction (all Fs(1,

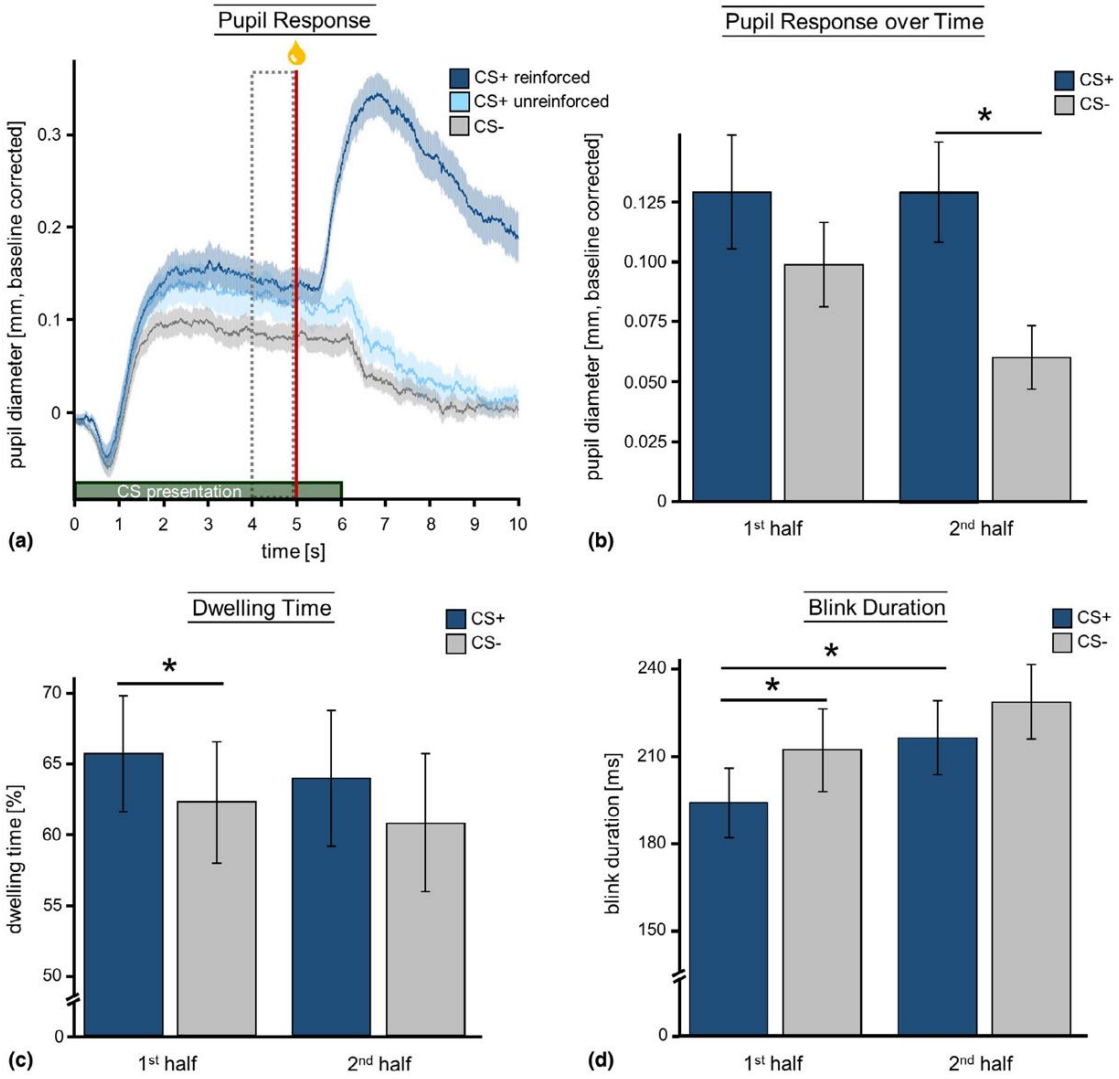

FIGURE 2 Ocular response measures. (a) Mean pupil diameter (baseline corrected) in reinforced/unreinforced CS+ and CS− trials over all participants. The CS+ elicited a stronger pupil dilation compared to the CS− in the predetermined time window (Second 4–5 after CS onset, dotted area). (b) Mean pupil diameter per condition in the first and second half of the experiment. The stronger pupil dilation to the reward-predicting stimulus is especially prominent in the second half of the experiment. (c) Average time participants' gaze fell on the displayed cue in the first and second half of the experiment. There was a longer gaze-dwelling time on the reward-predicting cue than on the control cue. (d) Blink duration contrasted by condition in the first and second half of the experiment. Blink duration was significantly shorter in CS+ trials and the first half of the experiment. All error bars represent *SEM*. $*p \leq .05$

25) ≤ 1.33, $p \geq .261$, $\eta_p^2 \leq .05$), indicating a heart period increase (heart rate deceleration) following CS+ compared to CS− presentations (Figure S2a,b; for computational results of the HPR, see Appendix S1, Figure S3). No conditioning effect was found in the SCR: The rmANOVA showed no significant main effects or Condition × Time interaction when contrasting CS+ with CS− (all Fs(1, 19) ≤ 2.80, $p \geq .111$, $\eta_p^2 \leq .13$). No significant startle potentiation difference was found between CS+ versus CS− in the EBR ($t = 1.30$, $p = .217$; paired t test) and PAR ($t = -0.61$, $p = .551$; paired t test).

3.6 | Correlations

Significant correlations were found neither between the ocular response measures showing significant conditioning effects (i.e., pupil diameter, gaze dwelling time, blink duration), nor between these ocular response measures, HPR, and dichotomous preference rating (all rs $\leq .28$, ps $\geq .117$). As expected, we found a significant positive correlation between the conditioning effect in pupil diameter and model-based PSR ($r = .82$, $p < .001$). We further explored associations between personality traits (extraversion and neuroticism) and

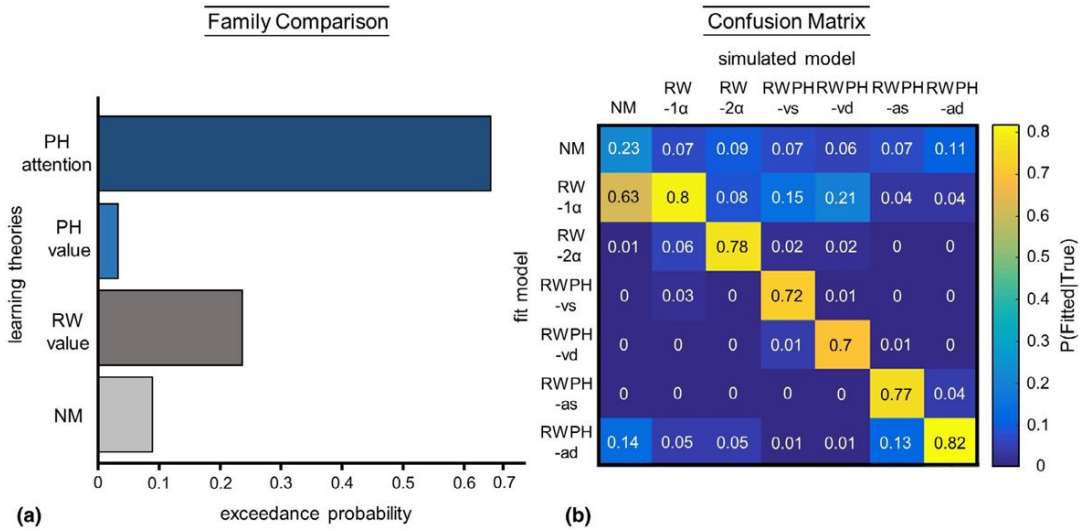

FIGURE 3 Modeling results. (a) Comparison of model families according to their exceedance probabilities. Pearce-Hall models that inferred the pupil response using the dynamic attention weights explained the data best. PH attention = Pearce-Hall models with attention weight (RW-PH-attention same, RW-PH-attention distinct); PH value = Pearce-Hall models with value weight (RW-PH-value-same, RW-PH-value distinct); RW value = Rescorla-Wagner value predicting pupil responses (RW-1α, RW-2α); NM = null model. (b) Confusion matrix: Recovery rates of models. NM = null model; RW-1α = Rescorla-Wagner with one fixed learning rate and expected value as predictor; RW-2α = Rescorla-Wagner with two fixed learning rates and expected value as predictor; RWPH-vs (value same) = Rescorla-Wagner Pearce-Hall hybrid with value as predictor and same parameters for both conditioned stimuli; RWPH-vd (value distinct) = Rescorla-Wagner Pearce-Hall hybrid with value as predictor and distinct parameters for the conditioned stimuli; RWPH-as (attention same) = Rescorla-Wagner Pearce-Hall hybrid with attention as predictor and same parameters for both conditioned stimuli; RWPH-ad (attention distinct) = Rescorla-Wagner Pearce-Hall hybrid with attention as predictor and distinct parameters for both conditions

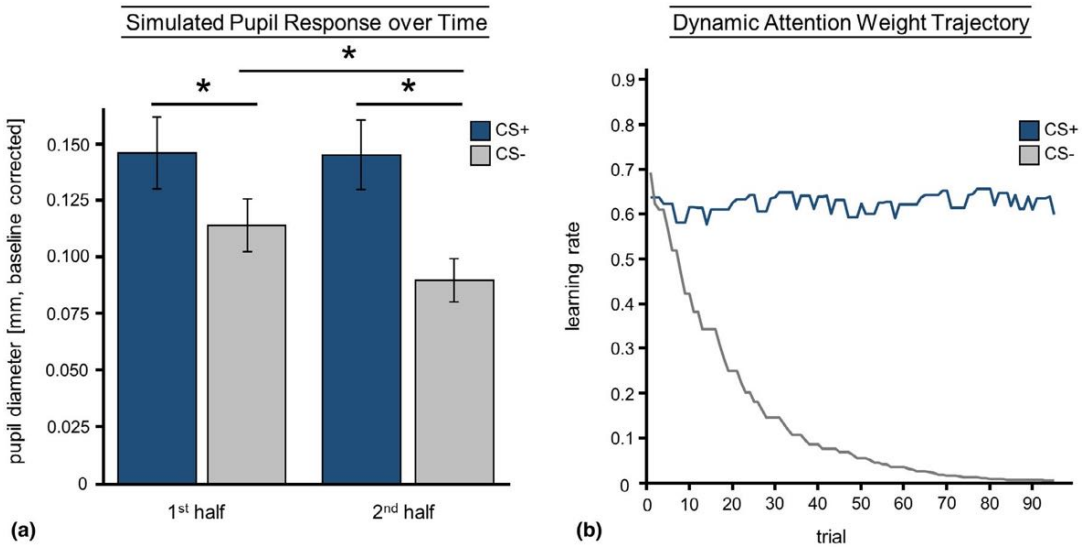

FIGURE 4 Modeling results. (a) Mean simulated pupil response per condition in the first and second half of the experiment. Results are comparable to the raw pupil data (Figure 2b). (b) Example attention weight trajectory of participant #26. The attention weight considers a general decay across accumulating trials along with the absolute prediction error of the previous trial. CS+ achieves relatively steady attention weights due to the high prediction error caused by a 0.5 reinforcement rate. Conversely, the attention weights for CS− slowly decrease as participants learn to not expect a reward following this stimulus. All error bars represent *SEM*. *$p \leq .05$

pupil diameter, HPR, and dichotomous preference applying Bonferroni correction (0.05/6 = 0.0083). We found that the dichotomous preference ratings correlated significantly with extraversion ($r = .48$, $p = .008$; Figure S4), while no other conditioned response was correlated with either extraversion or neuroticism (all rs $\leq .18$, ps $\geq .377$).

4 | DISCUSSION

The present study evaluated pupil diameter and other ocular response measures (gaze dwelling time, blink duration, and count) as psychophysiological indices of appetitive conditioned responding in humans. To this purpose, we designed a differential delay conditioning experiment, where two audiovisual stimuli were systematically paired with either a liquid primary reinforcer or no reward, while ocular response measures, as well as other psychophysiological (SCR, HPR, EBR, PAR) and behavioral (ratings, contingency awareness) parameters were acquired. We found that pupil diameter not only constitutes a sensitive index for representing appetitive conditioning, but also precisely reflects individual trial-by-trial learning mechanisms. Using different computational models and Bayesian model comparison to further elucidate the observed conditioned pupil response, we found that a Pearce-Hall attention-weighted learning model best explains the individual pupil responses. Moreover, we provide initial evidence that gaze dwelling time and blink duration are additional valuable psychophysiological indices of conditioning.

4.1 | Increased pupil dilation towards appetitive conditioned stimuli

We were able to initiate and extend evidence that the ocular response measures represent appetitive conditioning on a psychophysiological level. We specifically examined pupil dilation, which is associated with a variety of cognitive processes causing sympathetic nervous activation (Sirois & Brisson, 2014; van der Wel & van Steenbergen, 2018;). In the current study, participants showed a stronger pupil dilation in response to the conditioned reward-predicting cue (CS+) compared to the control CS−, and this differentiation trend-wise increased over time. Within a trial, we found an initial pupil constriction following CS onset that has been observed previously in paradigms using visual cues as CS (Reinhard, Lachnit, & Koenig, 2006). The differentiation between CS+ and CS− occurred approximately 2 s after CS onset and remained stable until US presentation (Figure 2a). In line with our findings, pupil dilation to appetitive conditioned stimuli has been described previously: Imaging studies using liquid primary reinforcers during Pavlovian conditioning reported pupil dilation to the appetitive conditioned CS in early trials of the experiment in a time window of 0–3 s after CS onset, which was not stable over time and consequently ascribed to potential habituation effects (O'Doherty et al., 2003). Another fMRI study using five different liquid primary reinforcers revealed increased pupil dilation to both the most and least preferred US in a time window of 0–5 s after CS onset (O'Doherty et al., 2006). Pupil dilation was also observed for an earlier time window after CS onset (0.5–1.5/2 s) during a Pavlovian task with a reversal component (Prévost et al., 2013) and for the proximal cue during a higher-order conditioning task (Pauli et al., 2015) using juice as US. In a mixed appetitive-aversive learning task with monetary reinforcement, differential pupil diameter responding was observed toward stimuli associated with rewards and losses (Seymour et al., 2007) using the peak light reflex after cue presentation in each trial (Bitsios, Szabadi, & Bradshaw, 2004). We found that learning about CS-US associations was expressed in stronger pupil dilation toward the CS+ relative to the CS− throughout the experiment. The assumption that change in pupil diameter is prone to early habituation was not observed in the present study (see also Leuchs, Schneider, & Spoormaker, 2018). Our finding showing increased pupil dilation to appetitive conditioned stimuli is therefore in accordance with previous findings, but the first to affirm pupil dilation as a conditioned response throughout the experiment in a design focused on appetitive classical conditioning using primary reinforcement in the established pre-outcome time window. This finding was substantiated by the conditioning effect observed in the PSR using psychophysiological modeling. This supports and complements previous evidence in that pupillary responding represents a promising measure for appetitive conditioning research.

4.2 | Gaze and blink duration as novel appetitive conditioned response measures

Besides pupil dilation, we observed a conditioning effect as participants' gaze remained on the CS+ longer and blink duration was shorter during CS+ compared to CS− presentations. Longer gaze dwelling time is likely explained in part by the attentional capture of reward-associated cues (Anderson, Laurent, & Yantis, 2011; Le Pelley, Pearson, Griffiths, & Beesley, 2015). Blink responding showed a trend of a greater blink rate for the CS− compared to the CS+. Previous studies have described a greater eyeblink rate to aversively conditioned stimuli compared to neutral stimuli (Pauli et al., 2015; Prévost et al., 2013), which may indicate that our CS− was perceived as qualitatively aversive in comparison with the appetitive cue as it was never associated with reward. As a novel measure, we found a significantly shorter blink duration on reward-associated stimuli and in the earlier phase of the experiment. Blink duration is commonly deemed an indicator of drowsiness and fatigue (Caffier et al., 2003; Stern et al., 1994), which would be compatible with the temporal component of our result. The differential responding toward both cues possibly indicates increased alertness or arousal to the reward-associated cue.

4.3 | Pearce and Hall's attention model predicts trial-by-trial pupil diameter change

We used computational models of trial-by-trial pupil diameter change to elucidate the cognitive process in more detail.

We tested whether the pupil responses were predicted more accurately by either the dynamic expected value or dynamic attention weight of the displayed stimuli. We observed that the Pearce-Hall learning model with distinct attention weights per CS type best predicted the pupil response. While this is an interesting result, it is important to recognize that XP only expresses the relative model fit within the considered model space. Pearce-Hall's learning theory describes the circumstances in which the attention given to a CS evolve in reaction to the experienced consequences, remaining high when the CS outcome is unpredictable and contrastingly decreasing when the CS outcome is highly predictable (Pearce & Hall, 1980). This is in accordance with our finding where we see steady attention weights to the CS+ where the outcome is uncertain in contrast to a declining attention weight in the CS− where the outcome (i.e., lack of reward) is certain. When examining appetitive and aversive higher-order learning, pupil diameter has shown to be modulated by an interaction of both CS value and prediction error (Pauli et al., 2015). An aversive learning experiment found that the trial-by-trial PSR predominantly reflects expected CS outcome (Tzovara et al., 2018). Interestingly, earlier studies have also found evidence in support of the Pearce-Hall learning theory in gaze-dwelling time, showing longer gaze durations on stimuli associated with appetitive and aversive uncertain outcome (Hogarth, Dickinson, Austin, Brown, & Duka, 2008; Koenig, Kadel, Uengoer, Schubö, & Lachnit, 2017), which is consistent with our result. Taken together, while pupil dilation was a sensitive measure of appetitive conditioning in our study, it seems to be more related to attentional processes rather than appetitive value.

In line with our modeling finding, pupil dilation has shown to be a robust measure for orienting attention toward cues that reliably predict an outcome (Lasaponara et al., 2019). Trial-by-trial pupil metrics have further been related to more complex learning processes such as change-point probability and relative uncertainty, which were associated with pupil change and pupil average, respectively (Nassar et al., 2012). Change in pupil diameter also distinctly reflects perceptual content and level of surprise (Kloosterman et al., 2015). Imaging (Murphy, O'Connell, O'Sullivan, Robertson, & Balsters, 2014) and translational animal model studies (Joshi, Li, Kalwani, & Gold, 2016; Rajkowski, Kubiak, & Aston-Jones, 1993; Varazzani et al., 2015) have associated pupil dilation with locus coeruleus activation and increased noradrenaline release (Aston-Jones & Cohen, 2005). Theories propose that noradrenaline is relevant for signaling unexpected uncertainty in a volatile environment (Yu & Dayan, 2005). Therefore, pupil diameter, as a proxy of locus coeruleus activation and noradrenaline release, presents a valuable outcome measure in the multi-dimensional learning and decision-making framework (Silvetti et al., 2018).

4.4 | Confirming successful conditioning through additional psychophysiological measures

The present study was able to corroborate the conditioning effect as differential CS responding was also observed in other independent parameters. Although the explicit valence rating was not a sensitive measure of conditioning, the more implicit dichotomous preference rating showed a clear conditioned preference increase to the reward-predicting stimulus. A possible explanation for the lack of a prominent valence differentiation is that we used neutral faces as CS, which are already afflicted with many social characteristics and contain a strong preference bias (Todorov, Olivola, Dotsch, & Mende-Siedlecki, 2015). As already established in fear-conditioning experiments (Castegnetti et al., 2016; Prévost et al., 2013), we observed conditioned bradycardia to the reward-associated stimulus, which is a novel finding in the appetitive conditioning domain. No conditioning effect was observed in the SCR or acoustic startle responses (EBR, PAR). Previous studies showed both significant SCR effects during appetitive conditioning (Andreatta & Pauli, 2015; Ebrahimi et al., 2019; Klucken et al., 2013, 2015, 2016; Tapia León et al., 2018), as well as no significant differential response to the conditioned CS (Ebrahimi et al., 2017; Klucken et al., 2009; Stussi et al., 2018; van den Akker et al., 2017b). Our non-significant finding may result from insufficient statistical power (especially due to the exclusion of eight participants from the SCR analysis) or habituation effects (i.e., a decrement in response amplitude with repeated CS presentation), which particularly afflicts experiments with a longer duration as used in our study (Leuchs et al., 2018; Lonsdorf et al., 2017). Although the acoustically evoked PAR has been suggested as a sensitive index of appetitive responding (Ebrahimi et al., 2019; Sandt et al., 2009; Stussi et al., 2018), the present study could not replicate this. We attribute the lack of a conditioned effect to the low sample size due to low data quality in this measure as well as the low sampling rate. Furthermore, the startle stimulus occurred comparatively early after CS onset, possibly conglomerating response effects.

Psychophysiological response measures with disparate results are common in conditioning research (Hermann et al., 2000; Stussi et al., 2018; Wardle et al., 2018). Interestingly, there was no discernible correlation between the different measures showing a conditioning effect. This is in accordance with prior findings theorizing that there are interindividual differences in the preferred response system or that the various measures are influenced by distinct psychological components of reward (Berridge, Robinson, & Aldridge, 2009; Wardle et al., 2018). The weak relationships among measures emphasize the importance of a multi-methodological

approach when investigating appetitive Pavlovian conditioning. Furthermore, it is would be desirable to standardize approaches of analysis, for instance, by using psychophysiological modeling techniques (Bach et al., 2018).

4.5 | Outlook

In conclusion, the present study highlights the potential value of ocular response measures when examining appetitive conditioning in humans. Although appetitive Pavlovian conditioning is a central learning mechanism and fundamental for understanding various pathological states, it remains vastly underexplored, largely due to the lack of a sensitive psychophysiological measure to represent conditioned responding. We propose the incorporation of eye-tracking measures when examining appetitive conditioning, as they provide multiple accurate, noninvasive measures with short reaction latencies that show clear conditioned differentiation. A further advantage is that ocular response measures have a high signal-to-noise ratio and are not susceptible to magnetic field artifacts, making them ideal measures in an fMRI environment. This could help expedite appetitive conditioning research and assist the exploration of neural correlates of appetitive learning derivatives like extinction and reinstatement (Konova & Goldstein, 2018) or reward prediction (Bach, Symmonds, Barnes, & Dolan, 2017). To conclude, our findings contribute evidence toward the establishment of a much-needed gold standard learning criterion in the human appetitive conditioning domain.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix 1
Tables S1, S2
Figures S1–S4

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Supplementary Material to:

Pupil dilation as an implicit measure of appetitive Pavlovian learning

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Self-report questionnaires

The five central dimensions of personality were assessed using the NEO-FFI. There was a significant sex difference in the personality category neuroticism (p = .027, t = -2.35), which was not apparent in the other factors. Extraversion and agreeableness correlated strongly (r = .603, p < .001), while extraversion and neuroticism correlated only moderately (r = -0.363, p = .053).

Carver and White's BIS/BAS questionnaire evaluating the behavioral inhibition and behavioral approach systems showed a significant sex difference in the total behavioral inhibition system (p = .011, t = -2.79) and behavior-approach reward-responsiveness system (p = .029, t = -2.33). All subscales of the behavioral approach system (drive, fun-seeking, reward-responsiveness) correlated considerably with the total behavioral approach system (all r > .6, p < .001).

The final State Trait Anxiety Inventory indicated that state and trait characteristics correlated moderately (r = .378, p = .043) with no significant difference between males and females.

Table S1. Sample characteristics of the NEO-FFI, BISBAS and STAI questionnaires

	Mean ± SD	Range	Variance
Neo-FFI			
Neuroticism	17.1±7.7	1-32	59.98
Extraversion	30.3±6.6	16-42	43.29
Openness	34.9±5.7	21-46	32.98
Agreeableness	33.2±6.3	19-42	39.46
Conscientiousness	32.5±5.4	23-46	29.40
BISBAS			
BIS-total	19.8 ± 4.2	12-26	17.62
BAS-total	41.1± 4.2	32-49	17.28
BAS-drive	12.2±2.1	9-16	4.46
BAS-fun-seeking	12.1± 2.1	7-15	4.60
BAS-reward-responsiveness	16.9± 1.9	12-20	3.50
STAI			
state	34.2 ± 5.5	23-44	30.48
trait	37.8 ± 9.4	25-51	88.31

Results of unreinforced CS+ with CS- trials

The rmANOVA of pre-outcome pupil diameter showed a significant main effect of condition ($F_{(1,24)}$ = 5.39, p = .029, η_p^2 = .18) when contrasting only unreinforced CS+ with CS- trials. However, no significant main effect of time or condition × time interaction was found (all $F_{(1,24)} \leq 2.16$, $p \geq .155$, $\eta_p^2 \leq .08$). Similarly, the rmANOVA of the model-based PSR showed a significant main effect of condition ($F_{(1,24)}$ = 10.69, p = .003, η_p^2 = .31) and time ($F_{(1,24)}$ = 4.34, p = .048, η_p^2 = .15), but no condition × time interaction ($F_{(1,24)}$ = 2.88, p = .103, η_p^2 = .11).

When contrasting only unreinforced CS+ with CS- trials, the rmANOVA of the HPR showed a main effect of condition ($F_{(1,25)}$ = 32.08, p < .001, η_p^2 = .56), but no main effect of time or condition × time interaction (all $F_{(1,25)} \leq 1.81$, $p \geq .190$, $\eta_p^2 \leq .07$)

No significant main effect or condition × time interaction was found in SCR (all $F_{(1,19)} \leq 2.37$, $p \geq .140$, $\eta_p^2 \leq .11$) when analyzing only unreinforced CS+ with CS- trials.

Modeling

Table S2. Means and variances of priors and fitted parameters from computational models of pupil diameter

Model	Free parameters	Priors (variance)	Mean fitted parameters (standard deviation)
1. Nullmodel	intercept β_0	0.1 (4)	0.05 (0.05)
	Cue coefficient β_1	0.1 (1)	0.06 (0.04)
	noise ζ	0.1 (1)	0.04 (0.03)
2. RW-1α	initial value $v^0_{CS^-}$	0.5 (1)	0.51 (0.09)
	learning rate α	0.2 (1)	0.18 (0.06)
	intercept β_0	0.1 (4)	0.05 (0.05)
	value coefficient β_1	0.1 (1)	0.11 (0.09)
	noise ζ	0.1 (1)	0.04 (0.03)
3. RW-2α	initial value $v^0_{CS^-}$	0.5 (1)	0.50 (0.09)
	learning rates α_{win} and α_{neutal}	0.2 (1); 0.2 (1)	0.17 (0.06); 0.20 (0.06)
	intercept β_0	0.1 (4)	0.05 (0.05)
	value coefficient β_1	0.1 (1)	0.10 (0.07)
	noise ζ	0.1 (1)	0.04 (0.03)
4. RW-PH value same	initial value $v^0_{CS^-}$	0.5 (1)	0.50 (0.02)
	initial attention weight α^0	0.7 (1)	0.69 (0.02)
	decay factor γ	0.1 (1)	0.10 (0.01)
	intercept β_0	0.1 (4)	0.06 (0.05)
	attention weight coefficient β_1	0.1 (1)	0.06 (0.05)
	noise ζ	0.1 (1)	0.04 (0.04)
5. RW-PH value distinct	initial value $v^0_{CS^-}$	0.5 (1)	0.50 (0.02)
	initial attention weights $\alpha^0_{CS^+}$ and $\alpha^0_{CS^-}$	0.7 (1); 0.7 (1)	0.69 (0.01); 0.70 (0.01)
	decay factors γ_{CS^+} and γ_{CS^-}	0.1 (1); 0.1 (1)	0.10 (0.004); 0.10 (0.002)
	intercept β_0	0.1 (4)	0.06 (0.05)
	attention weight coefficient β_1	0.1 (1)	0.06 (0.05)
	noise ζ	0.1 (1)	0.49 (0.04)
6. RW-PH attention same	initial value $v^0_{CS^-}$	0.5 (1)	0.70 (0.06)
	initial attention weight α^0	0.7 (1)	0.04

	decay factor γ	0.1 (1)	0.11 (0.04)
	intercept β_0	0.1 (4)	0.05 (0.04)
	attention weight coefficient β_1	0.1 (1)	0.10 (0.08)
	noise ζ	0.1 (1)	0.04 (0.03)
7. RW-PH attention distinct	initial value $v^0_{CS^-}$	0.5 (1)	0.49 (0.03)
	initial attention weights $\alpha^0_{CS^+}$ and $\alpha^0_{CS^-}$	0.7 (1); 0.7 (1)	0.69 (0.05); 0.70 (0.05)
	decay factors γ_{CS^+} and γ_{CS^-}	0.1 (1); 0.1 (1)	0.10 (0.01); 0.11 (0.05)
	intercept β_0	0.1 (4)	0.05 (0.04)
	attention weight coefficient β_1	0.1 (1)	0.10 (0.08)
	noise ζ	0.1 (1)	0.04 (0.03)

Dichotomous preference rating

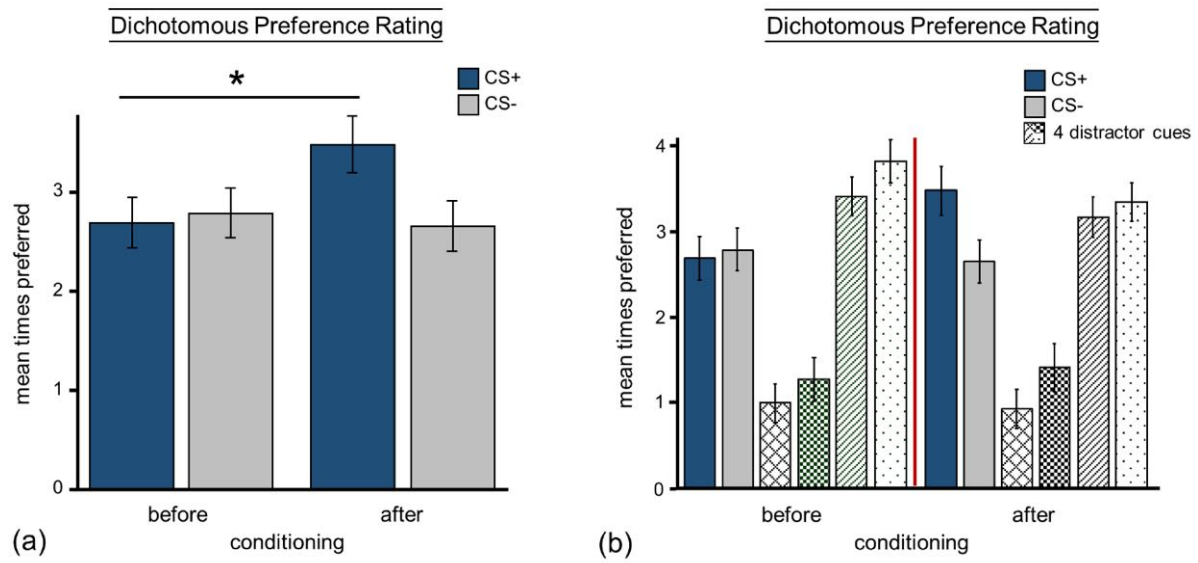

Figure S1(a) Average choice count per CS+ and CS- in the dichotomous preference rating before and after conditioning. **(b)** Average choice count per cue in the dichotomous preference rating before and after conditioning including CS+ and CS-, as well as four additional distractor images of young female faces most similar in attractiveness rating. All error bars represent SEM. *$p \leq 0.05$

US expectancy and reaction time

A significant condition × time interaction ($F_{(1,21)}$ = 6.51, p = .019, η_p^2 = .24) and main effect of condition ($F_{(1,21)}$ = 26.40, p < .001, η_p^2 = .56), but no main effect of time ($F_{(1,21)}$ = 1.69, p = .208, η_p^2 = .07) was found for US expectancy, supporting the conclusion of the CS-US contingency awareness rating that participants acquired a cue-reward association. Post-hoc t-tests (Bonferroni-corrected) show that the participants had a higher US expectation in CS+ compared to CS- trials (t = 5.138, p < .001, paired t-test), especially in the second half of the experiment (t = 5.444, p < .001, paired t-test).

For the reaction time, a main effect of condition ($F_{(1,21)}$ = 10.24, p = .004, η_p^2 = .33), time ($F_{(1,21)}$ = 15.46, p = .001, η_p^2 = .42) and a condition × time interaction ($F_{(1,21)}$ = 6.4, p = .02, η_p^2 = .23) was found. Bonferroni-corrected post-hoc t-tests show that, as expected, participants were generally faster at responding to the CS- (t = 3.201, p = .004, paired t-test), particularly in the second part of the experiment (t = 3.718, p = .001, paired t-test).

Heart period response

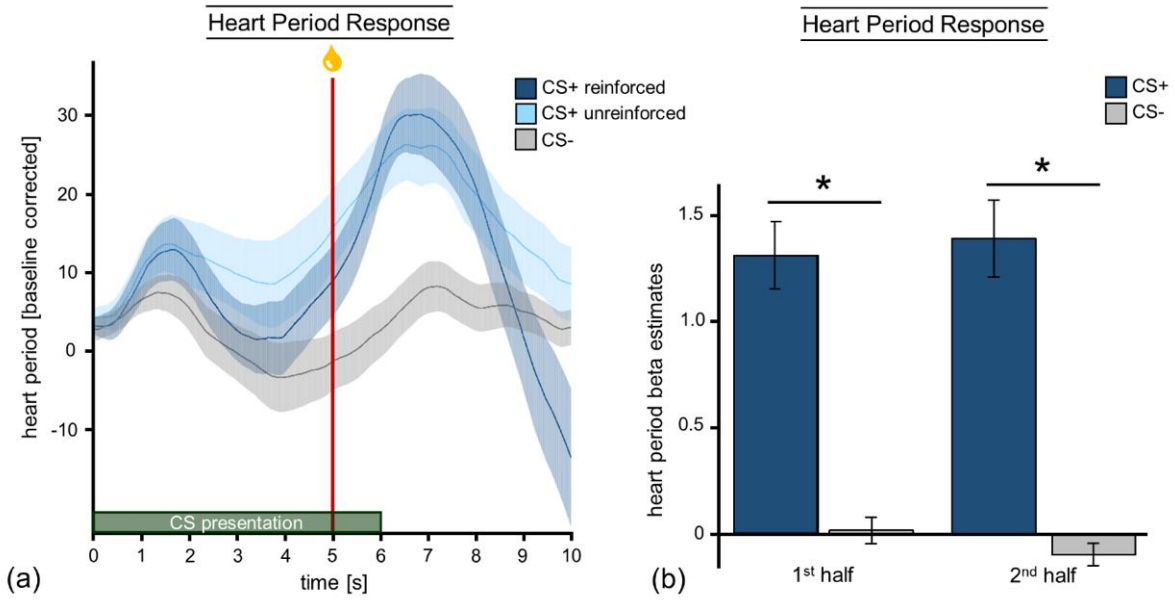

Figure S2(a). Mean heart period response in reinforced/unreinforced CS+ and CS- trials. The heart period response showed a strong initial increase (heart rate deceleration) in CS+ trials analog to the heart period US response, which was not as pronounced in CS- trials. Shaded area represents SEM. **(b)** Beta estimates of heart period response. All error bars represent SEM. *p ≤ 0.05

Computational modeling. Following a reviewer's suggestion, we applied the computational models from the pupil diameter analysis to trial-by-trial HPR data. For that, we extracted the mean HPR during the second half of the CS presentation (2.5 - 5 s after cue onset). This revealed that the Pearce-Hall model tracking the value explained the data best (exceedance probability for Pearce-Hall value models = .64, Fig. S3).

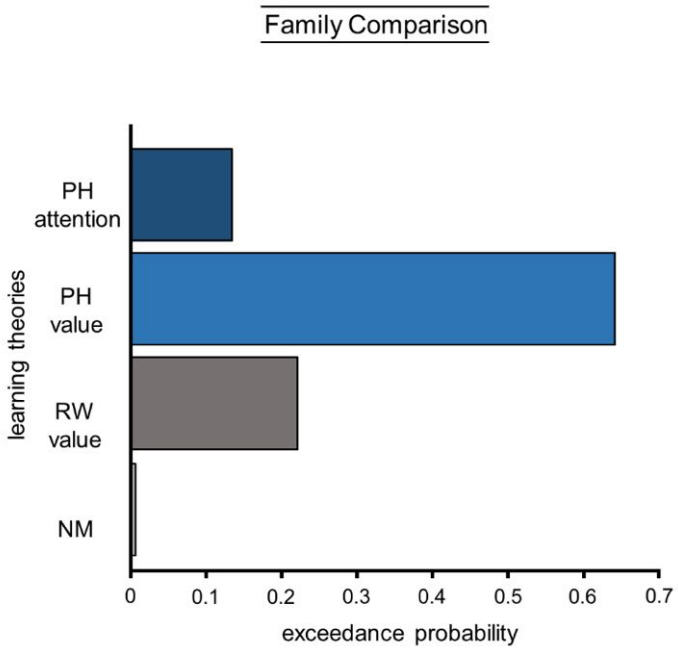

Figure S3. HPR Modeling results. Comparison of model families according to their exceedance probabilities. The Pearce-Hall model that inferred the HPR using the value weight explained the data best. PH attention = Pearce-Hall models with attention weight (RW-PH-attention same, RW-PH-attention distinct); PH value = Peace-Hall models with value weight (RW-PH-value-same, RW-PH-value distinct); RW value = Rescorla Wagner, value predicting pupil responses (RW-1α, RW-2α); NM = null model

Correlations

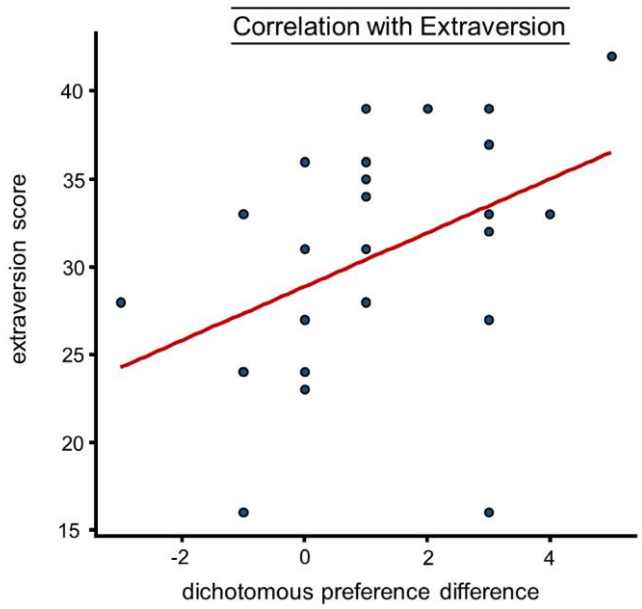

Figure S4. Correlation of dichotomous preference difference with the NEO-FFI personality trait extraversion. The higher the extraversion score, the greater the preference differentiation between CS+ and CS- from before to after conditioning.

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Combining D-cycloserine with appetitive extinction learning modulates amygdala activity during recall

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ABSTRACT

Appetitive Pavlovian conditioning plays a crucial role in the pathogenesis of drug addiction and conditioned reward cues can trigger craving and relapse even after long phases of abstinence. Promising preclinical work showed that the NMDA-receptor partial agonist D-cycloserine (DCS) facilitates Pavlovian extinction learning of fear and drug cues. Furthermore, DCS-augmented exposure therapy seems to be beneficial in various anxiety disorders, while the supposed working mechanism of DCS during human appetitive or aversive extinction learning is still not confirmed.

To test the hypothesis that DCS administration before extinction training improves extinction learning, healthy adults (n = 32) underwent conditioning, extinction, and extinction recall on three successive days in a randomized, double-blind, placebo-controlled fMRI design. Monetary wins and losses served as unconditioned stimuli during conditioning to probe appetitive and aversive learning. An oral dose of 50 mg of DCS or placebo was administered 1 h before extinction training and DCS effects during extinction recall were evaluated on a behavioral and neuronal level.

We found attenuated amygdala activation in the DCS compared to the placebo group during recall of the extinguished appetitive cue, along with evidence for enhanced functional amygdala-vmPFC coupling in the DCS group. While the absence of additional physiological measures of conditioned responses during recall in this study prevent the evaluation of a behavioral DCS effect, our neuronal findings are in accordance with recent theories linking successful extinction recall in humans to modulatory top-down influences from the vmPFC that inhibit amygdala activation. Our results should encourage further translational studies concerning the usefulness of DCS to target maladaptive Pavlovian reward associations.

1. Introduction

Drug addiction can be conceptualized as a disorder of persistent maladaptive memory: Environmental cues present during drug intake are associated with the rewarding properties of the drug and can trigger relapse even after long phases of abstinence (Everitt & Robbins, 2005). One way to target these persistent Pavlovian memories is extinction learning, where a previously conditioned cue (CS) is repeatedly presented without its associated reward (unconditioned stimulus, US). Extinction does not erase the maladaptive associations but represents an independent learning process that inhibits the expression of the original CS-US association (e.g., Myers & Davis, 2002). However, several conditions exist that impede extinction recall, causing the conditioned response to recover (Bouton, 2004). Pharmacological agents to enhance extinction learning are therefore of great clinical interest to improve the currently moderate effects of extinction-based addiction treatments (Conklin & Tiffany, 2002; Myers & Carlezon, 2012).

Animal studies using systemic administration of NMDA antagonists revealed an involvement of NMDA-dependent synaptic plasticity in the consolidation of Pavlovian extinction learning (Myers, Carlezon, & Davis, 2011). In line with this, animal models of fear extinction demonstrated that the NMDA receptor partial agonist D-cycloserine (DCS) facilitates extinction learning and

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deters some relapse effects when administered systemically or directly in relevant structures like the basolateral amygdala or hippocampus either before or immediately after extinction training (Fitzgerald, Seemann, & Maren, 2014). These results were replicated in animal models of drug addiction, where DCS facilitated the extinction of drug-paired cues and contexts (Nic Dhonnchadha & Kantak, 2011). Decreased effectiveness of the drug with increased time delay between extinction and post-training administration, as well as mixed effects on within-session extinction compared to long-term retention, suggest DCS to primarily support memory consolidation by enhancing NMDA receptor signaling (Botreau, Paolone, & Stewart, 2006; Ledgerwood, Richardson, & Cranney, 2003; Nic Dhonnchadha & Kantak, 2011). Anxiety research expanded these findings to clinical trials, demonstrating an overall beneficial effect for DCS-augmented exposure therapy in various anxiety disorders (Bontempo, Panza, & Bloch, 2012; Rodrigues et al., 2014; but see Ori et al., 2015). The few clinical studies combining DCS with cue exposure in addiction are less promising (for review, see Myers & Carlezon, 2012; Otto et al., 2015), although recently DCS-augmented cue exposure with 50 mg of DCS was shown to reduce cue-induced ventral striatal activation (Kiefer et al., 2015) and subjective craving (MacKillop et al., 2015) in alcohol-dependent subjects.

This raises the question of the precise working mechanism of DCS in human extinction learning. Experimental designs suitable to address this issue typically involve three phases: conditioning of CS-US associations, extinction learning, and extinction recall; all spaced at least 24 h apart (Guastella, Lovibond, Dadds, Mitchell, & Richardson, 2007; Klumpers et al., 2012). This allows learning to consolidate, manipulate extinction independent of conditioning, and test DCS effects during extinction recall in a drug-free state.

The proposed mechanism that DCS enhances extinction consolidation is not clearly confirmed in humans (Brom et al., 2015; Guastella et al., 2007; Klumpers et al., 2012; Kuriyama, Honma, Soshi, Fujii, & Kim, 2011); moreover, the neuronal changes that may underlie DCS-augmented extinction are currently unknown. While two human laboratory studies (Guastella et al., 2007; Klumpers et al., 2012) reported that DCS administration before extinction learning failed to attenuate conditioned fear responses during simple recall, that is, CS-presentations in the extinction context (spontaneous recovery), Kuriyama et al. (2011) found 100 mg of DCS to attenuate SCRs after a reactivation procedure (i.e., recall after a CS-US reactivation trial), while no group differences were observed during simple recall. Recently, Brom et al. (2015) administered 125 mg of DCS or placebo after extinction learning of conditioned sexual responses in females. While no group differences emerged during simple extinction recall, the DCS group showed attenuated conditioned responses when tested outside the extinction context, indicating that DCS reduced the context specificity of extinction learning. Especially in the appetitive domain, more research is needed to evaluate the usefulness of DCS as supporting pharmacological strategy to improve extinction-based treatments.

We therefore investigated the effect of 50 mg of DCS during extinction learning in a double-blind, placebo-controlled 3-day design, using a Pavlovian conditioning procedure with monetary wins and losses to probe appetitive and aversive extinction learning. To our knowledge, this is the first human study examining the neuronal correlates of DCS-augmented appetitive extinction learning. We assumed DCS to facilitate extinction of both the appetitive and aversive CS. We hypothesized attenuated SCRs and CS-evoked BOLD response after a reactivation procedure during extinction recall in areas implicated in Pavlovian conditioning, like the amygdala and hippocampus (Quirk & Mueller, 2008), in the DCS compared to the placebo group.

2. Materials and methods

2.1. Subjects

Forty-seven healthy, right-handed volunteers participated in this study. Subjects were examined by medical professionals and excluded in case of current or past psychiatric (DIAX-CIDI; Wittchen & Pfister, 1997), neurological or internal medical disorders (e.g., diabetes mellitus, increased blood pressure, or liver and renal dysfunctions). Further exclusion criteria were pregnancy, positive urinary drug screening, color blindness or weakness (Ishihara color-test; Ishihara, 1917), and abnormalities in hematology and resting electrocardiogram (ECG). Participants were instructed to refrain from alcohol on all days. The required learning criterion of explicit contingency awareness—shown to be necessary for trace conditioning, where CS and US are spaced by a time delay (Clark & Squire, 1998; Knight, Nguyen, & Bandettini, 2006; Weike, Schupp, & Hamm, 2007)—was met by 38 subjects immediately after conditioning on day 1. Of these, six participants were excluded from fMRI analysis due to slice misplacement or excessive signal loss, leaving 32 subjects with adequate data quality on all days (16 women, mean age = 27 ± 1 year SEM, range: 19–39 years; see also Supplementary Fig. S1 for a participant flow chart). Groups did not differ in terms of age, sex, education, or neuropsychological characteristics (see Supplementary Table S1). Participants provided written informed consent for study participation. The study was approved by the local ethics committee (LAGeSo, Berlin, Germany) and registered as a clinical trial at EudraCT (EudraCT-Nr.: 2006-004860-29).

2.2. Stimuli and procedure

Subjects underwent conditioning, extinction, and extinction recall on three consecutive days. They were randomized to receive either 50 mg of DCS or placebo 1 h before extinction under double-blind conditions (Fig. 1A). A Pavlovian trace conditioning and extinction paradigm with monetary outcomes was used (Fig. 1B + C).

Conditioning (day 1). In each trial, a CS was presented for 1.5 s followed by a fixed 3-s trace interval and a subsequent outcome stimulus for 1.5 s (100% reinforcement). The inter-trial interval (ITI) ranged from 3 to 10 s (exponentially distributed with mean 4.5 s; Fig. 1C). The paradigm included three conditions with 16 trials each:

(1) *appetitive condition:* CS ($CS+_{app}$) followed by appetitive US (US_{app}),
(2) *aversive condition:* CS ($CS+_{avers}$) followed by aversive US (US_{avers}), and
(3) *neutral condition:* neutral cue (CS−) followed by neutral outcome (noUS).

Geometric shapes (cycle, square, pentagon) combined with a tone (500, 550, 600 Hz) served as cues and were randomly assigned to conditions over participants. The US consisted of a 2€ coin image with plus or minus signs (US_{app}, US_{avers}), while the neutral outcome was a blurred coin image (noUS). Trial order was pseudo-randomized over subjects and sessions within the constraint of a maximum of three consecutive presentations of the same condition.

Participants were instructed to attend to the relations between cues and outcomes and were informed they would receive the cumulated money after the session. To maintain attention and obtain an additional measure of learning, participants engaged in a cued outcome discrimination task: In each trial, subjects discrim-

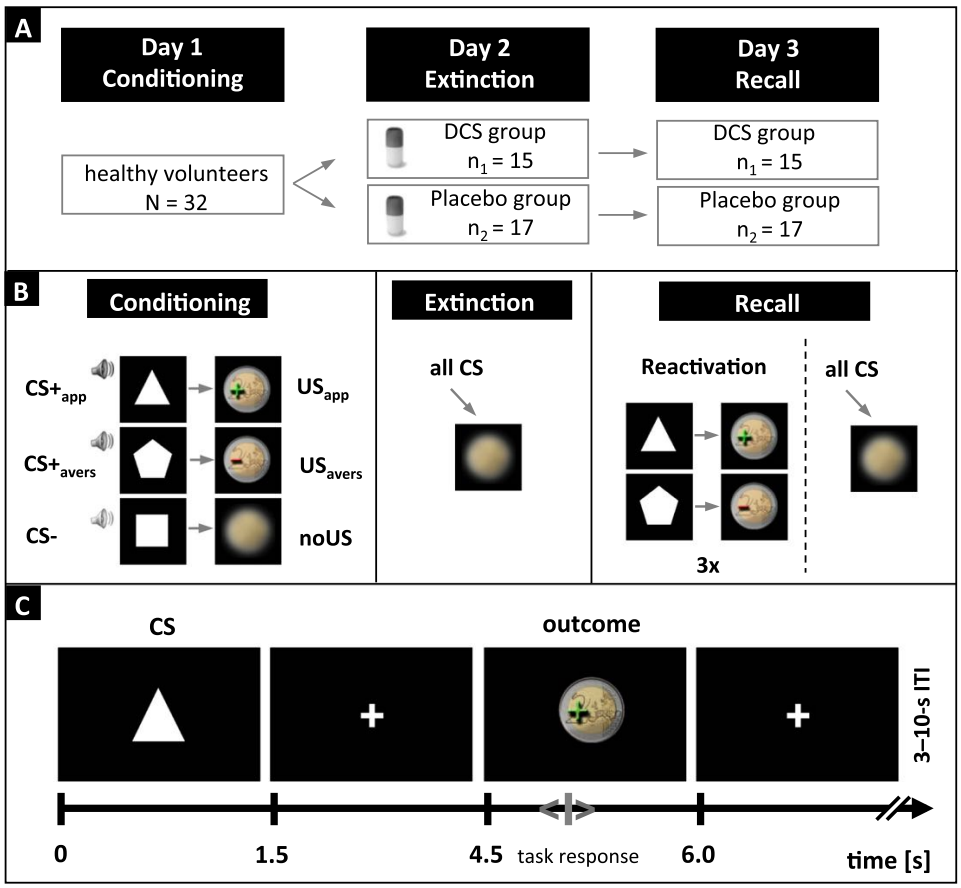

Fig. 1. Experimental design. (A) Double-blind, placebo-controlled design with three experimental phases—Pavlovian conditioning, extinction, and extinction recall—on three subsequent days. DCS or placebo was administered 1 h before extinction. Group sizes (N; n_1 and n_2 for subgroups) denote fMRI group samples. (B) During conditioning, neutral audiovisual stimuli (tones combined with geometric shapes) served as conditioned stimuli ($CS+_{app}$, $CS+_{avers}$) paired with unconditioned appetitive and aversive stimuli (US_{app}: 2€ win, US_{avers}: 2€ loss; 100% reinforcement schedule) or control cue (CS−) paired with a neutral outcome (noUS). During extinction and extinction recall, monetary stimuli were replaced by the neutral outcome. Before extinction recall three original CS-US couplings (reactivation procedure) were presented. (C) Representative appetitive trial showing the timing: The $CS+_{app}$ was presented for 1.5 s, followed by a fixed 3-s trace interval and a subsequent US_{app} for 1.5 s. In order to keep participants engaged in the task, subjects had to discriminate if they saw a coin (USs) or a circle (noUS) during outcome presentation (cued outcome discrimination task). Abbreviations: DCS, D-cycloserine; ITI, inter-trial interval.

inated via button press with the dominant hand (middle and index finger) between monetary (US_{app}, US_{avers}) and neutral outcomes (noUS). No explicit speed instruction was given for reaction to outcomes to avoid operant learning processes, where actions are reinforced or punished. Response mapping to US/noUS was counterbalanced across subjects. After conditioning, explicit knowledge about the CS-US relationships was assessed with one multiple-choice question per outcome, asking which of the three cues was followed by the respective outcome. Participants further rated CS valence prompted by the question "How pleasant or unpleasant do you find this stimulus?" on a 100-mm visual analogue scale ranging from 'very unpleasant' to 'very pleasant'.

Extinction & drug administration (day 2). During extinction, $CS+_{app}$ and $CS+_{avers}$ were followed by the neutral outcome (noUS), resulting in 16 unreinforced trials per condition. One hour before extinction, subjects received either a capsule of 50 mg of DCS (reformulated from 250 mg capsules, Seromycin®, USA) or a placebo, as plasma concentration reaches a peak level approximately 1 h after ingestion (van Berckel et al., 1997) and 50 mg of DCS was shown to be effective in augmenting exposure therapy for anxiety (Rodrigues et al., 2014) and cue exposure in alcohol use disorder (Kiefer et al., 2015; MacKillop et al., 2015). An external pharmacist prepared identical capsules, and randomization codes ensured balanced sex distribution between groups. Within the first 30 min after drug administration, subjects underwent neuropsychological assessment. No adverse side effects were reported in either the DCS or in the placebo group.

Extinction recall (day 3). Adapted from Kuriyama et al. (2011), three original CS-outcome pairings per condition were first presented (reactivation trials) followed by 16 trials of each condition in extinction. This procedure should reactivate the conditioning memory for both CS+, thereby making extinction recall more difficult and enhancing the probability of observing group differences in conditioned responses to both CS+.

The paradigm was implemented in Matlab (The Mathworks, Natick, United States) using Cogent (Wellcome Department of Imaging Neuroscience, Institute of Neurology, London, UK).

2.3. Data acquisition

Skin conductance and reaction times. The electrodermal signal was recorded from the thenar and hypothenar eminence of the left

hand at a rate of 50 Hz (MP150, Biopac Systems, Goleta, USA). RTs were collected using MR-compatible response buttons (Current Designs, Philadelphia, USA). RTs between 200 and 1500 ms were considered for analysis.

fMRI. Images were acquired with a 3-Tesla scanner (Trio, Siemens AG, Erlangen, Germany) using a GE-T2*-weighted echo planar imaging (EPI) sequence (TR = 2.09 s, TE = 22 ms, 90° flip angle, 64 × 64 matrix; 192 × 192-mm FOV). Volumes comprised 40 slices (2.5-mm thickness, 0.5-mm slice gab, 3 × 3-mm^2 in-plane voxel resolution), acquired interleaved with 25° rotation to AC-PC line. A T1-weighted structural scan (MPRage, 1-mm slice thickness) was acquired on day 2 and fieldmaps were collected on all days.

2.4. Data analyses

Skin conductance and behavioral measures. CS-related SCRs were estimated by means of Continuous Decomposition Analysis using Ledalab (Benedek & Kaernbach, 2010). Data were down-sampled (25 Hz) and smoothed (Gaussian window of 16 sample points). SCRs were quantified as sum of amplitudes exceeding 0.02 μS within 1.5–4.5 s after CS onset. Separately for each day, SCRs ≥ 4 SD from the individual mean response were replaced by the corresponding median SCR and then normalized by dividing all SCRs by the subject's maximum response. Three subjects were excluded from further analyses because of acquisition errors or absence of SCRs (>80% of responses below 0.02 μS), leaving 35 participants for statistical analyses (19 DCS/ 16 placebo). To analyze conditioning and extinction effects, SCRs were pooled into an early (first 8 trials) and late phase (last 8 trials) and entered into two separate mixed ANOVAs for day 1 and day 2 with within-factors condition (appetitive, aversive, neutral) and time (early, late phase), and between-factor group (DCS, placebo).

Square-root transformed RTs during the outcome discrimination task were analyzed with mixed ANOVAs analogous to SCR. Three subjects had to be excluded from analyses, as they also responded to the CS on at least one day, resulting in a sample of 35 subjects with correct task performance (17 DCS/18 placebo). As we expected the cued outcome discrimination task to also be sensitive to violations in expected contingencies, present at the beginning of extinction and following the reactivation trials, we additionally investigated RT differences using the first and last trial response in each session as time factor. Subjects with missing values in these trials were excluded from this analysis, resulting in a sample of 32 participants (16 DCS/16 placebo).

Reactivation effects during extinction recall were tested by analyzing the early recall phase (after the reactivation trials) using mixed ANOVAs with factors condition and group. ANOVAs were Greenhouse-Geisser corrected if sphericity was violated; followed by planned *t*-tests on an alpha level of 0.05. Statistical analyses were conducted using R (R Core Team, 2015).

fMRI. Data were analyzed using SPM8 (Wellcome Department of Imaging Neuroscience, London, UK). EPI images were corrected for delay in slice time acquisition and realigned to the mean volume across the three days. Images were corrected for distortion and movement-by-distortion interactions using acquired fieldmaps. The structural image was coregistered to the mean EPI and spatially normalized. These normalization parameters were applied to all EPIs (2-mm isotropic voxel resolution), which were finally smoothed with a 6-mm full-width at half maximum Gaussian.

An event-related analysis was applied using the SPM generalized linear model approach on two levels. On the subject level, the three trial types (CS+$_{app}$, CS+$_{avers}$, and CS−), the three outcome types, and the motor responses were defined for each session (conditioning, extinction, and extinction recall) and their onsets were included after convolution with the canonical HRF. Additionally for day 3, the initial reactivation trials were modeled separately as events of no interest. Movement parameters were entered as additional regressors to account for movement-related variance. The contrasts 'appetitive vs. neutral' (CS+$_{app}$ > CS−) and 'aversive vs. neutral' (CS+$_{avers}$ > CS−) were computed for each session.

On the group level, these two contrasts were entered into a flexible factorial model, with factors subject, day, and group. In order to test our main hypothesis, group differences between DCS and placebo were tested during extinction recall on day 3 for these two contrasts. Preclinical studies with animal extinction models provided evidence that the amygdala and hippocampus mediate DCS effects (Fitzgerald et al., 2014; Nic Dhonnchadha & Kantak, 2011). Furthermore, these structures have been involved in human fMRI studies of Pavlovian conditioning, extinction, and recall (Quirk & Mueller, 2008). Therefore, small volume correction using ROI masks derived from WFU Pickatlas (http://www.fmri.wfubmc.edu/download.htm) was applied at $p \leq 0.05$ family-wise error correction (FWE). Whole-brain analyses were performed at $p < 0.001$ uncorrected and clusters surviving $p < 0.05$ FWE correction at the cluster level were considered significant.

Following the notion that the prefrontal cortex modulates amygdala reactivity during recall (Milad et al., 2007; Phelps, Delgado, Nearing, & LeDoux, 2004), amygdala connectivity with frontal areas during appetitive extinction recall was further explored using psychophysiological interaction analysis (PPI; Friston et al., 1997). Time courses from the right amygdala were extracted within the anatomical ROI mask for the amygdala, ensuring independence from the main group analysis. Time courses were deconvolved using a Bayesian framework to generate the neuronal signal for the seed region (Gitelman, Penny, Ashburner, & Friston, 2003). The PPI was then defined as the element-by-element product of the neuronal times series of the seed region and a psychological variable coding for appetitive > neutral cue (CS+$_{app}$ > CS−). PPI analysis probes differences in regression slopes between a seed region (here amygdala) and other parts of the brain during a particular condition (here CS+ app) compared to a control condition (here CS−). Both the PPI and VOI term were entered into new first level statistics for the recall session otherwise similar to the aforenamed model. Contrasts for seed region and PPI were then entered into a flexible factorial design with factors subject and group to test for group differences in context modulated amygdala connectivity. Positive PPI betas indicate a more positive slope in the regression between seed and target region in the condition of interest relative to the control condition. A negative beta indicates a lower slope during the condition of interest compared to the control condition and not necessarily an inverse relationship of seed and target region.

3. Results

3.1. Trace conditioning and extinction (day 1 & 2)

Contingency awareness & valence ratings. After the conditioning session, 38 subjects met the required learning criterion of explicit awareness about the CS-US contingencies. CS valence ratings in contingency aware subjects further confirmed conditioning: A mixed ANOVA with factors group and condition showed that the CS+$_{app}$ was rated significantly more pleasant and the CS+$_{avers}$ significantly more unpleasant compared to the CS− across groups (main effect condition: $F_{1.38,49.76} = 21.88$, $p < 0.001$, no main or interaction effect of group: $p \geq .600$; post-hoc *t*-tests see Fig. 2A).

SCRs. We did not observe significant differential SCRs between the conditioned cues (CS+) and the CS− over the course of conditioning (no main effect of condition or condition by time interaction: $F_{2,66} \leq 0.69$, $p \geq 0.506$; Fig. 2A) or extinction (no main effect

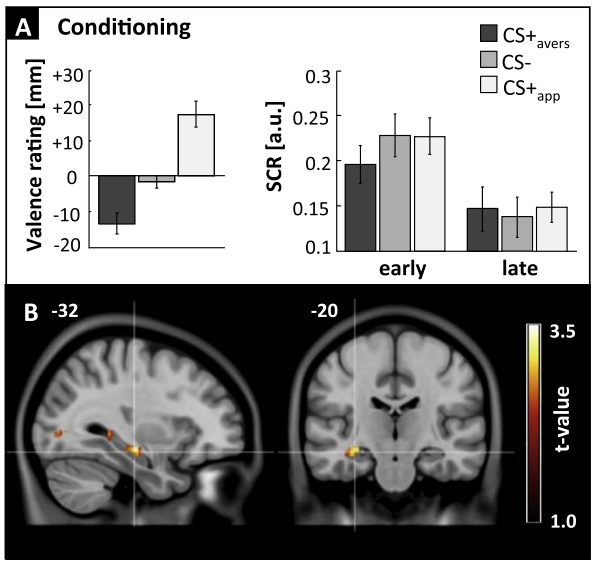

Fig. 2. Effects of Pavlovian conditioning. (A). Differences in valence ratings of conditioned stimuli (CSs) after conditioning (left panel; post-hoc *t*-tests for $CS+_{app}$ vs. CS−: $t_{37} = 4.165$, $p < 0.001$, $CS+_{avers}$ vs. CS−: $t_{37} = -3.826$, $p \leq 0.001$), but not in CS-evoked skin conductance responses (SCRs; right panel) during early or late phase of conditioning. (B) Activation of the left ventral hippocampus during appetitive trace conditioning (contrast '$CS+_{app}$ > CS−', cluster peak activation at MNI: [−32 −20 −14], Z = 3.405, FWE-corrected for hippocampal mask at $p < 0.05$). Activations displayed at $t \geq 2.35$, cluster extent $k > 50$. Bars represent means ± SEM.

of condition or condition by time interaction: $F_{2,66} \leq 0.69$, $p \geq 0.504$). No interaction effects with the factor group were present during conditioning or extinction ($F \leq 2.02$, $p \geq .141$). We observed a general decline in SCRs from early to late phase that might reflect habituation effects ($F_{1,33} \leq 17.16$, $p < 0.001$).

Cued outcome discrimination. During conditioning (day 1), participants discriminated the outcomes significantly faster in the second compared to the first half of the session (main effect of time: $F_{1,33} = 11.10$, $p = 0.002$). We also observed a main effect of condition ($F_{1,33} = 3.68$, $p = 0.030$) due to somewhat faster RTs towards the neutral cue. During extinction (day 2), no main or interaction effects were observed (all $p \geq 0.164$). No group effects were present during conditioning or extinction (all $p \geq 0.125$).

Learning the correct cue-outcome associations during conditioning likely contributed to the reduction in participants' RT responses to the outcomes on day 1. We also expected violations in learned contingencies to interfere with the required responses during extinction; an effect that may be transient and mostly present directly after an unexpected change in contingencies. In line with this, during extinction, participants showed significantly delayed RTs in the first appetitive and aversive compared to the neutral trial but not in the last trial, resulting in a condition by time interaction in the single-trial analysis ($F_{2,60} = 3.88$, $p = 0.026$, Fig. 3; see Supplementary Table S2 for further analyses). During conditioning, the single-trial analysis also revealed a main effect of time due to faster outcome discrimination in the last compared to the first trial ($F_{1,30} = 30.98$, $p < 0.001$).

Imaging. To evaluate brain regions recruited during appetitive and aversive Pavlovian trace conditioning, we assessed the differential contrasts 'appetitive vs. neutral' ($CS+_{app}$ > CS−) and 'aversive vs. neutral' ($CS+_{avers}$ > CS−). During appetitive conditioning, the left hippocampus but not the amygdala showed stronger activation in response to the $CS+_{app}$ compared to the non-reinforced CS− (MNI coordinates: [−32 −20 −14], $Z = 3.405$, $p_{FWE\ VOI} = 0.018$, Fig. 2B). In extinction, when the $CS+_{app}$ was no longer followed by monetary reward, the contrast '$CS+_{app}$ > CS−' did not reveal significant activation in the hippocampus or amygdala. Here, an exploratory analysis revealed activation in the dorsal ACC (MNI coordinates: [−6 24 32], cluster size $k = 18$, $Z = 4.09$, $p_{unc} < 0.001$).

Contrasting the $CS+_{avers}$ with the neutral CS− did not reveal significant activation differences within or outside our predefined VOIs during conditioning or during extinction learning.

3.2. Group differences during extinction recall (day 3)

SCRs. Analysis of SCRs for the early recall phase revealed no main effect of condition ($F_{2,66} = 0.01$, $p = 0.985$), no condition by group interaction ($F_{2,66} = 0.06$, $p = 0.939$), or main effect of group ($F_{1,33} = 0.09$, $p = 0.760$).

Cued outcome discrimination. We first probed behavioral effects of the reactivation procedure by analyzing RTs during the first half of the recall session, that is, during the first 8 unreinforced trials. A trendwise effect of condition ($F_{1,33} = 2.45$, $p = 0.094$) but not group by condition interaction was observed ($F_{2,66} = 0.19$, $p = 0.827$). As is the case during extinction, RT effects caused by violations in expected cue-outcome contingencies might be more prominent immediately after reactivation trials. Indeed, analyzing reactivation effects for the first unreinforced trial revealed a significant main effect of condition ($F_{1,30} = 5.32$, $p = 0.007$), with post-hoc *t*-tests confirming delayed RTs in both the appetitive ($t_{31} = 2.11$, $p = 0.043$) and the aversive condition ($t_{31} = 3.02$, $p = 0.005$) compared to the neutral one collapsed across groups (Fig. 3). In the single-trial analysis, we also observed a statistical trend for a main effect of group ($F_{1,30} = 3.15$, $p = 0.086$) due to somewhat faster RTs in the DCS group but again no group by condition interaction ($F_{2,60} = 0.06$, $p = 0.941$).

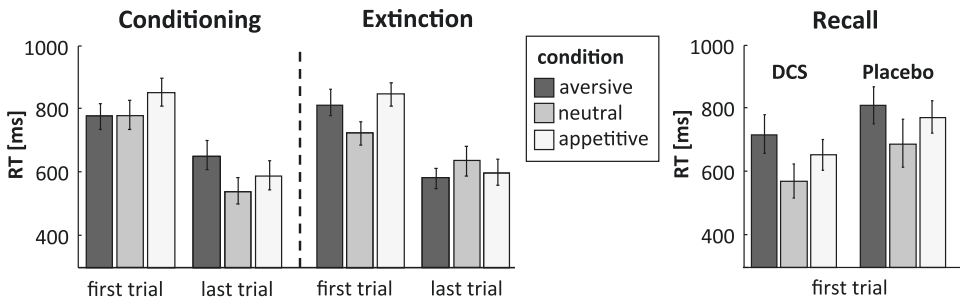

Fig. 3. Reaction times (RTs) during cued outcome discrimination. Conditioning: RT declines from first to last trial across conditions. Extinction: Delayed RTs in the appetitive and aversive condition compared to the neutral condition during the first but not last extinction trial. Recall: Significant reactivation effect in both the DCS and placebo group during the first recall trial. Note that in extinction and extinction recall, the task simplified to a one-button response to the noUS. Bars represent means ± SEM.

Imaging. For the contrast 'CS+$_{app}$ > CS−', the placebo group showed significantly stronger activation of the right amygdala compared to the DCS group (MNI coordinates: [26 −8 −24], Z = 3.30, $p_{FWE\ VOI\ Amy}$ = 0.021, Fig. 4A). This was due to significant activation in the placebo group (MNI coordinates: [22 −6 −22], Z = 4.81, $p_{FWE\ VOI\ Amy}$ < 0.001), which was not observed in the DCS group ($p_{FWE\ VOI\ Amy}$ > 0.14). No stronger activations were observed in the DCS group compared to the placebo group, and no significant group differences were observed in the hippocampus or in whole-brain analysis.

To further explore the activation difference within the amygdala, we employed a PPI analysis using the right amygdala as seed region and the contrast 'CS+$_{app}$ > CS−' as psychological modulator. Group comparison revealed larger context modulated functional connectivity between the right amygdala and a cluster within the vmPFC in the DCS compared to the placebo group ([6 34 −2], Z = 3.47, p_{un} < 0.001, k = 7, Fig. 4B). In contrast, the placebo compared to the DCS group showed stronger amygdala connectivity to widespread occipital and temporal but not frontal regions (see Supplementary Table S3).

Consistent with our negative findings regarding aversive conditioning and extinction, no group differences were observed during aversive extinction recall (contrast 'CS+$_{avers}$ > CS−').

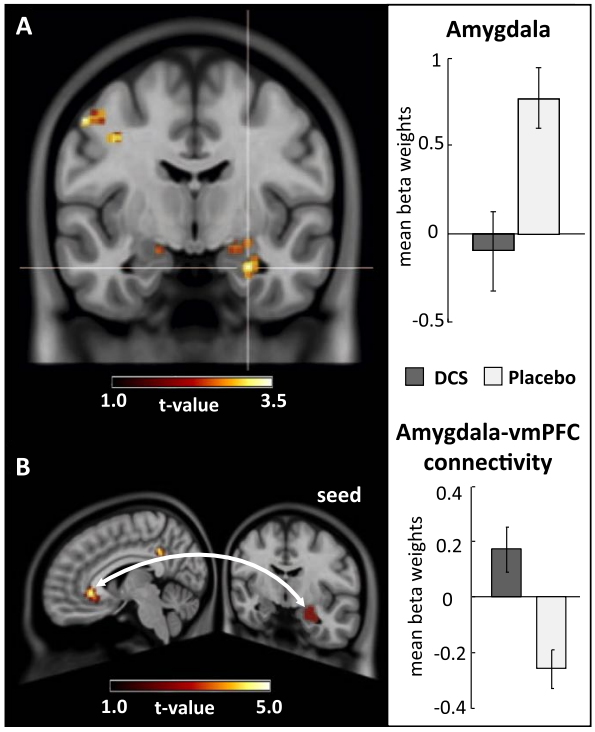

Fig. 4. DCS effects during appetitive extinction recall. (A) Significant amygdala activity during appetitive extinction recall (CS+$_{app}$ > CS−) in the placebo compared to the DCS group (MNI: [26 −8 −24], Z = 3.30, FWE-corrected for amygdala mask at p < 0.05) due to significant activation in the placebo group as shown in the right panel. (B) Exploratory PPI analysis with the right amygdala as seed region revealed stronger amygdala-vmPFC connectivity in the DCS compared to the placebo group (MNI: [6, 34, −2], Z = 4.32, cluster size = 56, uncorrected at p < 0.001). Mean beta estimates show that amygdala-vmPFC coupling during CS+$_{app}$ compared to CS− presentation is enhanced in the DCS group but attenuated in the placebo group. The seed region (anatomical amygdala mask) used for time course extraction is displayed in red. Activations displayed at t ≥ 2.35, cluster extent k > 50.

4. Discussion

This study investigated the hypothesis that DCS facilitates human extinction learning of appetitive and aversive Pavlovian cues using a double-blind, placebo-controlled design. We found that DCS administration before extinction training attenuated amygdala activation during recall of an extinguished appetitive cue. This points towards a facilitation of extinction memory recall in the DCS group, possibly via enhanced functional amygdala-vmPFC coupling.

Animal studies demonstrated that administering the partial NMDA receptor agonist DCS before or immediately after extinction facilitates extinction memory acquisition and/or consolidation, thereby reducing the number of required extinction trials, increases long-term retention, and prevents different relapse effects like spontaneous recovery or reinstatement of the conditioned response (Fitzgerald et al., 2014; Nic Dhonnchadha & Kantak, 2011). Here, after a reactivation procedure we observed significant amygdala activation to the extinguished appetitive cue compared to the neutral cue only in the placebo, but not in the DCS group, for which coupling of the vmPFC with the amygdala was higher. Preclinical studies demonstrated the central role of the amygdala for both appetitive and aversive Pavlovian conditioning, extinction, and recall of conditioned CS-US associations (Janak & Tye, 2015; LeDoux, 2000; Luo, Xue, Shen, & Lu, 2013), which has been widely confirmed with fMRI in human fear conditioning (e.g., Büchel, Dolan, Armony, & Friston, 1999; Kalisch et al., 2006; Phelps et al., 2004). More recent studies focusing on relapse phenomena after fear extinction also observed conditioned responses (SCRs) and amygdala activation to an extinguished cue when subjects were re-exposed to the US or a different context before extinction recall (Agren et al., 2012; Kalisch et al., 2006; Lonsdorf, Haaker, & Kalisch, 2014; Schiller, Kanen, LeDoux, Monfils, & Phelps, 2013). In line with our results, these findings highlight the involvement of the amygdala in relapse effects after extinction, suggesting it as an important site of CS-US memory storage (Agren et al., 2012). In our study, DCS administration during extinction learning seemed to attenuate the reactivation effect, although we have to caution that we could not provide an additional physiological marker such as SCR to confirm an attenuation of the conditioned response in the DCS group. Attenuation of conditioned responses by DCS after a change in context was recently shown in a sexual conditioning procedure (Brom et al., 2015). Women who received DCS after extinction showed attenuated subjective and genital conditioned responses to the CS+ when tested outside the extinction context 24 h later, relative to placebo, indicating that DCS reduced the renewal effect.

Our exploratory connectivity analysis (PPI) during appetitive extinction recall revealed stronger functional coupling between the right amygdala and the vmPFC in the DCS compared to the placebo group when exposed to CS+$_{app}$ compared to CS−. For Pavlovian extinction recall, the infralimbic cortex in animals is a necessary structure providing top-down control of the amygdala to inhibit the conditioned response (Peters, Kalivas, & Quirk, 2009; Quirk & Mueller, 2008). Likewise, activation and thickness of the vmPFC as a homologue region in humans has been linked to successful aversive extinction recall (Kalisch et al., 2006; Lonsdorf et al., 2014; Milad et al., 2005, 2007; Phelps et al., 2004). Although exploratory, our finding is in line with the proposed modulatory influence from the vmPFC on amygdala activity suggested in previous fMRI studies of fear extinction recall (Milad et al., 2007; Phelps et al., 2004). In contrast, the placebo group displayed higher connectivity compared to the DCS group between the amygdala and a widespread network including higher order

sensory areas, possibly indicating higher salience processing of the $CS+_{app}$.

In line with our reactivation procedure, it has been further suggested that DCS effects may be more reliably observed under conditions in which extinction recall is more difficult. This could explain why Kuriyama et al. (2011)—like Guastella et al. (2007) and Klumpers et al. (2012)—did not observe a DCS effect on spontaneous recovery of SCRs (i.e., during simple recall), but after a reactivation procedure. Likewise, Brom et al. (2015) found DCS to facilitate extinction recall in a renewal test, but not during simple recall. We used a similar reactivation procedure to Kuriyama et al. (2011) where initial pairings of CS+ and US precede extinction recall. Therefore, a more specific interpretation might be that DCS deters rapid reacquisition. This is consistent with animal studies on drug-cue extinction where DCS-augmented extinction slowed down the reacquisition of ethanol (Groblewski, Lattal, & Cunningham, 2009) or cocaine (Nic Dhonnchadha et al., 2010) conditioned place preference. Although rapid reacquisition does not explicitly modulate the context, this procedure is suggested to rely in part on an ABA renewal effect (Bouton, 2004). DCS may therefore affect the context dependency of extinction; either via enhanced extinction consolidation or interference with context encoding (Torregrossa, Gordon, & Taylor, 2013) and making it thus more generalizable and independent of the extinction context. This reduction of the context dependent renewal effect was also demonstrated in preclinical appetitive extinction paradigms (Torregrossa, Sanchez, & Taylor, 2010), while evidence from aversive paradigms is mixed (Bouton, Vurbic, & Woods, 2008; Ressler, Rothbaum, Tannenbaum, & Anderson, 2004; Woods & Bouton, 2006). Importantly, our reactivation procedure most likely captures both rapid reacquisition and spontaneous recovery effects that could not be differentiated, as no simple recall phase preceded the reactivation. It has been further proposed that DCS preferably acts at lower-level learning processes, leaving measures like expectancy ratings unaffected (Brom et al., 2015; Grillon, 2009). This is in accordance with our observation of group differences only on the neuronal level but not in the cued outcome discrimination task, which is most likely influenced by participants' expectations as well as higher order executive functions, such as response inhibition capacities.

At least some negative findings regarding DCS-augmented cue exposure therapy for addiction may be attributable to methodological challenges (Myers & Carlezon, 2012; Otto et al., 2015), for example, subjects need to show robust craving responses at baseline, but also experience significant reductions in cue reactivity during the exposure sessions (Hofmann, Hüweler, MacKillop, & Kantak, 2012; Watson et al., 2011). Moreover, abstinence before and after exposure sessions needs to be controlled in order to avoid conditioning effects (Kamboj et al., 2012). Kiefer et al. (2015) investigated only those abstinent alcohol-dependent patients showing robust neuronal activation in a cue reactivity paradigm at baseline and found cue-induced ventral and dorsal striatal activation significantly reduced after cue exposure with 50 mg of DCS compared to cue exposure with placebo. Positive effects of DCS on learning-dependent synaptic plasticity and reward-associated learning and decision making tasks have also been reported, although evidence in this field is still scarce (Forsyth, Bachman, Mathalon, Roach, & Asarnow, 2015; Scholl et al., 2014).

We did not observe differential SCRs between the $CS+_{app}$ and the CS− in our experimental sessions, and therefore cannot provide an additional physiological measure to evaluate the effect of DCS during appetitive extinction recall. This absence of significant SCRs is most likely due to much weaker physiological responses—including SCRs—in appetitive compared to aversive Pavlovian conditioning (Hermann, Ziegler, Birbaumer, & Flor, 2000) and may further be reduced by the use of monetary compared to primary reinforcers (Andreatta & Pauli, 2015) as well as a decreased signal-to-noise ratio during fMRI acquisition.

Regarding conditioning and extinction, several measures confirmed learning in both groups: First, only participants explicitly aware of all CS-US associations after conditioning were included, thus fulfilling a necessary condition for trace conditioning (Clark & Squire, 1998; Knight et al., 2006; Weike et al., 2007). In line with previous findings suggesting a vital role for contingency awareness in perceived CS valence (Klucken et al., 2009; Tabbert et al., 2011), participants showed reliable differential CS valence ratings reflecting the value of the associated outcomes.

The proportion of subjects unaware of the CS-US contingencies after conditioning (9 out of 47) seemed relatively high, although not unusual in such paradigms (e.g., Klucken et al., 2009). RT declines in the cued outcome discrimination task across conditions during conditioning further indicated the formation of CS-US and CS-noUS associations, while no differential effect of cue valence was observed. It is noteworthy that cue-outcome learning in this session cannot be distinguished from other task practice effects or motivational aspects unrelated to Pavlovian learning. However, the latter explanation is rather unlikely as participants were explicitly instructed that responses were unrelated to experimental procedures including timing or outcomes. In contrast, delayed RTs in the first extinction and recall trial in both extinction conditions provide an additional, rather cognitive, measure for acquisition recall and reactivation effects. On the neuronal level, appetitive trace conditioning was accompanied by enhanced hippocampal activation in response to the $CS+_{app}$ compared to the CS−, whereas during extinction, activity in the dACC was present at a more lenient threshold. Hippocampal activation has been observed during aversive trace rather than delay conditioning which has led to the hypothesis that this structure maintains a memory trace to bridge the temporal gab between CS and US in order to form an association (Büchel et al., 1999; Raybuck & Lattal, 2014). We specify this view by showing stronger ventral hippocampal involvement when forming a Pavlovian association (CS_{app}-US) compared to a neutral association (CS-noUS), in line with the proposed involvement of this part of the hippocampus in emotional learning processes (Fanselow & Dong, 2010). Moreover, hippocampal activity during context conditioning (Lang et al., 2009; Pohlack, Nees, Ruttorf, Schad, & Flor, 2012) highlights its role in encoding spatial contextual information. Consistent with the context specificity of extinction memory recall, imaging studies that—unlike our study—explicitly manipulated the context further revealed hippocampal involvement during recall and renewal in a novel context (Hermann, Stark, Milad, & Merz, 2016; Kalisch et al., 2006; Milad et al., 2007). As observed in context fear conditioning paradigms, differential hippocampal involvement seems to be specific during trace conditioning rather than extinction (Lang et al., 2009; Pohlack et al., 2012), while contextual shifts may engage the hippocampus during the recall phase.

Contrary to our expectation, we did not observe significant amygdala activity during appetitive conditioning. This negative finding agrees with a recent meta-analysis showing that amygdala activation was also not consistently observed in previous human fear conditioning studies (Fullana et al., 2016). This could be due to rapid habituation processes. Moreover, the use of a 100% reinforcement schedule as implemented in our study might have further reduced the BOLD signal by increasing US expectancy and promoting fast learning (Fullana et al., 2016; Sehlmeyer et al., 2009).

Finally, some limitations of this study have to be addressed. As already mentioned, the absence of significant differential SCRs during appetitive conditioning or extinction recall are a major limitation of our study, although this might be due to weaker conditioned responses in appetitive Pavlovian conditioning (Hermann et al., 2000). Future translational human studies should therefore

more closely investigate the psychophysiological effects of DCS-augmented appetitive extinction learning, using, for example, pupil dilation (Pauli et al., 2015) or startle responses (Andreatta & Pauli, 2015) as physiological indicators for appetitive Pavlovian learning. Second, valence ratings were only acquired after conditioning, but not in extinction or extinction recall sessions which might have provided an additional although subjective measure of extinction and recall. A disadvantage of the implicit nature of the cued discrimination task without explicit speed instruction or feedback might be increased variability in RTs that could limit the ability to detect learning-related RT changes. Third, our sample size was small and may not have been adequately powered to detect small or medium effects associated with the pharmacological manipulation with appropriate confidence (post-hoc power calculation revealed a power of 0.71 for a medium to large group effect (d = 0.8) between DCS and placebo in our study). Fourth, this study was designed to investigate DCS effects on appetitive extinction, given its relevance in addiction (Everitt & Robbins, 2005), and included the aversive condition as a comparison, with monetary stimuli for comparability across modalities. However, we did not observe neuronal or physiological effects for the aversive contrast '$CS+_{avers}$ > CS−', as found in previous studies (Fullana et al., 2016; Hermann et al., 2000; Sehlmeyer et al., 2009). This might be due to differences in task design, as aversive Pavlovian conditioning is typically studied using a mild electric shock as US, which might be a more effective US than monetary loss. Furthermore, our stimulus material for the US_{avers} (a 2€ coin with a minus sign) may have positively biased the aversive condition.

More work is also needed to elucidate the optimal dosage of DCS to facilitate extinction. Studies suggest that higher doses and/or chronic administration of DCS reduce its effectiveness and may even have antagonistic effects on the NMDA receptor (Hofmann, Wu, & Boettcher, 2013). A meta-analysis by Rodrigues et al. (2014) concluded that low doses of DCS (50 mg), administered a limited number of times and immediately prior to (1–2 h) or after exposure sessions are effective in augmenting exposure therapy for anxiety disorders, which corresponds to our selected DCS dosage and timing. However, whether 50 mg of DCS is in fact the ideal dose to enhance extinction remains unclear. Meta-regression could not detect a dose dependent effect of DCS in clinical trials (dose range: 50–500 mg; Rodrigues et al., 2014). However, the majority of analyzed trials used 50 mg of DCS, hence studies directly comparing different doses are crucial.

To summarize, we showed for the first time that DCS application during appetitive extinction learning caused attenuated amygdala response during an extinction recall procedure, an effect possibly mediated by enhanced functional vmPFC-amygdala coupling. The absence of further physiological markers indicative of appetitive extinction recall precludes a final evaluation of the effectiveness of DCS to facilitate extinction learning of Pavlovian reward cues. However, our results add new evidence to the hypothesis that DCS acts by facilitating extinction consolidation in humans; possibly by rendering subjects less vulnerable to relapse phenomena after an encounter with the reward. Translational human studies are thus recommended to further evaluate the potential of DCS to improve extinction-based therapies for addiction.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nlm.2017.05.008.

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Neurobiology of Learning and Memory

Supplemental Material for

Combining D-cycloserine with appetitive extinction learning modulates amygdala activity during recall

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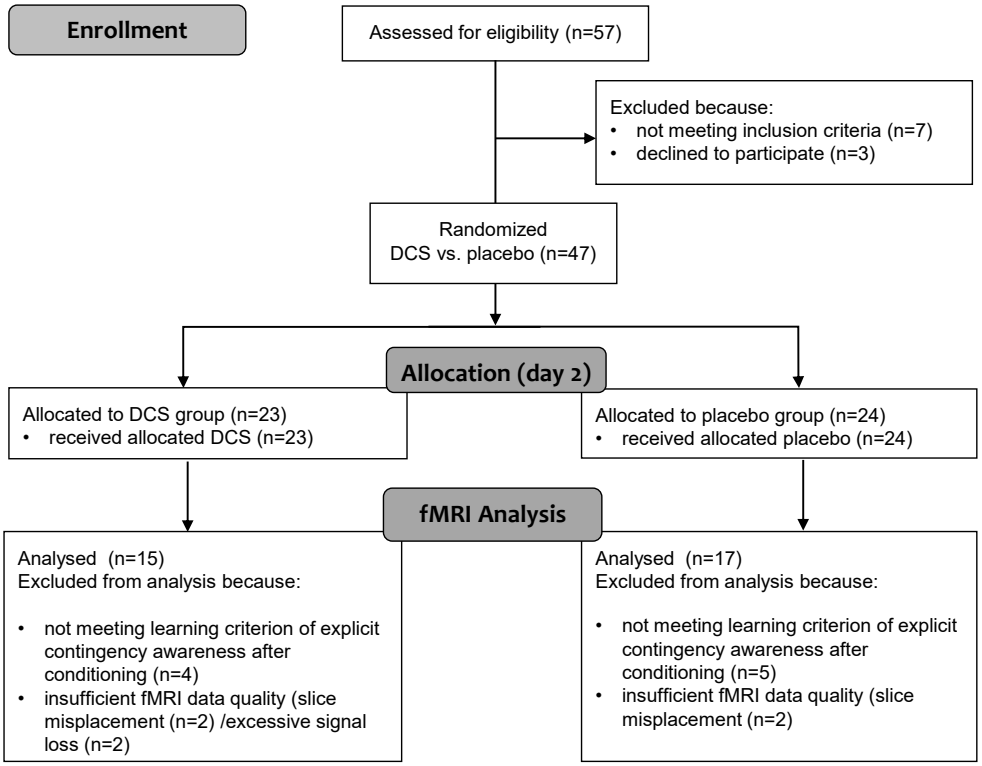

CONSORT (Consolidated Standards of Reporting Trials) flow chart. A total of 57 subjects were assessed for eligibility; seven of them did not met the inclusion criteria and 3 dropped out before (n=2) or after (n=1) the first experimental session, leaving 47 subjects that randomly received either DCS (n=23) or placebo (n=24) 1 hour before extinction on day 2. A total of n=15 subjects from the DCS group and n=17 subjects from the placebo group were included in the fMRI analyses. Subjects had to be excluded because of insufficient fMRI data quality on one or more days (DCS: n=4, placebo: n= 2) or unawareness about the conditioned contingencies assessed immediately after the conditioning session on day 1 (DCS: n= 4, placebo: n=5).

Table S1: Demographic and neuropsychological sample characteristics

Characteristic	DCS (n=15) mean (SD)	Placebo (n=17) mean (SD)	DCS vs. placebo statistic	p-value
DEMOGRAPHICS				
Sex (n: female/male)	8f /7m	8f /9m	0.13[a]	.723
Age (years)	26.3 (4.7)	27.8 (5.7)	-0.81	.425
NEUROPSYCHOLOGY				
TMT-A	23.4 (5.7)	25.2 (4.3)	-0.96	.339
TMT-B	48.2 (16.8)	49.1 (14.9)	-0.16	.875
Digit span (foreward)	8.2 (2.0)	8.8 (1.8)	-0.84	.410
Digit span (backward)	7.6 (1.4)	8.4 (1.7)	-1.38	.178
MWT	27.3 (3.0)	26.7 (3.3)	0.50	.619

[a] χ^2-test statistic, df=1; all other test statistics refer to Welch's two-sample t-test, two-sided

Abbrevations: standard deviation (SD), Trial Making Test A and B (TMT; Reitan, 1992), Multiple-Choice Vocabulary Intelligence Test (MWT; Lehrl, 2005), digit span forward/backward (subtest from Wechsler-Adult Intelligence Test, WAIS-III; von Aster *et al.*, 2006)

Table S2: Post-hoc t-tests for single-trial analysis of RTs in cued outcome discrimination during conditioning (day 1) and extinction (day 2)

Session	Comparison	t_{31}-value	*p*-value
CONDITIONING	**first vs. last trial (t1 vs t2)**		
	$CS+_{app}$ t1 vs. $CS+_{app}$ t2	6.64	<.001
	$CS+_{avers}$ t1 vs. $CS+_{avers}$ t2	2.12	.042
	CS- t1 vs. CS- t2	4.90	<.001
EXTINCTION	**first trial (t1)**		
	$CS+_{app}$ vs. CS-	2.76	.010
	$CS+_{avers}$ vs. CS-	1.96	.058
	last trial (t2)		
	$CS+_{app}$ vs. CS-	0.62	0.540
	$CS+_{avers}$ vs. CS-	-0.99	0.330
	first vs. last trial (t1 vs. t2)		
	$CS+_{app}$ t1 vs. $CS+_{app}$ t2	5.42	<.001
	$CS+_{avers}$ t1 vs. $CS+_{app}$ t2	5.34	<.001
	CS- t1 vs. CS- t2	1.95	.060

Additional results cued outcome discrimination task. Post-hoc analysis of the time effect during conditioning showed that participants got significantly faster in outcome discrimination in all conditions. We further observed a trendwise differentiation at the end of conditioning (condition by time interaction: $F(2,60) = 3.03$, $p = .056$) and no difference between groups

(main or interaction effects group: $p \geq .165$). In extinction all three cues were followed by the noUS, so that the same button response was required across conditions. Post-hoc comparisons to differentiate the significant time by condition interaction indicated that on the first trial participants were significantly slower in the appetitive and trendwise in the aversive compared to the neutral condition; an effect no longer present at the end of extinction learning. Further, significant RT-decreases over time were only present in both extinction conditions but not the neutral condition. We also observed a statistical trend for a group effect due to faster RTs in the DCS group, irrespective of condition type (main effect of group: $F(1,30) = 4.03$, $p = .054$; group interactions: $p \geq 0.352$).

Table S3: Brain activations revealed by PPI analysis for contrast CS+$_{app}$ > CS- during appetitive extinction recall

Contrast	Brain structure	x	y	z	*Z*	Cluster size
DCS > Placebo	anterior cingulate gyrus (BA 32)	6	34	-2	4.32	56
	middle cingulate gyrus (BA 31)	6	-38	36	4.01	36
	middle temporal gyrus (BA 21)	58	-48	4	3.99	38
Placebo > DCS	lingual gyrus	-18	-66	-4	5.35	423
	cuneus (BA 31)	14	-72	24	5.18	536
	lingual gyrus (BA 18)	-28	-90	-16	5.14	188
	postcentral gyrus (BA 40)	-20	-40	56	4.93	149
	superior temporal gyrus (BA 22)	-52	-8	-10	4.86	59
	fusiform gyrus (BA 19)	40	-66	-18	4.85	360
	cuneus (BA 18)	-10	-78	24	4.74	929
	sub-gyral (BA19)	38	-54	0	4.63	217
	superior temporal gyrus (BA 41)	-50	-24	8	4.39	55
	middle occipital gyrus (BA 18)	-38	-86	-2	4.37	77
	superior temporal pole (BA 38)	44	14	-30	4.36	51
	postcentral gyrus (BA 3)	18	-40	58	4.35	152
	lateral posterior nucleus	-24	-18	22	4.27	35
	middle temporal pole (BA 38)	-40	18	-30	4.22	35
	superior temporal gyrus (BA 41)	56	-26	6	4.15	40
	middle cingulate gyrus (BA 24)	12	2	40	4.13	45
	middle temporal gyrus (BA 21)	54	-16	-16	4.10	100
	superior temporal gyrus (BA 41)	38	-32	16	3.98	42
	supplementary motor area (BA 6)	2	-20	72	3.72	33
	substantia nigra (brain stem)	12	-18	-16	3.68	30

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Augmenting extinction learning with D-cycloserine reduces return of fear: a randomized, placebo-controlled fMRI study

Running title: DCS-augmented fear extinction

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Abstract

D-cycloserine (DCS), a partial NMDA-receptor agonist, seems to be a promising enhancer for exposure therapy in anxiety disorders. It has been tested successfully in animal models of fear extinction, where DCS enhanced extinction learning. Applied in clinical studies, results of DCS-augmented exposure therapy remain ambiguous, calling for a deeper understanding of the underlying mechanisms of DCS and its exact effect on extinction learning and return of fear in humans.

In the present study, we investigated the effect of DCS-augmented extinction learning on behavioral, psychophysiological, and neural indices of return of fear during a 24 h delayed recall test. Thirty-seven participants entered a randomized, placebo-controlled, double-blind, three-day fear conditioning and delayed extinction fMRI design. One hour before extinction training, participants received an oral dose of 50 mg DCS or a placebo.

Behavioral arousal ratings revealed a generalized return of fear during extinction recall in the placebo but not DCS group. Furthermore, participants receiving DCS compared to placebo showed attenuated differential BOLD responses in left posterior hippocampus und amygdala from extinction learning to extinction recall, due to increased hippocampal recruitment in placebo and trendwise decreased amygdala responding in DCS subjects. Our finding that DCS reduces return of fear in arousal ratings and neural structures subserving defensive reactions support a role for NMDA receptors in extinction memory consolidation and encourage further translational research.

Introduction

Anxiety disorders are among the most common mental disorders, with a 12-month prevalence of 14% in the EU [1]. Although exposure-based techniques are effective in treating anxiety disorders, relapse after successful therapy is frequently observed [2,3]. Therefore, various lines of translational research aim to establish new behavioral and pharmacological augmentation strategies that could improve long-term retention and reduce relapse rates [4].

D-cycloserine (DCS), a partial N-methyl-D-aspartate (NMDA) receptor agonist, might show promise as a cognitive enhancer in humans, as glutamatergic activation of the NMDA receptor influences long-term potentiation dependent forms of learning and memory [5]. NMDA receptors are widely spread in amygdala, hippocampus and other brain regions critically involved in fear processing and associative learning [6–9]. While animal studies found DCS pre- or post-learning administration to facilitate extinction retention [10,11], meta-analyses from clinical trials investigating DCS-augmented exposure therapy in anxiety disorders remain inconclusive, with results ranging from no evidence [12] to medium effects, especially when DCS was administered at low doses and in close proximity to exposure sessions [13].

Human fear conditioning and extinction represent a laboratory model for the development and treatment of anxiety disorders [4,14,15] and provide an important tool to investigate the pharmacological effects of DCS in a controlled setting. During fear conditioning, a neutral stimulus is conditioned (CS+) by repeated coupling with an unconditioned stimulus (US), signaling potential threat or danger and triggering a conditioned fear response (CR), while another stimulus (CS-) is never paired with the US. During fear extinction training – the experimental analogue of exposure therapy – the CS+ is no longer followed by the US, resulting in a reduced CR. According to the inhibitory model of fear extinction [16], extinction learning leaves the initial excitatory CS-US association intact, while establishing a new,

competing inhibitory association. This duality explains why fear often returns after successful extinction learning [14], thereby challenging the long-term success of exposure therapy.

Fear expression and inhibition are mediated by partly different neural circuits. Neuroimaging studies revealed a network involved in fear conditioning, comprising amygdala, hippocampus, anterior insula, and dorsal anterior cingulate cortex (dACC) as key structures [17,18]. Activation of dACC and insula has been related to fear expression, threat anticipation and interoceptive processing [18]. Amygdala and hippocampus show rather time dependent activation patterns [19,20], indicating their relevance for initial acquisition of CS-US associations [21]. However, involvement of these latter structures was not consistently confirmed across fear conditioning studies [18]. Accumulating evidence points towards a specific role of the ventromedial prefrontal cortex (vmPFC) in long-term extinction retention [22], mediating the inhibition of conditioned responding [23–25]. Supporting the dual-model [16], return of fear following reinstatement has been associated with increased blood-oxygen-level dependent (BOLD) activation in structures like amygdala and hippocampus, while decreasing vmPFC involvement [26,27].

The few laboratory studies directly examining the effect of DCS on fear extinction retention yielded mixed results. While Kuriyama and colleagues [28] found DCS to facilitate extinction recall in terms of attenuated differential skin conductance responses (SCRs) after a reactivation procedure, two other studies observed no effect of DCS [29,30]. A neuroimaging study found DCS to enhance fear memory consolidation, as post-learning administration of DCS compared to placebo enhanced SCRs and neural activation in posterior hippocampus/collateral sulcus and medial prefrontal cortex/ACC during delayed fear recall [31]. First promising results have been reported in appetitive conditioning studies, with DCS administration compared to placebo after extinction being associated with reduced renewal of conditioned sexual responses in healthy females [32] and attenuated amygdala activation during extinction recall [33].

To further shed light on the neurobehavioral effects of DCS, we administered 50 mg of DCS

one hour before extinction learning in a double-blind, placebo-controlled, three-day fMRI design. We hypothesized that DCS would facilitate extinction learning consolidation, thereby reducing the return of fear on a behavioral (subjective ratings), psychophysiological (SCRs), and neural (BOLD) level by contrasting extinction recall (day 3) with extinction learning (day 3).

from day 2 to day 3. We anticipated that DCS would attenuate BOLD activation in fear-associated structures (amygdala, hippocampus, anterior insula, dACC), while increasing activation in inhibitory structures like vmPFC.

Materials and Methods

Participants

Thirty-seven healthy participants (n=20 DCS/n=17 placebo), providing complete and quality-controlled fMRI data for all three days, were included in the study (Figure S1). Participants were recruited via advertisement at two study sites (Berlin/Dresden) as part of a registered, multi-center clinical trial of the national research initiative Panic-Net. Exclusion criteria comprised current or past psychiatric (confirmed via standardized clinical interview [34]), neurological or internal medical disorders, pregnancy, positive urinary drug screening, and color blindness or weakness [35]. Experimental groups did not differ in sociodemographic or neuropsychological measures nor subclinical levels of anxiety sensitivity (ASI [36]; Table S1). Participants provided written informed consent and received 75€ for participation. A subsample (n=6 DCS/n=9 placebo) also underwent an appetitive conditioning task previously published [37]. The study was approved by the ethics committees of Berlin (LAGeSo; EudraCT-Nr.: 2006-004860-29) and Technische Universität Dresden (EK 62022010).

Experimental protocol

Subjects underwent a three-day fear conditioning and delayed extinction paradigm [37] to investigate the effect of DCS-augmented extinction training on return of fear during extinction recall. To this end, subjects received either 50 mg of DCS or placebo one hour before extinction training on day 2 in a randomized, double-blinded trial (Figure 1A).

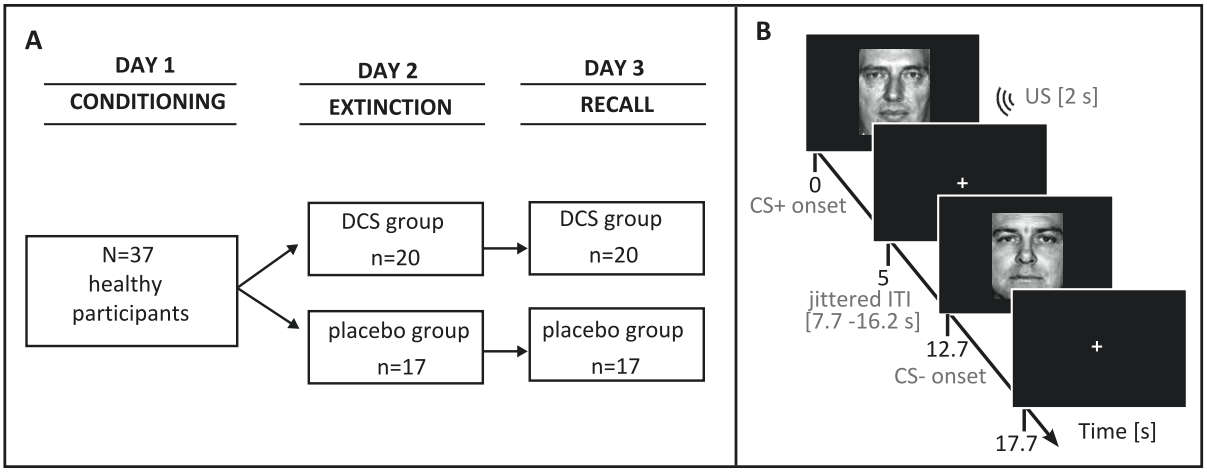

Figure 1 Experimental design and paradigm. A Healthy participants were enrolled in a double-blind, placebo-controlled design with three experimental sessions — Pavlovian conditioning, extinction, and extinction recall — spaced approximately 24 h apart to allow consolidation between sessions. An oral dose of 50 mg of DCS or placebo was administered 1 h before extinction training. **B** Exemplary trial: In each trial, a CS was presented for 5 s, followed by a jittered ITI (range: 7.7-16.2 s). In case of a CS+ trial during acquisition, the US (aversive panic scream, 2 s duration) appeared simultaneously with CS+ offset (100% reinforcement schedule).

Fear conditioning and delayed extinction paradigm

The paradigm comprised three sessions: habituation and fear conditioning (day 1), extinction learning (day 2), and extinction recall (day 3). Two male faces from the Ekman series [38] served as cues, with picture-cue assignment counterbalanced across subjects. Each session comprised three phases with eight CS+ and eight CS- trials in pseudo-randomized order, resulting in 48 trials per session (day 1: habituation, early and late acquisition; day 2: early, middle, and late extinction training; day 3: early, middle, and late extinction recall). During both acquisition phases, the CS+ was followed by an auditory US (aversive panic scream, 100% reinforcement) presented using MR-compatible headphones, while the CS- was never reinforced (Figure 1B). In the remaining phases (habituation, extinction learning, extinction recall), only unreinforced CS+ were presented. US intensity (volume) was individually

adjusted prior to conditioning using a 9-point Likert scale (1=not aversive; 9=very aversive) to meet a predefined criterion (US rating≥8).

Stimulus presentation was controlled via Presentation 14 software (Neurobehavioral Systems; https://www.neurobs.com).

Drug administration (day 2)

Participants received an oral dose of 50 mg of DCS (reformulated from 250 mg capsules, Seromycin®, USA) or placebo one hour before extinction [13]. An external pharmacist prepared identical capsules in blocks of four, each containing 2 DCS/placebo pills in random order. None of the participants reported adverse events.

Data acquisition and preprocessing

Contingency knowledge

Participants' awareness of the CS-US contingency was assessed in a post-experimental interview outside the scanner on day 1 (for details see [37]). Thirty-four participants were classified as aware, while three (2 DCS/ 1 placebo) were classified as unaware.

Valence and arousal ratings

Subjective ratings of CS valence and arousal were obtained immediately before and after acquisition, extinction learning, and extinction recall using an MR-compatible button box. Participants rated each cue on two 9-point Likert scales ranging from -4='negative' to 4='positive' and 1='not at all' to 9='very much', respectively.

SCRs

Skin conductance was recorded continuously from the non-dominant hand during all sessions. The electrodermal signal was recorded using two Ag/AgCl electrodes attached to either the thenar and hypothenar eminence sampled at 50 Hz in Berlin (MP150, Biopac Systems, Goleta, USA) or the second phalanx of middle and index finger sampled at 1000 Hz in Dresden (MR-compatible BrainAmp ExG amplifier, Brain Products, Munich, Germany).

Preprocessing and statistical analysis of single-subject data was performed within the PsPM toolbox, using the general linear model (GLM) approach (4.0.2; http://pspm.sourceforge.net; see Supplementary Materials). Data from two participants were lost due to technical failure on one of the days, leaving 35 subjects for analysis.

fMRI

Structural and functional images were acquired on 3-Tesla scanners (Trio, Siemens AG, Erlangen, Germany). Functional images were acquired in an interleaved fashion using a standard EPI sequence (voxel size=3x3x3 mm, 41 slices, TR=2.5 s, TE=25 ms, 64x64 matrix; 192x192-mm FOV; 368 volumes on day 1, 356 volumes on days 2 and 3). Images were angled 20° to the anterior-posterior commissure. The first five volumes per session were discarded to avoid T1 equilibrium effects. Furthermore, a T1-weighted structural scan (MPRage, voxel size= 1x1x1 mm) was acquired.

Imaging data were analyzed within SPM8 (https://www.fil.ion.ucl.ac.uk/spm/). Preprocessing included slice time correction, realignment to the mean EPI, coregistration and segmentation of the structural image, spatial normalization to MNI space (3-mm isotropic voxel resolution), and iterative smoothness equalization to a target smoothness of 10-mm full-width at half maximum Gaussian kernel to account for differences in intrinsic smoothness between scanners [39].

Statistical analyses

Behavioral and psychophysiological measures

All analyses included study site as a covariate and were performed using R software (v3.4.3; [40]). Conditioning effects in valence and arousal ratings were analyzed in separate repeated measures ANCOVAs (rmANCOVA) with within-subject factors cue (CS+/CS-) and time (pre-/post-acquisition on day 1). DCS effects on return of fear, i.e. increased conditioned responding from extinction learning to extinction recall, were assessed by contrasting

participants' post-extinction (day 2) with pre-recall (day 3) ratings in two mixed ANCOVAs with within-subject factors cue (CS+/CS-) and time (post-extinction/pre-recall) and between-subject factor group (DCS/placebo). SCRs were analyzed analogously, whereby 'time' refers to early/late acquisition to assess conditioned responses on day 1 and to late extinction/early recall for evaluation of return of fear. Significant effects were followed up by planned FDR-corrected [41] post-hoc t-tests.

fMRI

Individual subject data were modeled using a GLM including each day as separate session. Event onsets (CS+, CS-, US) for each of the nine phases were modeled as stick functions and convolved with the canonical HRF. Rating phases (modeled as box-car functions) and six movement parameters per session were entered as additional regressors. Baseline contrasts for CS+ and CS- were computed for each phase and entered into two random-effects flexible factorial models on the group level, both including study site as a covariate.

The neural signatures of fear conditioning (day 1) were investigated in a flexible factorial model including CS+ and CS- regressors for both acquisition phases. The main effect of conditioning was assessed with the differential contrast 'CS+>CS-' during acquisition. Possible time effects over the course of conditioning were investigated by assessing interactions with conditioning phase ($early_{[CS+>CS-]}$ vs. $late_{[CS+>CS-]}$).

The hypothesis that DCS facilitates extinction recall through enhanced extinction learning consolidation was investigated in a flexible factorial model including CS+ and CS- regressors for the three extinction learning (day 2) and recall (day 3) phases and the experimental group factor (DCS/placebo). Paralleling the behavioral and psychophysiological analyses, return of fear 24 h post-extinction was evaluated by comparing each individuals' differential BOLD responses during extinction learning on day 2 with extinction recall on day 3, examining the contrast $extinction\ recall_{[CS+>CS-]}$>$extinction_{[CS+>CS-]}$ over subjects and the critical interaction with experimental group. This analysis incorporated all trials per session to maximize

statistical power. To specify the direction of observed group differences, post-hoc analyses were conducted for each group separately. As an additional exploratory analysis, we also investigated group differences during the first recall phase only (first eight CS+/CS- presentations).

Our analyses focused on predefined regions of interest (ROIs) using small volume correction (SVC) at p<.05 FWE-corrected, specifically, insula, dACC, amygdala, hippocampus and vmPFC [17,18,22]. ROI masks for insula, amygdala and hippocampus were derived from the WFU PickAtlas (http://www.fmri.wfubmc.edu/download.htm). ROIs of dACC and vmPFC were created using a 10-mm sphere on the midline-centered coordinates [x=0, y=18, z=34] and [x=0, y=34, z=-6] derived from a meta-analysis on fear extinction recall in healthy participants [22]. We also performed whole-brain analyses, using an initial cluster-forming threshold of p<.001 uncorrected and 10 contiguous voxels; clusters surviving p<.05 FWE correction at the cluster level are reported. For plotting purposes, mean beta estimates within a 6 mm sphere surrounding peak activations were extracted.

Results

Fear conditioning

Behavioral and psychophysiological measures

Valence ratings showed a main effect of cue ($F_{1,36}$=4.47, p=.042, η^2p=.11) and a cue×time interaction ($F_{1,36}$=16.30, p<.001, η^2p=.31; Figure 2A). Post-hoc t-tests revealed that CS+ valence significantly decreased (t_{36}=4.23, p_{FDR}<.001, d=.70) and CS- valence increased (t_{36}=-2.47, p_{FDR}=.024, *d*=.41) from pre- to post-conditioning, causing a significant differentiation between cues after acquisition (t_{36}=3.65, p_{FDR}=.002, d=.60) but not at baseline (t_{36}=-0.23, p=.817). Analyzing arousal ratings, significant main effects of cue ($F_{1,36}$=13.92, p=.001, η^2p=.28) and time ($F_{1,36}$=4.69, p=.037, η^2p=.12), as well as a significant cue×time interaction emerged ($F_{1,36}$=33.36, p<.001, η^2p=.48; Figure 2B). Post-hoc t-tests confirmed that arousal

increases towards the CS+ (t_{36}=-4.27, p_{FDR}<.001, d=.70) and decreases towards the CS- (t_{36}=3.104, p_{FDR}=.004, d=.51) led to differential ratings post-conditioning (t_{36}=-5.01, p_{FDR}<.001, d=.82) in absence of baseline differences (t_{36}=0.53, p_{FDR}=.597).

SCRs showed a main effect of cue with higher SCRs towards the CS+ compared to the CS- ($F_{1,34}$=7.20, p=.011, η^2p=.17; Figure 2C) and a main effect of time due to general declines in SCR amplitudes over phases ($F=_{1,34}$=9.08, p=.005, η^2p=.21), but no significant cue×time interaction ($F_{1,34}$=3.99, p=.054, η^2p=.11).

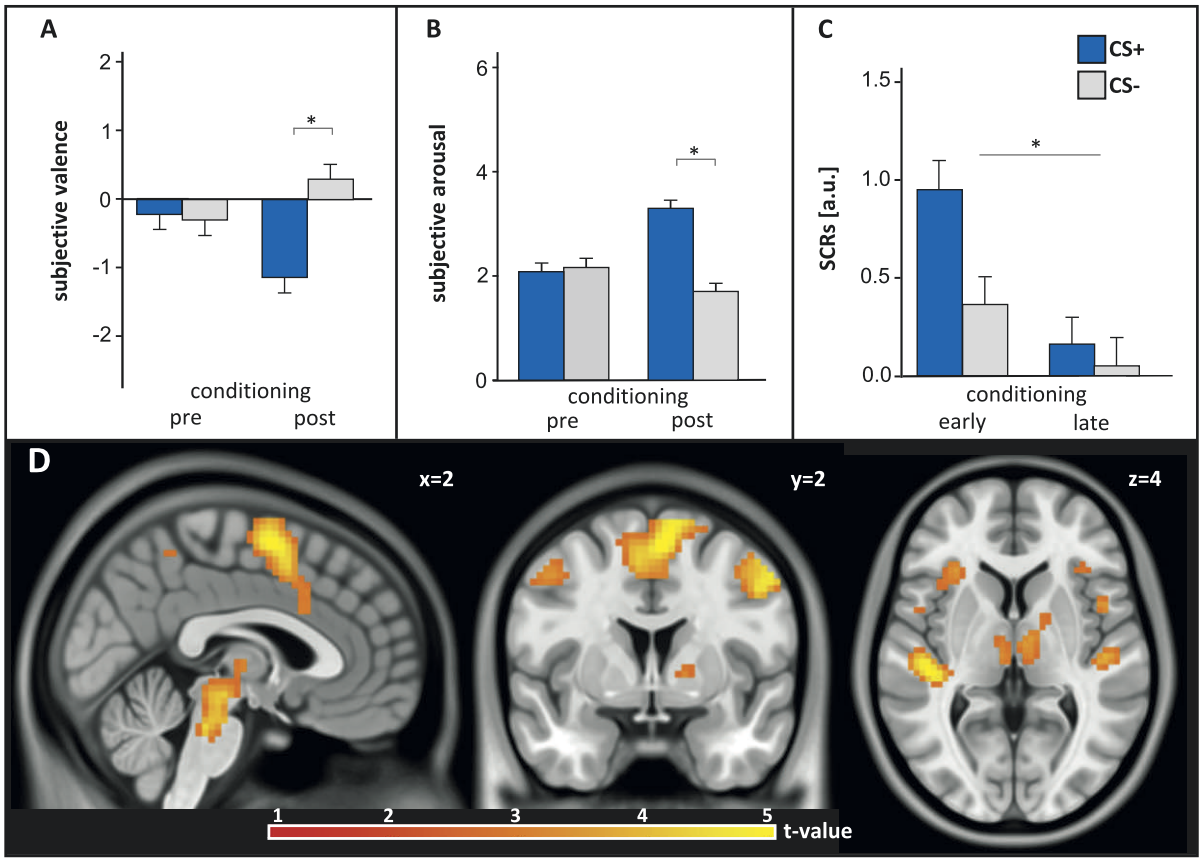

Figure 2 Behavioral, psychophysiological, and BOLD responses during fear conditioning. A-C Conditioning was associated with decreased valence ratings and increased arousal ratings towards the CS+ compared to the CS- from pre to post conditioning, and induced differential SCRs (CS+>CS-) across both phases of conditioning. Bar graphs represent the mean ± within-subject SEM [42,43]. **D** Differential BOLD responses during conditioning in brain structures of the fear network (i.e. bilateral insula, dACC, SMA, midbrain). T-maps are displayed on a visualization threshold of p<.005 uc. with k≥5 cluster extent.

fMRI

Differential BOLD responses during acquisition were observed in bilateral insula (left: x=-33, y=-31, z=19, Z=3.83, $p_{FWE\ ROI}$=.013; right: x=48, y=8, z=1, Z=3.57, $p_{FWE\ ROI}$=.013) and dACC (left: x=-3, y=14, z=31, Z=3.02, $p_{FWE\ ROI}$=.041; right: x=9, y=14, z=37, Z=3.53, $p_{FWE\ ROI}$=.009). Moreover, whole-brain analyses revealed increased BOLD responses towards CS+ compared to CS- in midbrain, bilateral supplementary motor area (SMA), transverse temporal gyrus (TTG), and precentral gyrus (Table 1, Figure 2D). No significant amygdala or hippocampal activation was observed. Time-based analyses over acquisition phases revealed no significant time-dependent activation changes but only a trendwise increase in differential responding from early to late acquisition in the right amygdala (x=24, y=2, z=-22, Z=2.55, $p_{FWE\ ROI}$=.090).

Table 1: Whole-brain results during conditioning for the contrast CS+>CS- across participants

Region	Side	Voxel	Peak voxel MNI			Z_{max}	p_{FWE}[a]
			x	y	z		
Midbrain	R/L	270	9	-22	-8	5.65	<.001
TTG	L	238	-39	-31	7	5.22	.001
SMA	R	316	6	2	61	5.14	<.001
SMA	L		-6	5	52	4.48	
TTG	R	101	45	-28	10	4.72	.029
Precentral gyrus	L	107	-36	-7	49	4.68	.024
Precentral gyrus	R	109	54	2	46	4.50	.022

[a]Clusters are cluster-level family-wise error corrected for multiple comparisons at p<.05 (cluster forming threshold at p<.001 uncorrected with 10 contiguous voxels). TTG: transverse temporal gyrus; SMA: supplemental motor area; L: left hemisphere, R: right hemisphere.

DCS effects on return of fear

Behavioral and psychophysiological measures

Return of fear in valence and arousal ratings was investigated by comparing post-extinction with pre-recall ratings. With respect to valence (Figure 3A), no cue×time interaction was present (p=.658), indicating no return of fear in this measure across subjects. The cue×time×group interaction was only marginally significant ($F_{1,35}$=2.96, p=.094, η^2p=.08) due to numerically decreasing valence ratings from post-extinction to pre-recall in the placebo group (corresponding to return of fear) but increasing valence ratings in the DCS group. We further observed a significant main effect of group due to overall lower valence ratings in the placebo compared to the DCS group ($F_{1,35}$=5.70, p=.023, η^2p=.14). No further effects were significant (all p≥.508). Analyzing return of fear in arousal ratings revealed a main effect of cue due to higher arousal towards the CS+ compared to the CS- across phases ($F_{1,35}$=7.43, p=.010, η^2p=.18), indicating incomplete extinction of subjective arousal that persisted until extinction recall. No cue×time or cue×time×group effects were observed (p≥.169), indicating no differential return of fear. However, a significant group×time interaction ($F_{1,35}$=7.98, p=.008, η^2p=.19) yielded evidence for a rather generalized return of fear in the placebo group (Figure 3B). Post-hoc t-tests confirmed a significant increase in overall arousal ratings from post-extinction to pre-recall only in the placebo group (t_{16}=-2.73, p_{FDR}=.030, d=.66; DCS: t_{19}=1.17, p_{FDR}=.259); an effect mainly driven by increases towards the CS+ rather than the CS-. No significant main or interaction effects were observed in the analysis of SCRs (p≥.259).

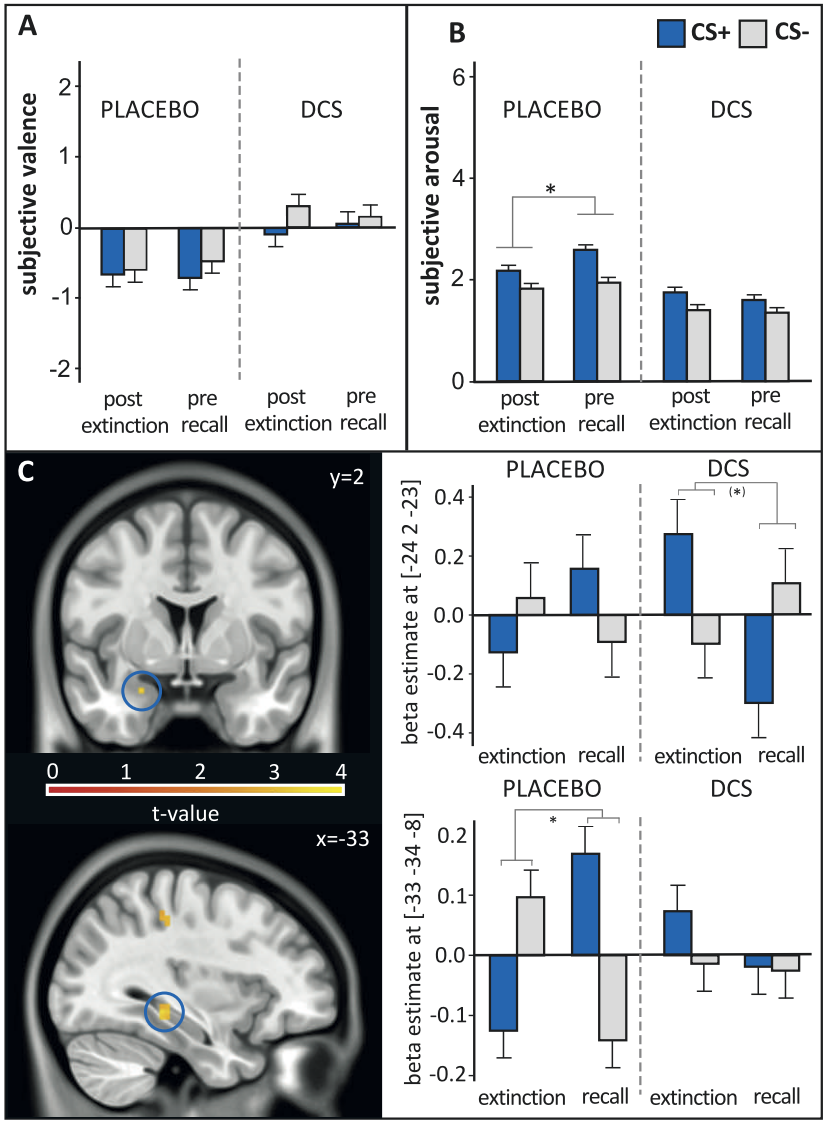

Figure 3 Group differences in behavioral and neural measures of return of fear. **A+B** While no significant differential return of fear was observed across or between groups in rating measures of CS valence or arousal, subjects in the placebo but not DCS group showed non-differential return of fear with increased arousal ratings from extinction training to extinction recall. Bar graphs represent the mean ± within-subject SEM [42,43]. **C** Participants in the placebo compared to the DCS group showed stronger return of fear on a neural level, that is increased differential BOLD responses from extinction learning to extinction recall in left amygdala and posterior hippocampus. Bar graphs represent mean parameter estimates from a 6-mm sphere surrounding peak voxel activation ± within-subject SEM [42,43]. T-maps are displayed on a visualization threshold of p<.005 uc. with k≥5 cluster extent.

fMRI

While we did not observe an overall return of fear effect in differential BOLD responses when examining the contrast recall$_{[CS+>CS-]}$>extinction$_{[CS+>CS-]}$, group comparisons revealed that

placebo compared to DCS subjects showed significant increases in differential BOLD responses in the left amygdala (x=-24, y=2, z=-23, Z=3.15, $p_{FWE\ ROI}$=.019) and left posterior hippocampus from extinction learning to recall (x=-33, y=-34, z=-8, Z= 3.39, $p_{FWE\ ROI}$=.033; Figure 3C). Separate post-hoc analyses showed that the amygdala effect was driven by a marginally significant decrease towards the CS+ from extinction learning to recall in the DCS group (x=-24, y=2, z=-23, Z=2.63, $p_{FWE\ ROI}$=.075). In contrast, participants in the placebo group exhibited significant increases in differential BOLD responses in the posterior hippocampus from extinction learning to recall (x=-33, y=-31, z=-8, Z=4.07, $p_{FWE\ ROI}$=.003).

As an additional exploratory analysis, we also investigated spontaneous recovery effects by analyzing the first phase of the recall session only. Group comparisons revealed stronger BOLD responses towards CS+ compared to CS- in the placebo compared to DCS group in the right dACC (x=6, y=14, z=40; Z=3.14, $p_{FWE\ ROI}$=.033) and left insula (x=-33, y=-16, z=7; Z=3.53, $p_{FWE\ ROI}$=.037; x=-27, y=17, z=10; Z=3.30, $p_{FWE\ ROI}$=.074; Figure 4). The reverse contrast (DCS>placebo) revealed no significant effects.

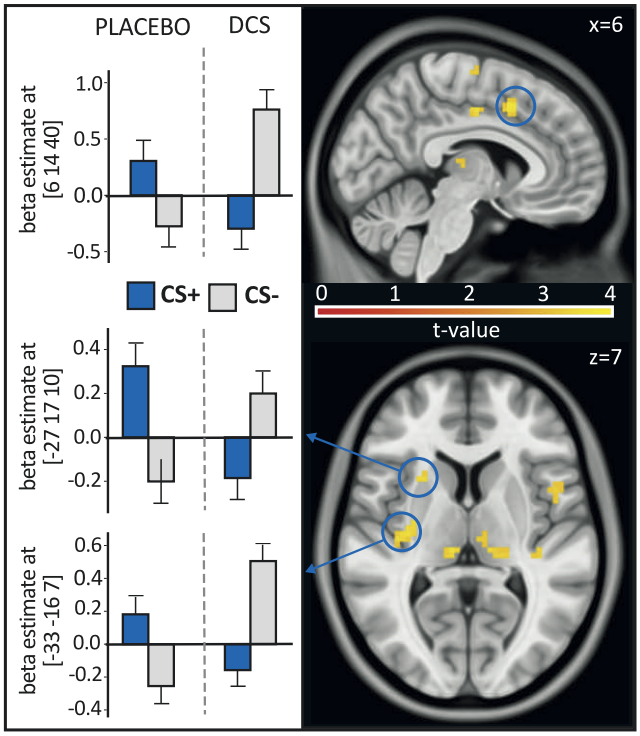

Figure 4. Exploratory group differences during early extinction recall. Increased differential BOLD responses in the placebo compared to the DCS group in right dACC and left insula when investigating the first recall phase

only. Bar graphs represent mean parameter estimates from a 6-mm sphere surrounding peak voxel activation ± within-subject SEM (63,64). T-maps are displayed on a visualization threshold of p<.001 uc. with k≥5 cluster extent.

Discussion

The present study investigated the effect of DCS-augmented extinction learning on behavioral, psychophysiological, and neural indices of return of fear by applying a three-day fear conditioning and delayed extinction paradigm that allowed for consolidation between learning sessions. We found that 50 mg of DCS facilitated long-term extinction retention, as only participants in the placebo but not the DCS group experienced a generalized return of fear in arousal ratings. This was accompanied by relative down-regulation of amygdala activation in the DCS group from extinction training to recall, while placebo subjects displayed increased posterior hippocampus activation. Furthermore, exploratory analyses showed a down-regulation in dACC and insula following DCS administration during the early recall trials.

Fear acquisition: characteristics of the conditioned response

The data demonstrate successful fear acquisition across participants, as reflected by reduced subjective valence and increased arousal ratings towards the CS+, as well as increased differential SCRs and a pattern of activation within bilateral insula, dACC, SMA and midbrain including thalamus, key regions of the fear network [18,37]. Activation within the TTG (also called Heschl's gyrus), known to be involved in auditory processing [44], likely reflects US anticipation, in line with previous findings using this paradigm [37]. Although animal studies clearly demonstrate the central role of the amygdala in fear acquisition [45], we did not observe significant amygdala activation during conditioning, conforming to a recent meta-analysis of human fear conditioning [18]. Besides methodological difficulties when measuring amygdala activity [17], it has also been argued that human fear conditioning experiments might not primarily engage the basic threat detection circuit, but instead recruit

an extended 'autonomic-interoceptive network' for threat appraisal [18]. The temporal sensitivity of this structure observed in some studies [19,20] might further contribute to the mixed evidence.

DCS prevented the return of fear in arousal ratings

Participants receiving placebo but not DCS experienced a generalized return of fear in arousal ratings, mainly driven by increases towards the CS+ from post-extinction to pre-recall. Generalization of the CR towards the CS- is commonly observed in studies investigating return of fear following reinstatement [46], and may further represent a characteristic feature during conditioning in anxiety disorders [47]. Our result suggests that DCS facilitates extinction memory retention and thereby prevents generalized return of fear. While previous studies on DCS-augmented fear extinction did not evaluate stimulus ratings, using an appetitive sexual conditioning paradigm Brom et al. [32] found post-learning DCS administration to attenuate differential valence and arousal ratings as well as conditioned physiological responses in a delayed combined renewal and reinstatement test, suggesting DCS facilitates extinction learning by reducing its context-sensitivity. Although we observed conditioned SCRs during fear acquisition, there was no evidence for return of fear in terms of increased SCRs from extinction learning to extinction recall across participants or in either group, thus no effect of DCS could be observed in this measure.

Using SCRs as primary outcome measure, two laboratory studies failed to find differences between DCS and placebo during extinction recall [29,30], while one study found DCS to attenuate differential SCRs after a reactivation procedure [28]. Moreover, DCS administration after fear acquisition and immediate extinction resulted in increased SCRs during fear recall 72 hours later, in line with a facilitation of fear memory consolidation [31]. The less robust SCR findings could be due to the method itself, as SCRs represent a rather noisy measure and appear to habituate quickly [48]. Moreover, the scanner environment negatively affects the signal-to-noise ratio [49], making it more difficult to detect rather transient psychophysiological return of fear effects [46]. Future research would therefore benefit from

a combination of several psychophysiological response measures, i.e. assessing conditioned pupillary responses via eye-tracking could be especially suited for neuroimaging [50].

DCS attenuated neural activation patterns of fear-associated brain regions

Our finding that DCS prevented the return of fear in subjective arousal ratings was corroborated by significant group differences in BOLD response shifts in amygdala and posterior hippocampus from extinction learning to recall. Specifically, we observed a relative increase in differential amygdala activation in placebo compared to DCS that was mainly driven by a deactivation towards the CS+ in the DCS group. Several studies highlight the pivotal role of the amygdala in human fear conditioning as well as in extinction learning and recall [17,51,52]. Higher amygdala activity has been associated with stronger fear memory reconsolidation and return of fear [53,54]. Increased amygdala activity in the placebo compared to DCS group therefore suggests a stronger recall of the original CS-US association in the placebo group, which was abolished in the DCS-treated participants. Using a similar three-day design, Ebrahimi et al. previously found 50 mg of DCS during extinction learning to attenuate amygdala activity during appetitive extinction recall, while groups did not differ on a behavioral level (reaction times; [33]). The deactivation observed during extinction recall in the DCS group potentially suggests active inhibition of amygdala activity towards the CS+. While the vmPFC is a prime candidate to explain the observed group difference in amygdala activity – as vmPFC activity has been linked to the successful extinction recall and top-down control of the amygdala [21,25,55,56] – we did not observe significant group differences in vmPFC BOLD response. Preliminary evidence for a mediating role of the vmPFC in DCS-augmented appetitive extinction learning comes from Ebrahimi et al. [33], who observed increased amygdala-vmPFC functional connectivity during CS+ compared to CS- presentations in DCS compared to placebo.

Our finding that only the placebo group showed increased activation in the posterior hippocampus is in line with studies associating return of fear with increased BOLD responses in this area [57], often together with increased amygdala activity [25,26]. In animal studies,

inactivation of the hippocampus reduces the expression of the CR and prevents the return of fear after extinction [58,59]. Previous work indicates a differential role of the anterior and posterior part of the hippocampus in human fear conditioning, where activation of the anterior hippocampus has been mainly associated with extinction memory recall [24,25], whereas the posterior hippocampus has been related to return of fear phenomena [25,31]. In the same vein, posterior hippocampal activation has been shown to correlate with SCRs during return of fear after reinstatement [26]. Increased posterior hippocampal activation under placebo therefore likely reflects recall of the original fear memory, which was attenuated under DCS.

In an exploratory analysis, we focused on the first phase of extinction recall and observed relatively increased dACC and insula activation towards the CS+ in the placebo but not the DCS group. These brain regions are associated with fear acquisition, threat anticipation and CR expression [17,60]. The dACC mediates fear responses [61], whereas the insula plays an important role in interoception and experience of subjective feelings [62,63]. As such, robust activation of these areas during fear conditioning and recall has been ascribed the subjective experience of fearful states, possibly in terms of interoceptive awareness [22]. Our finding of heightened dACC and insula activation in placebo compared to DCS during the early recall phase might reflect stronger threat anticipation in the placebo group and therefore provides further evidence that DCS attenuates return of fear during extinction recall.

Conclusions and perspectives

Our results support the hypothesis that DCS-augmented extinction learning enhances long-term extinction retention, thereby preventing return of fear. DCS attenuates differential BOLD responses in key structures of the fear network, including the amygdala. Anxiety disorders are characterized by amygdala hyperactivity and heightened fear generalization [64]. As such, the present findings provide new insights regarding the underlying mechanisms of DCS as a potential augmentation strategy for exposure therapy. To support this approach, multi-modal studies with bigger sample-sizes are needed. Furthermore, future research should investigate the impact of psychopathology on this basic mechanism, as well as moderating

factors that may determine which patients will benefit most from DCS augmentation. A deeper understanding of these relationships could help to optimize exposure therapy and prevent relapse in anxiety disorders.

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Supplementary Material to

Augmenting extinction learning with D-cycloserine reduces return of fear: a randomized, placebo-controlled fMRI study

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[*] These authors contributed equally to this work.

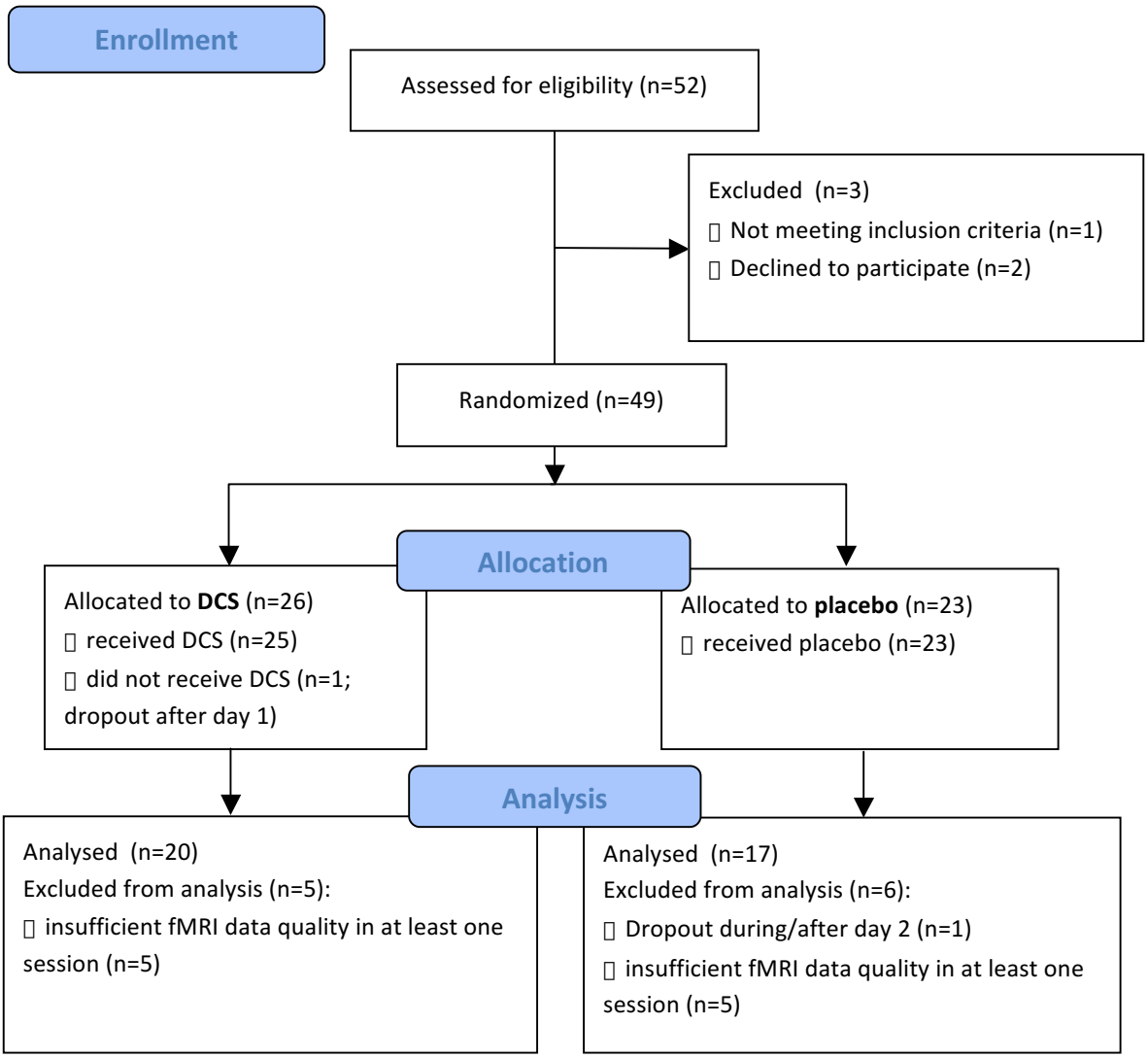

Figure S1. CONSORT flow diagram. A total of 52 participants were assessed for eligibility and 49 were enrolled. Recruitment took place between March 2013 and March 2014. We assumed a strong effect of f=0.25 (1, 2), so that detection of a group (DCS vs. placebo) x time (extinction vs. recall) interaction at a significance level of $\alpha = 0.05$ with a power of 0.8 required a minimum of 17 subjects per group (G*Power 3 (3)). Taking into account a drop out of approx. 20% the required sample size per group was 21 participants per group. Two subjects dropped out before completing all experimental sessions (n=1 after day one, n=1 after day 2). Only subjects providing high quality fMRI data in all experimental sessions (conditioning, extinction, extinction recall) were included in the analyses, resulting in a final sample of n=20 participants in the DCS group and n=17 participants in the placebo group. Reasons of fMRI data exclusion were excessive head movement (n=1), low signal-to-noise ratio (n=6; i.e. no activation in primary visual and/or auditory cortex at $p<.05$ uncorrected for visual and auditory (day 1 only) baseline contrasts), and excessive signal loss in predefined regions of interest (n=1).

Table S1. Sample characteristics

Characteristics		DCS n = 20	Placebo n = 17	*chi²/F*	*p*
gender (m/w)		9 /11	7/10	0.055	.815
age		25.85 (5.67)	27.82 (5.24)	1.194	.282
smoking status* (sm/nsm)		7/12	5/11	0.121	.728
neuroticism		1.58 (0.36)	1.56 (0.26)	0.045	.833
Anxiety Sensitivity Index		8.75 (6.33)	8.69 (5.4)	0.001	.975
Trail making test	TMT A	22.80 (6.0)	23.93 (5.71)	0.318	.577
	TMT B	46.85 (11.14)	50.47 (13.57)	0.750	.393
Regensburger word fluency test	P words	11.15 (3.8)	13.53 (3.56)	3.537	.069
	K words	14.95 (3.14)	15.27 (4.74)	0.057	.814
digit span	forward	8.60 (1.85)	8.80 (1.2)	0.133	.718
	backward	8.25 (1.88)	8.53 (2.32)	0.158	.693

Data reported as mean (SD), except for gender and smoking status, where *n* is reported. All participants were right-handed and had the highest secondary school qualification (12-13 years). *information available for n= 19 DCS/ n=16 placebo. **Abbreviations**: sm=smoker, nsm=non-smoker

Preprocessing of skin conductance data and first level GLM

Raw data were downsampled to 50 Hz and preprocessed within the PsPM toolbox (4.0.2; http://pspm.sourceforge.net), comprising visual inspection of the raw signal, linear interpolation of movement related artefacts, and median filtering to remove short spikes due to the scanning environment. Afterwards, the skin conductance time series was band pass filtered (first-order Butterworth filter with cut-off frequencies of 0.05 Hz and 5 Hz), downsampled (10 Hz) and normalized to remove between-subject variance in response amplitudes (4). Single subject SCR data were then analyzed using the general linear convolution model (GLM) approach as implemented in PsPM (4). Paralleling the fMRI analysis, the first-level GLM comprised each day as separate session, and event onsets (CS+, CS-, US) for each phase were modeled as stick functions and convolved with a canonical SCR function. The resulting estimates of the SCR amplitude were then analyzed on the group level.

Supplementary References

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