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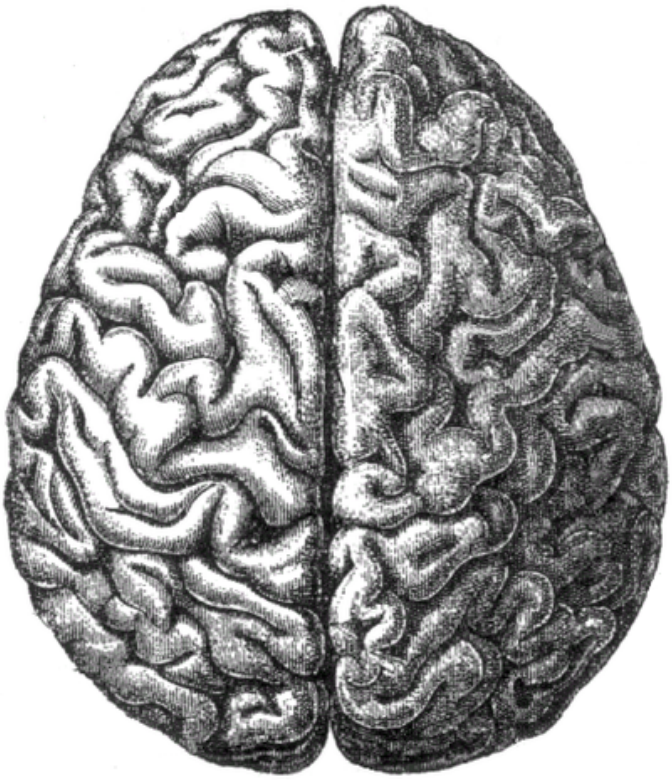
DOPAMINERGIC AUGMENTATION of HUMAN FEAR EXTINCTION



DOPAMINERGIC AUGMENTATION of HUMAN FEAR EXTINCTION

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Abbreviations

e.g. =	exempli gratia (for example)
US =	unconditioned stimulus
UR =	unconditioned response
CS =	conditioned stimulus
CR =	conditioned response
CN =	cortical nucleus
CE =	central nucleus
MEA =	medial nucleus
BLA =	basolateral complex
NMDA =	N-Methyl-D-aspartic acid
DCS=	D-Cycloserine
fMRI =	functional magnetic resonance imaging
SCR =	skin conductance response
RT =	reaction time
mPFC =	medial prefrontal cortex // vmPFC = ventral mPFC
ACC =	anterior cingulate cortex // sgACC = subgenual ACC
PAG =	periaqueductal grey
OFC =	orbitofrontal cortex
IL =	infralimbic cortex
MAPK =	mitogen-activated protein kinase
LTP =	long-term potentiation
CBT =	cognitive behavioural therapy
DSM =	Diagnostic and Statistical Manual of Mental Disorders
PD =	panic disorder
PTSD =	posttraumatic stress disorder
SAD =	social anxiety disorder

GAD =	generalised anxiety disorder
BT =	behaviour therapy
SSRIs =	selective serotonin reuptake inhibitors
SNRIs =	selective noradrenalin reuptake inhibitors
TCAs =	tricyclic antidepressants
MOAIs =	monoamineoxidase Inhibitors
AMPA =	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
cAMP =	cyclo-adenosyl-mono-phosphate
PKA =	cAMP-dependent protein kinase
CREB =	Ca2+/cAMP responsive element binding protein
IEG =	immediate early genes
BDNF =	brain-derived neurotrophic factor
VTA =	ventral tegmental area
DOPA =	dihydroxyphenylalanin
MAO =	monoamineoxidase
COMT =	catechol-o-methyl-transferase
SNP =	single nucleotide polymorphism
L-DOPA =	3,4-dihydroxyphenylalanine (INN: levodopa)
INN =	International Nonproprietary Name
BOLD =	blood-oxygenation-level-dependent
HRF =	haemodynamic response function
SPM8 =	Statistical Parametric Mapping 8
NMI =	Montreal Neurological Institute
DARTEL =	Diffeomorphic Anatomical Registration using Exponentiated Lie algebra
GLM =	general lineal model
FWE =	family wise error
SVC =	small volume correction
ROI =	region of interest
i.e. =	id est (that is)

1. Introduction

1.1 General Introduction

Fear is an emotion that is good for us.

In terms of the emotional experience of fear, this statement does not seem to be true. Everybody experienced emotional states of fear during their lifetime and no one has enjoyed this situation. Nevertheless, fear is an essential part of our emotional sensations (e.g. Öhman et al. 2004) and has crucial influence on our behaviour and thinking in our "emotional brain" (e.g. LeDoux 1998). If we think of our ancestors, we can imagine why fear is so important to us: Fear reactions in dangerous situations were central to the mammalian evolution (Marks 1969, Seligman 1971, Öhman & Mineka 2001). Thus, theories of emotions suggests that evolution shaped a highly developed "fear-system" that helped us to survive by detecting and avoiding (through changed perception, memory and behaviour) situations that could have been perilous (e.g. Öhman & Mineka 2001, LeDoux 2000, LeDoux 2012). Fear is therefore an emotion that is good for us and protects ourselves from dangerous situations.

Moreover, the feeling of fear seems to be a source of information and motivation that helps us reflect upon threatening situations and our own behaviour and to develop successful coping strategies (Epstein 1972). Even from philosophical a perspective, emotions (as fear) can be seen as intrinsic motivations that can non-inferentially adjust judgements and integrate our emotional experience into reasoning (Döring 2007, Döring 2009).

Besides all these adaptive functions, problems emerge if the fear-system does not work properly. Outside homeostatic processes, fear looses its protective properties, but still influences physiological, behavioural and cognitive processes (Rosen & (Rosen & Schulkin 1998, Öhman 2000). So, today, anxiety disorders have the highest prevalence of mental disorders in the USA and Western Europe (Alonso et al. 2004, Kessler et al. 2005). The persistence of fear memories often diminishes effects of psychotherapeutical treatment and leads to relapse. Pharmacotherapeutical options are only symptomatic and none of them augments effects gained during psychotherapy.

In order to investigate the biological underpinnings of anxiety related disorders and their exposure based treatment, laboratory models of classical fear conditioning and extinction have received much interest in the last decades (Milad & Quirk 2012). The translation of neurobiological and pharmacological results has led to new clinical pharmacotherapeutic treatment strategies. The present thesis focuses on learned "safety memories" that inhibit fear and, thus, prevent relapse. In order to extend our knowledge on the neurotransmitters that are involved in safety memory formation and retrieval, this doctoral thesis specifically examines dopaminergic neurotransmission. Two human placebo-controlled randomised pharmacological neuroimaging studies investigate the strengthening of safety memories through enhanced dopaminergic neurotransmission.

1.2 Conditioning

Pavlov discovered classical conditioning in 1927. He rang a bell before he delivered food to a dog. Upon receiving the food, the dog secreted saliva. After a few pairings of the bell and the food, the dog already salivated to the sound of the bell (Pavlov 1927).

This is the concept of classical conditioning:

An unconditioned stimulus (US, the food) evokes an unconditioned response (UR, salivation). After a few pairings of a neutral stimulus (the bell) with the US, the neutral stimulus evokes a response that prepares the organism for the US. Through these pairings, the neutral stimulus becomes the conditioned stimulus (CS, the bell after conditioning), which evokes a conditioned response (CR, salivation). This procedure of conditioning can also be performed with an aversive US rather than an appetitive US. Pairings of a neutral stimulus with an aversive US such as a painful electric shock make the neutral stimulus a CS that evokes a fear CR. This procedure is therefore called classical fear conditioning (Pavlov 1927).

Fear conditioning can be categorised into "cue" and "context" conditioning. In cue conditioning distinct cues as geometric symbols or flashing lights are associated with the US. When the US is not paired to distinct stimuli, the context becomes associated with the US. Cued fear conditioning results in phasic fear responses to the

presentation of the cue CS, whereas the responses to the contextual CS in contextual fear conditioning are more sustained.

Classical conditioning is a form of associative learning, in which a subject learns the prediction of the US through the CS. This association is acquired and then consolidated as a memory. This associative memory is considered a “fear memory” comprising the prediction of the aversive US by the CS. Presentation of the CS retrieves the memory of the US, which leads to the CR. The context where the cue CS is presented and paired with the US is associated with the US and therefore gates retrieval of the fear memory, as well (for review Bouton 2002).

1.3 Extinction

When the CS is no longer paired with the US, the CR slowly declines. This decline (and the procedure itself) is called extinction. This decline suggests two hypotheses: Either the association between the CS and the US is erased or new learning inhibits the association. Behavioural observations after extinction suggest that the latter is true. After extinction, the conditioned fear memory is not deleted, but can still be recalled through CS presentation. Three different forms of this “return of fear” are known:

-Renewal, when the context of CS presentation is different from the context of extinction.

-Reinstatement, when the US is presented alone before CS presentation

-Spontaneous Recovery, when a CS presentation elicits a CR after some time has elapsed since extinction.

These phenomena speak against an erasure of the conditioned memory. Extinction therefore creates another form of associative memory. It is learned and consolidated as a memory of the “CS – no US” association. This memory is thought to inhibit the conditioned “CS-US” memory and is thus expressed as an absent CR. Presentation of the CS leads to retrieval of both: the conditioned fear memory and the extinction memory. The ensuing memory competition leads to either an inhibition of the CR, or a renewal, reinstatement or spontaneous recovery of the CR.

In the case of fear conditioning, the extinction memory has inhibitory effects on the conditioned fear memory. The contextual environment of the CS in extinction learning has an important influence on the extinction memory. Unlike conditioning, extinction learning is context dependent (Bouton 2002). Outside of the extinction context, the fear memory dominates over the inhibitory extinction memory, leading to return of fear (as described above). This return can be seen as a result of the contextual dependency of the extinction memory (Bouton 2004): In the case of renewal, it is obvious, that the context of extinction could not be transferred into the context of CS presentation. Reinstatement leads to a mental "retrieval" of the conditioning context. Return of fear is observed, if the CS then occurs in the reinstated context. In spontaneous recovery, the different time points of extinction learning and CS presentation can be seen as different contexts.

1.4 Neural systems mediating fear extinction

Investigation of the biological systems that mediate fear conditioning and extinction are highly important to understand these basic emotional responses. Furthermore, this basic research enables understanding of disorders of emotional responding, such as anxiety related disorders.

Learning and recall of conditioned fear is mediated through distinct neuronal networks in the human brain (for review, see Sehlmeyer et al. 2009). Extinction of conditioned fear is distributed across neuronal systems, as well. Each of the structures inside the network, however, may contribute to different functions (Quirk & Mueller 2008).

1.4.1 Amygdala

The **amygdala** is one important structure in the acquisition, consolidation and retrieval of conditioned fear as well as in extinction learning (for review Pape & Pare 2010).

The corpus amygdaloideum is located in the medial temporal lobe (see figure I1) and consists of different nuclei, namely the cortical nucleus (CN), the central nucleus (CE), the medial nucleus (MEA) and the basolateral complex (BLA) (Amunts et al. 2005, Solano-Castiella et al. 2010, Trepel 2011).

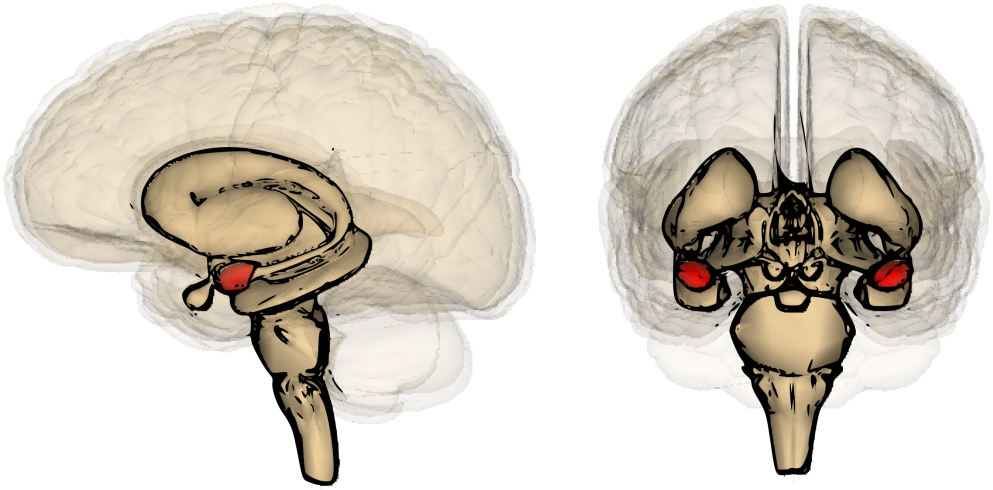

Figure I1. Schematic illustration of the location of the amygdala (red) in the human brain (modified from Wikimedia Commons. No known restriction on publication.

The involvement of the amygdala in the brain networks mediating fear conditioning, fear memory consolidation and fear memory recall is well known from studies in rodents (for review LeDoux 2000). A role of the amygdala in extinction was also found in animal studies (for review Pape & Pare 2010). Herry and colleagues described two distinct neuronal populations in the basal nuclei within the BLA: one encoding for states of fear and another for extinction (Herry et al. 2008). In addition, the amygdala is involved in the consolidation extinction memories.

N-Methyl-D-aspartic acid (NMDA) receptors in the lateral BLA are involved in extinction learning and memory consolidation. Injection of an NMDA antagonist (AP5) into the amygdala was found to impair extinction consolidation (Falls et al. 1992), whereas the partial agonist D-Cycloserine (DCS) facilitated extinction memory consolidation (Davis et al. 2003, Mao et al. 2006). The pathways of NMDA dependent synaptic plasticity in extinction learning are not fully understood, but it is suggested that NMDA receptors in the amygdala are majorly involved (Herry et al. 2010). Besides this, extinction learning leads to induction of the immediate early gene c-fos, which plays a role in memory consolidation (see chapter 1.6 Memory consolidation) in the basal nuclei of the BLA (Herry & Mons 2004).

In translation of these animal findings, studies of fear conditioning acquisition in humans revealed amygdala activity in some studies (for review, see Sehlmeyer et al.

2009), but other metaanalysis found no involvement of the amygdala (Mechias et al. 2010). In contrast to the animal literature, no human imaging study revealed significant activity of the amygdala during recall of fear (Kalisch et al. 2006, Milad et al. 2007, Kalisch et al. 2009, Milad et al. 2009, Spoormaker et al. 2010, Spoormaker et al. 2011).

During extinction learning, neuroimaging studies in humans revealed activity in the amygdala (LaBar et al. 1998, Gottfried & Dolan 2004, Knight et al. 2004, for review, see Sehlmeyer et al. 2009). However, in all of these studies, amygdala activity declined during extinction learning. For example, Phelps and colleagues reported amygdala activation in a human functional magnetic resonance imaging (fMRI) study where this activation was positively correlated to the conditioned responses (measured as skin conductance responses (SCRs), see chapter 3.1 SCR) during acquisition of fear conditioning and extinction learning (Phelps et al. 2004). In the recall of extinction memory 24 hours later, activity of the amygdala was diminished. These results suggest that the amygdala may express a remaining state of fear during extinction learning.

Gottfried and Dolan (2004) extended these findings, revealing different amygdala regions during conditioning and extinction. They estimated increased heamodynamic responses for conditioning and extinction learning in one region of the amygdala. Another region of the amygdala responded exclusively to extinction learning. These different sites of activity might thus reflect two different processes during extinction learning: processing of the conditioned “CS-US” association as well as mediation of new “CS-noUS” memory formation.

In sum, rodent studies describe an important role for the amygdala in extinction learning, and synaptic plasticity after learning. Human studies revealed involvement of the amygdala during extinction learning mostly due to remaining fear processing and only one study implied the amygdala in the processing of a new association that might be related to extinction learning.

1.4.2 Medial Prefrontal Cortex

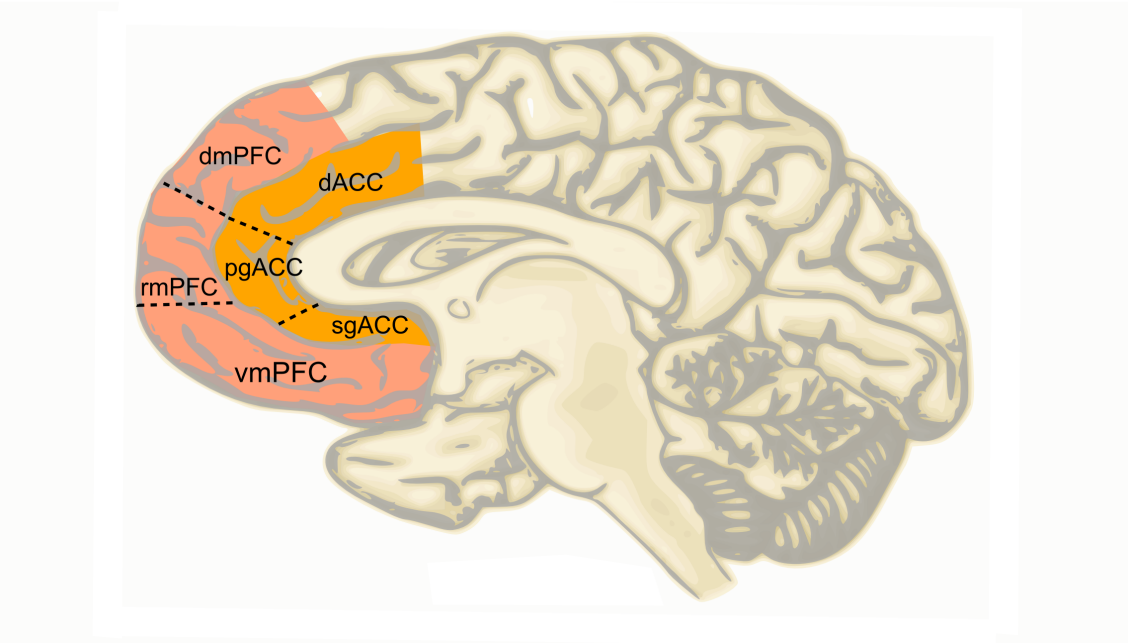

Figure I2. Regions of the anterior cingulate cortex (ACC, orange) and mPFC (red) (own illustration after Etkin et al. (2011). Abbreviations: sg=subgenual, pg=pregenual, d= dorsal, vm=ventromedial, rm=rostromedial, dm=dorsomedial

Another important neural structure in extinction is the **medial prefrontal cortex (mPFC)**. The region occupies the median wall of the (pre)frontal lobes adjacent to the anterior cingulate cortex (ACC). The mPFC is important in regulation of emotional behaviour and is connected to the amygdala, hypothalamus, periaqueductal grey (PAG), orbitofrontal cortex (OFC) and ACC (for review, see Etkin et al. 2011).

Lesions of the mPFC impair fear extinction, but leave fear conditioning intact (Morgan et al. 1993, Morgan & LeDoux 1995). Interestingly, a lesion of only a discrete part of rodent mPFC, the infralimbic cortex (IL), corresponding to the human ventral mPFC (vmPFC) left extinction learning intact, but impaired recall of extinction 24 hours later (Quirk et al. 2000). In line with this, neurons in this region showed CS evoked potentials only during recall of extinction memory, but not during extinction learning (Milad & Quirk 2002). Additionally, the recall of extinction is correlated to neuronal plasticity in the IL (Herry & Garcia 2002).

Inhibition of neuronal plasticity (see chapter 1.6 Memory consolidation) through post-training blockade of NMDA receptors (Burgos-Robles et al. 2007) or mitogen-

activated protein kinase (MAPK) inhibition (Hugues et al. 2004) in the IL impaired recall of extinction memory. These studies consistently showed, that extinction learning leads to extinction memory consolidation events within IL, necessary for the recall of extinction memory.

The inhibitory properties of extinction memory on the conditioned fear memory are paralleled by the projections of the IL to the amygdala. Stimulation of connections from the IL to the amygdala were shown to downregulate activation of amygdala subregions that are associated with fear responses (Quirk et al. 2003, Rosenkranz et al. 2003).

This line of research in rodents suggests that neuronal activity during extinction memory recall in the IL exerts an inhibitory influence on structures necessary for conditioned fear memory recall. in a human fMRI study, Phelps and colleagues revealed activity in mPFC regions during acquisition of conditioned fear, extinction learning and extinction memory recall (Phelps et al. 2004). But only the subgenual anterior cingulate (sgACC), a structure adjacent to the vmPFC (see figure I2), reflected extinction learning. Subjects with reduced conditioned responses (measured as SCR) during extinction learning had less deactivation of the sgACC on the next day during extinction memory recall.

Moreover, the recall of extinction is associated with correlated activity in the vmPFC and the hippocampus (Kalisch et al. 2006, Milad et al. 2007).

A recent review of medial prefrontal areas in the regulation of fear found that the dorsal and ventral ACC (dorsal ACC and ventral ACC) and regions of the dorsal mPFC are involved in extinction learning (Etkin et al. 2011). The authors note, that more dorsal activations in the ACC and mPFC are involved in generating conditioned fear responses as well. These regions might therefore reflect remaining fear processing during extinction learning. In line with this, extinction recall involves only more ventral structures in the ACC and mPFC (Etkin et al. 2011).

In sum, the ventral mFPC and its connections to other regions is an important part of the neural system in consolidation and recall of extinction memory.

1.4.3 Hippocampus

The **hippocampus** is located in the medial temporal lobes. It can be generally separated into ammon's horn and the dentate gyrus. It has a profound role in memory processes as well as spatial orientation and in the processing of contextual environmental information (e.g. Gazzaniga 2004). As such, it is an important structure in the contextual aspects of conditioning and extinction.

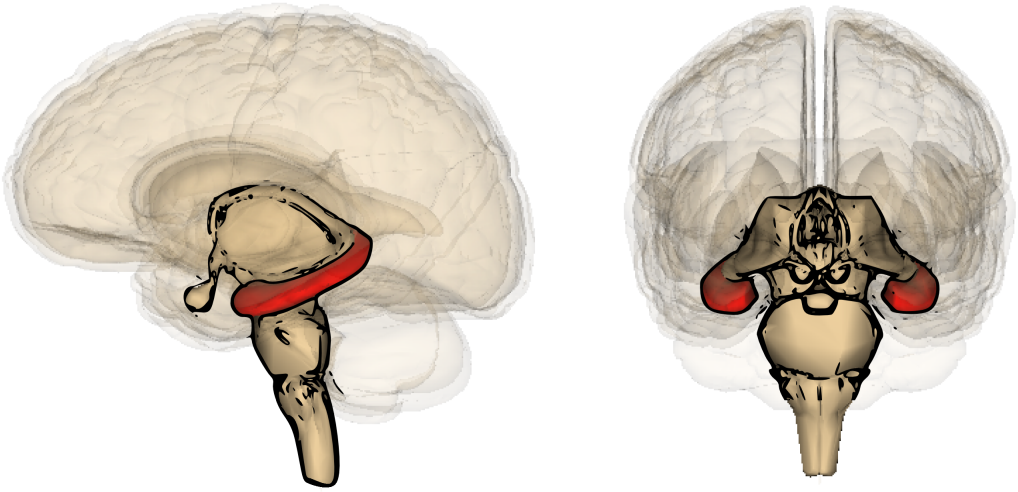

Figure I3 Schematic illustration of the location of the hippocampus (red) in the human brain (modified from Wikimedia Commons. No known restriction on publication).

The hippocampus is known to be involved in contextual fear conditioning in rodents (e.g. Kim & Fanselow 1992) and humans (e.g. Marschner et al. 2008).

In extinction, the hippocampus also has a strong influence on contextual modulation (Bouton et al. 2006). Studies in rodents showed that inactivation of the hippocampus (through the inhibitory ($GABA_A$ agonist) agent Muscimol) before extinction learning led to delayed extinction learning. But more interestingly, this inactivation diminished the return of fear through renewal (Corcoran & Maren 2001). In a subsequent study, Corcoran generated an inactivation of the hippocampus prior to extinction recall and found the same behavioural result (Corcoran et al. 2005). More specifically, inactivation of only the dorsal hippocampus after extinction led to decreased neuronal responses associated with the renewal of fear (Maren & Hobin 2007).

Extinction learning furthermore induced long-term potentiation (LTP, see chapter 1.6 Memory consolidation) in the connections of mPFC with the dorsal (Farinelli et al. 2006) and ventral (Hugues et al. 2006) hippocampus. Following this pattern, impairments of this LTP diminish extinction recall and conversely, facilitation restores it (Farinelli et al. 2006). Maren (2011) supposed that the different hippocampal regions gate either the recall of fear or extinction memory.

The suggested role of the hippocampus for the contextual control of conditioned fear extinction and extinction recall in animals is in agreement with results in humans (Kalisch et al. 2006, Milad et al. 2007, Lang et al. 2009). The human posterior hippocampus corresponds to the rodent's dorsal hippocampus and the human anterior hippocampus is probably homologous to the rodent's ventral hippocampus. Two studies of Kalisch and co-workers observed activity in the posterior hippocampus during the recall of fear memory (Kalisch et al. 2006), (Kalisch et al. 2009). More important, they provided evidence for anterior hippocampal activity during a context specific extinction memory recall. In addition, activation in the ventral mPFC during this recall was correlated with this hippocampal activity (Kalisch et al. 2006). These findings of context depended recall of extinction were replicated by Milad et al. (2007).

In sum, the hippocampus probably mediates the context-dependent influences during extinction recall and plays an important role in the consolidation of extinction in a network involving the mPFC.

1.5 Anxiety related disorders

1.5.1 General

Fear conditioning and extinction are widely used as a model for the aetiology of anxiety related disorders and their exposure based treatment (Milad & Quirk 2012). In order to prepare a potential clinical application of results gained in this doctoral thesis, the parallels of anxiety related disorders and cognitive behavioural therapy (CBT) with the model of fear conditioning and extinction will be discussed in this chapter.

Following the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV (DSM-IV-TR 2000), anxiety disorders include the following:

1) Panic disorder (PD): recurrent, unexpected attacks of multiple somatic and cognitive fear symptoms, which can occur with or without agoraphobia (fear of experiencing panic in situations with no opportunity for escape).
2) Posttraumatic stress disorder (PTSD): intrusive, distressing memories of a traumatic event, avoidance of activities and other cues related to the trauma, and persistent hyperarousal.
3) Social anxiety disorder (SAD): avoidance of social situations owing to fear of negative evaluation.
4) Specific phobias: excessive fear and avoidance of a circumscribed class of objects and/or contexts.
5) Generalised anxiety disorder (GAD): chronic pattern of excessive, uncontrollable worry, muscle tension and related physical features.
6) Obsessive–compulsive disorder: intrusive obsessions and compulsive behaviours.

All anxiety related disorders share exaggerated responding to threat as a common feature of the disorder. Differences between the disorders exist, for example, in the category and the range of objects that the patients respond to.

GAD respond to a broad range of different life events with excessive and uncontrollable worry, together with symptoms of motor tension and vigilance (Craske & Waters 2005).

Patients suffering from PD react to a narrower range of objects and cues with panic attacks. These cues are mostly body sensations (Craske & Waters 2005). Moreover, PD is accompanied by persistent thoughts about symptoms and consequences of these panic attacks. PD can be accompanied by agoraphobia.

Threat responding in phobias is narrowly related only to discrete objects or circumstances. In the case of responding to social cues, the phobia is definded as social phobia or SAD (Craske & Waters 2005). In this disorder, excessive fear is related to social performance situations, where judgments of others could be negative or embarrassing. Although phobias have a narrow threat responding, they can generalise, which includes fear responding not only to distinct cues but related situations in general.

PTSD is generally associated to one distinct experience of a traumatic situation, but the responding to threat in normal life situations can vary. Patients respond with physiological reactions and strong distress to trauma related cues. Moreover, intrusive recollections and dreams of the trauma as well as flashbacks are part of the disorder and patients then show threat responses without any obvious trigger (Keane et al. 2006).

Anxiety related disorders are of high relevance for society and the health system with a lifetime prevalence of 28.8% in the US-American population (Kessler et al. 2005). Woman have a overall higher prevalence of anxiety disorders (e.g. (Pigott 2003), and female gender is a significant predictor of develop an anxiety disorder (odds ration female = 1.6 ; male = 1.0) (Kessler et al. 2005). Phobias have the highest lifetime prevalence among the anxiety disorders, with 12.1% for social phobia and 12.5% for all other phobias. GAD has a prevalence of 5.7% and PD of 4.7%. The lifetime prevalence for PTSD is 6.8% (Kessler et al. 2005). Lifetime prevalence of anxiety disorders in Germany the was estimated at 14.4% (Wittchen et al. 1998) in the last decade. To date, the life time prevalence of anxiety disorders is about 13.6% in Western European countries (Belgium, France, Germany, Italy, the Netherlands and Spain) (Alonso et al. 2004).

1.5.2 Associative learning in acquisition of anxiety related disorders

Risk factors for developing anxiety related disorders are diverse. They include temperament, genetic factors, parental influences and biological corollaries of threat responses, such as cardial vagal tone or anticipatory arousal (Craske & Waters 2005). Besides this, associative learning is thought to play an important role in the acquisition of anxiety related disorders.

One important factor is experiential learning during processes of direct aversive conditioning (Craske & Waters 2005). Rachman and Wolpe (1960) extended this with two additional pathways:

-Vicarious acquisition, through observation of others responding fearfully towards objects or situations

-Informational processing, that is, instructed acquisition of fear towards objects or situations through fear eliciting information

In patients with specific phobias, unpleasant experience with the object of fear is often self-reported. This could support theories of experiential associative learning mechanisms, even though these self-reports are not reliable and biased by the disorder itself (de Jongh et al. 1995). Different variables such as life history, contextual and post-event factors also influence the acquisition of a phobia (Mineka & Zinbarg 1996). In addition, it seems as if individuals with more experience of certain unpleasant or dangerous situations have less risk to develop a phobia, compared to individuals that avoid the unpleasant situation (Mineka & Cook 1986, Craske & Waters 2005). Vicarious acquisition can be a factor for expression of fear in children, as well. For example, one study found that the fearfulness of the child was correlated with the fear that mothers expressed in the presence of their children (Muris et al. 1996).

In social phobias or SAD, the rejection by a social group is an aversive stimulus learned in different social situations and interactions (Craske & Waters 2005). Besides this, mis-appraisal mechanisms and parental influences are thought to have influences on the acquisition of social as well as other phobias (Öhman & Soares 1998).

In PD, the experience of a panic attack itself works as the unconditioned stimulus leading to interoceptive conditioning: Occurrence of an unexpected first panic attack is associatively connected with body sensations before the attack. This mechanism leads to over-interpretation (or mis-appraissal) of normal somatic responses (e.g. a faster heartbeat is interpreted as a sign for a heart attack), which leads to increased fear and increased somatic responses that end in a panic attack, via a self-reinforcing vicious circle (Craske & Waters 2005). Moreover, fear towards body symptoms can be associatively learned through informational processing. Misinterpretation of medical advice or diagnoses can result in enhanced fear-sensitivity or expectancy of fear, which is a risk factor for developing panic disorder (Reiss 1991).

Acquisition of PTSD is thought to be influenced by three factors according to Kean and Barlow (Barlow 2004). This includes pre-existing psychological variables, biological variables and the experience of a trauma. The mechanisms of associative learning in the traumatic experience are of importance in the development of the disorder (Keane & Kaloupek 1982, Friedman et al. 2010).

In sum, mechanisms of associative learning during traumatic and unpleasant events play a role in the acquisition of an anxiety related disorder. Different factors modulate and moderate the onset of these disorders and enhance or diminish the individual risk of developing anxiety disorders. In addition, the uncontrollable recall of traumatic

events presumably reflects the disinhibited aversive memory (Elzinga & Bremner 2002).

Fear conditioning as a form of associative learning that leads to an aversive memory is therefore a useful and valid model for mechanisms in the acquisition of anxiety related disorders.

1.5.3 Treatment of anxiety related disorders

1.5.3.1 Cognitive-behavioural therapy (CBT) and extinction learning

CBT is a form of psychotherapy that uses exposure-based elements as well as methods of cognitive restructuring to change maladjusted behaviour.

CBT is the major treatment in anxiety related disorders and has proven efficacy in PD (e.g. Clum et al. 1993), GAD (e.g. Stanley et al. 2009), phobias (e.g. Ost et al. 2001) including social phobia (e.g. Feske & Chambless 1995) as well as PTSD (e.g. Foa et al. 1999). However, reviews of placebo-controlled studies showed there is room for improvement in the effects of exposure-based therapies (e.g. Hofmann 2007).

In the mid 1950s, behaviour therapy (BT) started to emerge. BT has it roots in Pavlov's –aforementioned- work (Pavlov 1927, Rachman 2009), which revealed that the effects of the exposure to conditioned stimuli in the absence of the US, which leaded to extinction of the CR. Wolpe confirmed in animal studies, that fear could be reduced through repetitive exposure to the fear eliciting stimulus (for review Shin & Liberzon 2010). He consequently treated patients with an analogous approach: He exposed patients to mental images of their fear (Wolpe 1958). This was later extended to exposure to actual objects or situations of fear (Rachman 2009).

Cognitive therapy developed in the 1960s, pioneered by Beck and Ellis who proposed that psychological disturbances mostly arise from maladapted cognitive processing. They regarded behavioural therapy as the gathering of new, corrective information about the fear stimulus or situations, which then leads to a change in behaviour. This stood in contrast to behaviourist thinking, which held that the change in behaviour during exposure is in itself the key to achieve a therapeutic effect.

The synthesis of behavioural and cognitive aspects into one form of therapy emerged in the 1980s. Cognitive-behavioural therapy (CBT) combines behavioural exposure elements and cognitive restructuring. CBT in the treatment of anxiety disorders is based on new learning processes: Patients experience the object or situation of fear and break through the vicious circle of avoidance. In addition, patients experience their own weakening fear response, which is based behaviourally on extinction of conditioned fear. Moreover, these parallel mechanisms of extinction and exposure based therapies (as CBT) already imply the sources of relapse: renewal, reinstatement and spontaneous recovery (Bouton 2002). The relapse after successful therapy is a major problem in anxiety disorders. A longitudinal study reported reoccurring fear symptoms in over 50% of the patients in 2 to 14 years after successful therapy (Durham et al. 2005). Furthermore, this return of fear was not predicted by the initial success of therapy.

1.5.3.2 Neuroimaging of CBT and extinction

The neural systems mediating extinction in healthy volunteers have been described above. If CBT and extinction share common learning mechanisms, the question is whether they rely on the same biological processes, as well.

Already healthy volunteers with high trait anxiety have a diminished connectivity of structures that have been implicated in extinction memory recall (see chapter 1.4 Neural systems mediating fear extinction), namely ventral mPFC and the hippocampus during experimental fear conditioning and extinction (Indovina et al. 2011).

A meta-analysis of neuroimaging studies of negative emotional processing in anxiety related disorders revealed increased activity in brain regions known from fear conditioning (e.g. (Etkin & Wager 2007, Engel et al. 2009)). Interestingly, the authors noticed decreased activity in the mPFC in patients with anxiety related disorders. Furthermore, Milad and co-workers showed that this decreased activation in patients is accompanied with impairments of extinction memory recall for conditioned cues (Milad et al. 2009) and contexts (Rougemont-Bücking et al. 2011). In addition, a meta-analysis of neuroimaging effects of CBT (contrasting before and after therapy) in anxiety related disorders revealed decreased activity in brain regions known from fear conditioning and an increase in activity in the mPFC (Porto et al. 2009).

A recent review concluded that the insights gained about neurobiological systems of extinction are related to the neurobiology of anxiety related disorders and its treatment (Milad & Quirk 2012).

In sum, neuroimaging of extinction in healthy volunteers reveals neurobiological systems and mechanisms related to dysfunction in anxiety related disorders and effects of CBT. The research on neurobiological systems of extinction therefore has strong implications on the neurobiological understanding of exposure-based therapies and anxiety related disorders.

1.5.3.3 Pharmacological therapy of anxiety disorders

Different classes of drugs are used in the treatment of anxiety related disorders, in general. These drugs include antidepressants such as selective serotonin reuptake inhibitors (SSRIs), selective noradrenalin reuptake inhibitors (SNRIs) or tricyclic antidepressants (TCAs) in the first-line treatment.

Guidelines and expert reviews prefer non-drug treatment in PTSD patients, because there is no evidence for a strong efficacy of medication (Alderman et al. 2009, Stein et al. 2009). Nevertheless, Stein and co-workers (2009) reviewed SSRIs as the first choice in pharmacological treatment of PTSD (Stein et al. 2009), in line with other reviews (Keane et al. 2006, Bandelow et al. 2008, Janicak et al. 2010). Other options include anticonvulsants (e.g. lamotrigine, carbamazepine, topiramate, and valproic acid) that reduce symptoms in patients with PTSD (Keane et al. 2006, Mula et al. 2007). But still, these reviews advise further research. Benzodiazepines seem to have no effect in PTSD (Nutt 2005, Bandelow et al. 2008).

While patients with PD benefit from various medications, psychotherapy (alone or in combination with pharmacotherapy) has comparable effects (Hofmann & Smits 2008). One third of patients with PD that stop psycho- or pharmacotherapy relapse within two years (Yonkers et al. 2003). In line with this, SSRIs are effective in preventing acute panic attacks, but may not alter mechanisms of anxiety and fear (Janicak et al. 2010) but see (Karpova et al. 2011). Nevertheless, SSRIs are recommended as the first-line drug treatment in the American Psychiatric Association guidelines (Baldwin & Birtwistle 1998, Practice guideline for the treatment of patients with panic disorder (2nd Edition) 2009). Studies of TCAs in treatment of PD revealed anti-panic effects

(Lydiard & Ballenger 1987, Schweizer et al. 1993), but symptoms reoccurred when medication was stopped; moreover, the therapeutic gain was lost (Liebowitz 1997). Furthermore, anti-cholinergic side effects of TCAs can lead to bodily sensations similar to panic symptoms (Noyes et al. 1989, Janicak et al. 2010). Monoamineoxidase Inhibitors (MOAIs) are anti-panic agents with benefits compared to placebo, but again no substantial benefit after discontinuation of medication (Janicak et al. 2010). Benzodiazepines showed beneficial effects in patients with PD (Janicak et al. 2010). However, administered in patients with panic disorder, benzodiazepines have a smaller effect-size than antidepressants and high drop-out rates, due to their side effects (Gould et al. 1995).

Guidelines recommend SSRIs in the first-line treatment of GAD, with evidence in different placebo-controlled clinical trials (Bandelow et al. 2008). Other antidepressants such as SNRIs or TCAs showed superior effects compared to placebo in GAD patients, but with reduced evidence compared to SSRIs (Bandelow et al. 2008). Benzodiazepines reduce anxiety in GAD patients (Janicak et al. 2010), but limitations in their prescription time, due to addictive properties, prevent them from being used as a chronic treatment. Pregabaline and Quetiapine showed evident efficiency in GAD patients, nevertheless both drugs were not superior to antidepressant treatment (Bandelow et al. 2008, Mula et al. 2007).

Specific phobias are preferably treated with exposure therapy (Janicak et al. 2010), due to lacking evidence for pharmacotherapeutic effects (Zitrin et al. 1983). In contrast, evident effectiveness for SSRIs has been revealed in different meta-analyses for social phobia (Blanco et al. 2003, Hedges et al. 2007), making them the first-line treatment (Bandelow et al. 2008). Other options are SNRIs and MAOIs, but the latter have more common interactions with food and reduced evidence for effective treamtent (Janicak et al. 2010). Given that the onset of social phobias is early in life, the risk of suicide as a side-effect is important to consider in pharmacotherapy with SSRIs (March et al. 2007). Anticonvulsants are not the first choice in treatment, but Pregabalin has been shown to be effective in patients with social anxiety disorder (Mula et al. 2007). Symptomatic relief through administration of beta-blockers is seen with scepticism, due to lacking evidence of efficacy in treatment of social phobia (Davidson 2006).

In summary, there are pharmacotherapeutic options in the treatment of anxiety related disorders. Mainly SSRIs reveal good evidence for effects in a wide range of anxiety related disorders (Bandelow et al. 2008). But still, this evaluation of evidence focused

on short-term outcomes and there is less evidence for the prevention of relapse, which should be important for a rational treatment (see Durham et al. (2005). For example, side effects influence the compliance of pharmacotherapy, thus are important to consider in the evaluation of effective long-term treatment.

Atypical antipsychotics, for example, were reported as an effective treatment for a variety of anxiety disorders in pilot studies. A recent metaanalysis, however reviewed them negatively due to the abundance of effects in trials comparing them against standard therapy and high drop out rates because of side effects (Vulink et al. 2011).

The long-term outcomes of pharmacotherapy point towards no lasting benefit after discontinuation. Furthermore, augmentative effects of combined pharmacotherapy and CBT are inconsistent (Bandelow et al. 2008, Foa et al. 2002). In addition, in specific phobias, PTSD and PD, there seems to be no augmentative pharmacotherapeutic options for psychotherapy. Many recent reviews therefore demand novel strategies of pharmacotherapeutic research in the treatment of anxiety related disorders. These should try to address the problems of pharmacotherapeutic treatment resistance and relapse after CBT (Hofmann 2007, Janicak et al. 2010, Ganasen et al. 2010, (Ravindran & Stein 2010). Much hope is currently placed in treatment strategies arising from translational research (Hofmann et al. 2006, Davis et al. 2006). As one example, the partial NMDA receptor agonist DCS was found to enhance extinction memory consolidation in rodents (Walker et al. 2002, Ledgerwood et al. 2003) and to enhance the effects of CBT in patients with phobia (Ressler et al. 2004) and other anxiety disorders (e.g. Panic disorder (Otto et al. 2010), PTSD (de Kleine et al. 2012), SAD (Hofmann et al. 2006), for metaanalysis Bontempo et al. (2012)). However, DCS therapy has limitations, because it may also affect the processing of aversive events (Kalisch et al. 2009). On the one hand, the consolidation of this aversive memory could be enhanced, while under DCS (for example a car accident after leaving the CBT session). On the other hand, an aversive event leads to high levels of glutamate at NMDA-receptors and, in turn, the partial agonistic properties of DCS decreases NMDA receptor transmission (e.g. Davis et al. 2006), which could diminish the effect of DCS on exposure-based therapy (Langton & Richardson 2010, Hofmann et al. 2011).

Furthermore, DCS seems to have no influence at higher cognitive levels in extinction therapy, which could be disadvantageous in more cognitive based therapies (Grillon 2009).

Another candidate resulting from translation of research of emotional memory consolidation in animals (Roozendaal 2000) is hydrocortisol. Acute administration during CBT diminished fear responses during exposure (Soravia et al. 2006), but more importantly, enhanced CBT effects in the follow up after one month (de Quervain et al. 2011). Despite these encouraging results, more studies still have to be done in order to evaluate and improve new pharmacotherapeutic strategies of anxiety disorders.

1.6 Memory consolidation

The consolidation of emotional memories is a key aspect in this doctoral thesis. Therefore, this chapter conceptualises the important steps during memory consolidation with a focus on extinction memories.

The acquisition of an association (e.g. fear extinction) takes place within seconds, which directly induces memory formation (Rogan et al. 1997, Izquierdo & McGaugh 2000). Consolidation refers to the transfer of a labile memory into a (more) stable state after learning within a time-window of several hours (e.g. Bliss & Collingridge 1993). One neuronal correlate (beside others) of this phenomenon of learning and a stable memory is LTP.

1.6.1 LTP

Memory can be viewed as a lasting change in synaptic efficiency. Cajal proposed that neurons are not in cytoplasmic continuity and could communicate with each other (Cajal 1928). This communication is expressed as spatio-temporal neural activity patterns, which themselves cause changes in synaptic efficiency. Hebb and Konoski observed that a synapse that connects two cells gets strengthened if the cells are active at the same time (Konorski 1948, Hebb 1949). Furthermore, brief high frequent stimulation of monosynaptically linked excitatory cells resulted in a sustained increase of synaptic efficiency, an effect that is termed LTP and was for the first time observed in the hippocampus (Bliss & Gardner-Medwin 1973, Bliss & Lomo 1973).

The induction of LTP is important in the formation of memory traces, leading to stabilised memory, which can be recalled behaviourally after learning (Bliss & Collingridge 1993). LTP is accompanied by cascades of molecular events on the cellular level which in their entirety build up the recallable memory (e.g. Bliss & Collingridge 1993, Izquierdo & McGaugh 2000). LTP is a major aspect in the consolidation of extinction memories where it has been observed in the amygdala (e.g. Rogan et al. 1997) the hippocampus (e.g. Hugues et al. 2006, Farinelli et al. 2006) and the mPFC (e.g. Herry & Garcia 2002).

1.6.2 Cellular steps in extinction memory consolidation

At the molecular level, a first step in the consolidation of a newly formed memory is the activation of glutamate receptors, namely: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), metabotropic and in particular NMDA receptors. Extinction learning activates NMDA receptors in the amygdala, prefrontal cortex and the hippocampus. Subsequently, NMDA receptor activation stimulates the cyclo-adenosyl-mono-phosphate (cAMP)-dependent protein kinase (PKA) and the MAPK. PKA and MAPK were revealed to be involved in both the consolidation of fear (Brambilla et al. 1997, Huang et al. 2000) and extinction memories (Davis 2002, Orsini & Maren 2012). Activated MAPK triggers the phosphorylation of transcription factors such as Ca^{2+}/cAMP responsive element binding protein (CREB), thus regulating the expression of proteins that are important for long-term memory formation (Silva et al. 1998). Again, the phosphorylation of CREB was found to be necessary for both fear (Bourtchuladze et al. 1994) and extinction memory consolidation (Mamiya et al. 2009, Herry & Mons 2004).

The observation of LTP and of the activation of molecular consolidation events are in agreement with the behavioural observation described earlier that extinction does not erase the fear memory but instead generates a new, inhibitory memory trace. Interestingly, however, extinction can also reduce CREB phosphorylation (through enhanced levels of calcineurin) (Lin et al. 2003). This dephosphorylation is observed in the context of depotentiation of fear-responsive neurons through extinction, that is reversal of the LTP induced through fear conditioning (Kim et al. 2007, Hong et al. 2011, for review Orsini & Maren 2012). One could interpret this as a reversal or erasure of fear conditioning. However, a recent review of fear extinction consolidation

suggested this might also reflect a redistribution of the fear memory (Orsini & Maren 2012).

Extinction learning was also found to induce CREB-mediated gene expression and other immediate early genes (IEG) such as c-fos and zif268 in the amygdala and IL (Mamiya et al. 2009, Herry & Mons 2004). These genes regulate protein synthesis, which is crucial for extinction memory recall (Berman & Dudai 2001, Myers & Davis 2006). One of the regulated genes codes for brain-derived neurotrophic factor (BDNF). An increase of BDNF mRNA in the amygdala is observed in a time window of up to 2h hours after extinction learning (Chhatwal et al. 2006). Interestingly, BDNF was shown to facilitate extinction memory recall when infused into the IL in rats and to reduce fear memory recall even without intervening extinction training (Peters et al. 2010). In addition, studies of genetic variants of the pro-domain in the human BDNF gene (BDNFval66met) could reveal altered associative learning during fear conditioning and extinction (Lonsdorf et al. 2010).

In sum, extinction learning is followed by consolidation of the extinction memory. This process involves molecular cascades that lead to a stable and recallable memory. Consequently, changes on the transmitter or second messenger level may affect this process, as will be shown for dopaminergic transmission.

1.7 Dopamine

1.7.1 Dopamine in the human body and brain

Dopamine in the central nervous system is distributed in three major pathways (e.g. Bentivoglio & Morelli 2005):

-The nigro-striatal pathway cell bodies lie in the substantia nigra and axons terminate in the corpus striatum. This pathway accounts for 75% of dopamine in the human brain.

-The mesolimbic/mesocortical pathway originates in the ventral tegmental area (VTA) and projects to the nucleus accumbens in the ventral striatum, to the amygdala and to frontal cortical regions.

-The tubero-hypophyseal pathway consists of short neurons running from the ventral hypothalamus to the median eminence and pituitary gland, regulating secretion of prolactin, for example.

Dopamine is synthesised through the vicinal hydroxylation of the aminoacid tyrosine, catalyzed by tyrosine hydroxylase. The product, dihydroxyphenylalanin (DOPA), is decarboxylated to dopamine through DOPA decarboxylase. Dopamine is inactivated like all catecholamines in the synaptic cleft through a specific dopamine transporter, a Na+ cotransporter. Metabolising steps are the oxidation of the primary amine to an aldehyde and oxidation of the aldehyde to dihydroxyphenylacetic acid through monoamineoxidase (MAO), located on the surface of the mitochondria in the axon. If dopamine or dihydroxyphenylacetic acid is transported into the glia, catechol-o-methyl-transferase (COMT) catabolises the methylation of the meta-hydroxy-group to the ethylamine side chain leading to homovanillinic acid or metoxythyramine.

Dopamine receptors are G-protein coupled receptors of two different classes. Activation of receptors of the D1 class, containing the D1 and D5 receptor subtypes, stimulates adenylyl cyclase. This activates the synthesis of cAMP. Activation of receptors of the D2 class, containing the D2, D3 and D4 receptor subtypes, by contrast, decreases cAMP levels. All dopamine receptors can be located on the post-synaptic side of the synaptic cleft, but only D2 receptors can also be found on the pre-synaptic side where they function as autoreceptors, inhibiting the presynaptic release of dopamine.

The three major dopaminergic pathways are involved in transmission of important functions of the central nervous system: motor control, behavioural functions and endocrine control. The behavioural functions of the mesolimbic/mesocortical pathway have received much interest, in particular its role in learning and motivation. The model of appetitive conditioning revealed dopaminergic influences in this domain of behaviour.

1.7.2 Dopamine in appetitive conditioning and motivational control

Dopaminergic neurons transmit in two different modes: a ‘‘tonic’’ and a ‘‘phasic’’ mode (Grace 1991, Grace et al. 2007). Tonic dopaminergic transmission generates a steady level of dopamine, enabling the normal functioning of dopaminergic neural circuits

(Schultz 2007). In phasic transmission, dopamine neurons sharply increase or decrease their firing rates for 100–500 ms, causing large changes in dopamine concentrations in target structures that last for several seconds (Schultz 1998).

Schultz and colleagues (Schultz et al. 1997, Schultz 1998) found this phasic dopaminergic firing of dopaminergic cells to unexpected rewards as well as to reward predicting stimuli. Furthermore, neurons did not fire when an expected reward failed to occur. This observation fits into prediction error-based learning models in animals and humans (Schultz 1998, Fiorillo et al. 2003, D'Ardenne et al. 2008). Put simply, the basic idea of those models is that learning results in the formation of a prediction of an event (US) based on the occurrence of a signalling stimulus (CS). If a US occurs unpredictably or if an established US prediction is violated (e.g., because the expected US does not follow the CS), a prediction error occurs and the prediction for the next CS presentation is adjusted. An unexpected CS that has been established as a good US predictor also generates a prediction error, just like an unexpected US itself. This prediction error-based model for classical conditioning was first proposed by Wagner und Rescorla (1972) and later extended by (Schultz et al. 1997, Sutton & Barto 1998 to the temporal difference learning model. There is now evidence from animal studies that phasic dopamine release in the ventral striatum encodes reward prediction errors (Schultz 2006). In humans, striatal neural prediction error signals can be modulated by dopaminergic drugs (Pessiglione et al. 2006).

Recently, Matsumoto and Hikosaka (2009) found a population of neurons in the monkey midbrain that responded to both aversive and appetitive conditioned stimuli in a manner compatible with prediction error coding. New theories of dopaminergic neuronal coding of outcome prediction involve appetitive, aversive and alerting stimuli (integrating them to signals of motivational outcome and salience) (e.g Bromberg-Martin et al. (2010) for review).

1.7.3 Dopamine in fear conditioning and fear memory consolidation

The influence of dopaminergic transmission during fear conditioning was revealed through different pharmacological animal studies (Pezze & Feldon 2004). In this review, the authors reported that a general increase of dopaminergic transmission as well as D1 receptor agonism were followed by enlarged fear responses in contrast to activation of D2 receptors which led to inhibition of fear expression. Subsequent

studies replicated these findings for dopaminergic transmission in the BLA (de la Mora et al. 2010) and VTA (de Oliveira et al. 2011).

Pezze and Feldon also highlighted the importance of dopaminergic signals in the mPFC and nucleus accumbens during the acquisition of fear conditioning. Besides these effects on acute learning, they suggested dopaminergic modulation of fear memory consolidation as well (Pezze & Feldon 2004). However, there is no direct evidence so far that dopamiergic transmission during fear conditioning explicitly follow the proposed model of prediction error signalling.

Human neuroimaging studies show that brain activation time courses are in accord with prediction error-based learning models in fear conditioning acquisition. Different studies revealed activity in the ventral striatum as an indirect indication of dopaminergic influence in the acquisition of fear conditioning (Seymour et al. 2004, Gläscher & Büchel 2005, Li et al. 2011), in accordance with a review of the striatal influence on fear conditioning and aversive learning (Delgado et al. 2008). Interestingly, Klucken and colleges found heamodynamic responses in the ventral striatum in subjects that learned the aversive prediction of the CS in contrast to unaware or informed subjects (Klucken et al. 2009).

An fMRI study analyzing brain activation during fear conditioning with a prediction error-based model (temporal differences) and administering the dopaminergic enhancer amphetamine, the D2 receptor antagonist Haloperidol or placebo, found dopaminergic modulation of activity in the ventral striatum (Menon et al. 2007). Amphetamine enhanced prediction error related activity in the ventral striatum (for both, the CS+ and CS-) compared to placebo and Haloperidol. However, the administration of Amphetamine and Haloperidol interferes with general attention, and the results might therefore reflect general attentional effects rather than learning-specific changes. Furthermore, Haloperidol and Amphetamine change local blood flow (e.g. Lavyne et al. 1977, Corson et al. 2002), leading to changes in the heamodynamic responses that may impact task-related signals.

The prediction error based analysis of fear conditioning imaging data reveals brain regions that overlap with appetitive associative learning, however only indirect evidence for a dopaminergic influence on these processes has been shown. Involvement of the dopaminergic system in fear conditioning, that is evident in rodents, is therefore only speculative in humans.

Concerning memory consolidation, dopamine is known to play an important role in the promotion of LTP induction. Studies by Frey and co-workers could show that blockade of dopamine D1 transmission impaired LTP in the hippocampus (Frey et al. 1990). Electrophysiological studies revealed that stimulation of the nucleus accumbens modulated LTP induction in the hippocampus (López et al. 2008). More specifically, dopaminergic blockade in the nucleus accumbens impaired LTP in the dentate gyrus (Kudolo et al. 2010). Moreover, this influence of dopaminergic midbrain signalling on hippocampal memory induction was in agreement with results from a human neuroimaging study (Wittmann et al. 2005).

Modulation of fear memory consolidation through a dopaminergic manipulation was elegantly demonstrated in genetically dopamine-depleted mice. Only restoration of dopamine transmission directly after fear conditioning permitted fear memory recall, suggesting a dopaminergic influence on memory consolidation (Fadok et al. 2009). A recent study by Zweifel and co-workers revealed NMDA receptors on dopaminergic neurons to be necessary for the consolidation of the CS-US association and for the expression of CS-associated fear (Zweifel et al. 2011). In addition, a study by Fadok could show that dopaminergic transmission in the nucleus accumbens and the amygdala is necessary for long-term fear memory (Fadok et al. 2010). In line with this, dopamine gates LTP induction in the amygdala after fear conditioning in animals (Bissière et al. 2003).

There are no human pharmacological studies looking at the influence of dopamine on fear consolidation, but genetic analyses give some first hints.

For example, a functional COMT single nucleotide polymorphism (SNP) leads to substitution of valine by methionine in codon 158 (COMTval158met), resulting in four times lower activity of COMT in carriers of the met/met genotype (Männistö & Kaakkola 1999). The activity of COMT is relevant for dopaminergic catabolism and, due to its distribution (Matsumoto et al. 2003), for prefrontal dopaminergic functions in humans (Egan et al. 2001). It could be shown, that this COMTval158met SNP has implications for fear conditioning and extinction in humans and patients with anxiety related disorders. Carriers of the met/met genotype showed impaired extinction learning behaviourally, in an experimental study (Lonsdorf et al. 2009) and less symptom relief in patients with panic disorder during CBT (Lonsdorf et al. 2010), compared to val allele carriers. A recent review by (Lonsdorf & Kalisch 2011) of genetic influences in fear conditioning and extinction suggested that these effects of lower metabolism in the met allele carriers contribute to a stronger fear memory

consolidation. This would explain that met/met carriers show unaffected immediate extinction learning following upon fear conditionig (that is, without an intervening consolidation phase) (Raczka et al. 2011), but were impaired when extinction learning was conducted 24 hours later (Lonsdorf et al. 2009).

In sum, the animal studies provide evidence for a dopaminergic influence on the consolidation of fear memories. Human studies used genetic methods and therefore give only indirect evidence for a dopaminergic influence on fear consolidation.

1.7.4 Dopamine in extinction and extinction memory consolidation

There is comparatively less known about the potential influence of dopamine neurotransmission in extinction and extinction memory consolidation.

Early studies of dopaminergic involvement in fear extinction administered drugs of abuse such as Cocaine (Willick & Kokkinidis 1995) or Amphetamine (Borowski & Kokkinidis 1998), which enhance extracellular dopamine levels, during the learning of extinction and observed deficits in learning and recall of extinction memories. Further evidence of this dopaminergic influence came from studies with the specific D1 dopamine receptor agonist SFK 38393 (Borowski & Kokkinidis 1998) and the D2 receptor agonist Quinpirole (Nader & LeDoux 1999), replicating these findings. In line with this, systemic dopaminergic D2 antagonism through Sulpiride could be shown to facilitate extinction recall, when administered at the beginning of extinction learning (Ponnusamy et al. 2005).

All these studies manipulated the extinction learning phase, thus providing limited information on extinction consolidation. In addition, results were most likely confounded by the drugs' main and side effects (majorly locomotion) (e.g. Adams et al. 2001, Wood & Anagnostaras 2009). All studies measured fear responding as freezing, that is complete immobility, which is clearly influenced by locomotion. Furthermore, the testing phase (in all studies 24 hours after drug administration) was considered to be drug free, which hardly can be true if Quinpirole has a half-life of 9.5 hours in rats (Whitaker & Lindstrom 1987) and Amphetamine has a half-life of 5-9 hours in rats (Kuhn & Schanberg 1978).

Recent studies that tried to account for these effects failed to replicate the results of Borowski & Kokkinidis (1998). Amphetamine administration during extinction learning

showed no effects on the recall of extinction two days (drug-free) after administration (Mueller et al. 2009) or with dosages that were devoid of locomotor side effects (Carmack et al. 2010).

In the same vein, one recent study by Mueller et al. (2010) tried to control for the cataleptic side effects of a dopaminergic D2 antagonist (Raclopride) during extinction learning. The authors used reduced dosages in systemic administration or microinjection directly into the infralimbic cortex. Both administration routes did not change extinction learning, but the recall of extinction memory was deficient after infralimic injection (Mueller et al. 2010). This study conforms to a former study, that used microinjections of a dopamine D4 receptor antagonist (L-741) into the IL, and revealed the same effect (Pfeiffer & Fendt 2006). These studies imply that dopaminergic antagonism might impair the consolidation of extinction memories.

In line with this, intracerebroventricular administration or microinjection into the nucleus accumbens of Haloperidol administered before extinction learning impairs the recall of the extinction memory, tested 48 hours after learning (Holtzman-Assif et al. 2010), while not affecting extinction learning itself. The authors concluded that dopamine transmission in the nucleus accumbens during extinction learning is critical for the later recall of the extinction memory. They suggested that this dopaminergic transmission is due to prediction error signalling (see above) in extinction, signalling the unexpected omission of the US (Dickinson 1980,Rescorla 1988).

Further evidence comes from a very recent study that administered the combined dopamine and noradrenalin transporter inhibitor Methylphenidate directly after extinction learning (Abraham et al. 2012). The recall of contextual extinction memories tested up to three days after learning was enhanced. Interestingly, this effect disappeared if methylphenidate was administered 4 hours after extinction learning, suggesting there is a critical time window for dopaminergic effects on extinction consolidation. Together with the previous studies, this study provides evidence for a possible augmenting influence of dopaminergic agonism on extinction memory consolidation in animals.

Human studies of genetic polymorphisms are the only studies that give an insight into the dopaminergic influences on extinction learning.

A study in our group investigated effects of a polymorphism in the dopamine transporter (DAT) gene on extinction learning (Raczka et al. 2011). The shorter 9-tandem repeat allele (9R) of the 40 base-pairs long 3`-untranslated region in the DAT

gene is presumably associated with reduced DAT expression in the striatum (Fuke et al. 2001, VanNess et al. 2005, Heinz et al. 2000, but there are contradictory findings (Jacobsen et al. 2000) and studies revealing no differences (Mill et al. 2005)). Theoretical models of DAT function predict this should be coupled with amplified phasic dopamine signalling (Cragg & Rice 2004). The analyses of Raczka et al. (2011) were based on the idea that unexpected US omission during extinction corresponds to a positive surprise or unexpected reward and should therefore generate reward-type, dopamine-mediated prediction error signals in the ventral striatum. The study revealed that 9R carriers learned extinction more quickly compared to homozygous carriers of the 10R allele. In the fMRI analysis, the ventral striatum showed prediction error-related activation specifically during extinction, which was amplified in 9R carriers. This study thus suggests that a dopaminergic genetic polymorphism influences prediction error-based learning of extinction in a dominant dopaminergic brain region.

In sum, animal data suggests an influence of dopaminergic neurotransmission in the consolidation of extinction memory, but direct evidence is still missing. One human study showed the involvement of genetic polymorphisms in the dopaminergic system in prediction error based extinction learning. Nevertheless, these results suggest that dopaminergic neurotransmission in the phase of extinction consolidation may be a pharmacological target in the augmentation of extinction memory.

2. Study design

The aim of this doctoral thesis is to evaluate the influence of dopaminergic agonism during the consolidation phase of the extinction memory on extinction memory recall.

To induce global changes in dopaminergic transmission, the L-isomere of 3,4-dihydroxyphenylalanine (L-DOPA, International Nonproprietary Name (INN): levodopa) was chosen as the study drug. L-DOPA is a prodrug that is decarboxylated through DOPA-decarboxylase to dopamine. Combination with a peripheral DOPA-decarboxylase inhibitor (Benserazide) avoids peripheral side effects and enriches the active drug in the brain. L-DOPA passes the blood-brain barrier and enriches after decarboxylation majorly in the terminals of nigrostriatal dopaminergic neurons (Kumakura & Cumming 2009), where it leads to higher levels of extracellular dopamine (Rodríguez et al. 2007) . Peak level concentration after oral administration are expected in the brain after 70-90 minutes (Olanow et al. 1991, Fachinformation MADOPAR(12/2009) . Furthermore, the half-life of this drug is short (1,5h (Brunton et al. 2007, Fachinformation MADOPAR(12/2009)) and subjects can be tested 24 hours later without any acute effects of the drug. To avoid delayed absorption of the drug, the subjects were in fating state for at least 90 minutes before drug intake (Nutt & Fellman 1984, Fachinformation MADOPAR(12/2009)). L-DOPA or placebo were administered in a double-blind fashion using a randomised, parallel (between-subject) design.

Two studies were conducted with behavioural paradigms appropriate for testing the return of fear or, in other words, failed extinction memory recall.

The paradigm in Study A incorporated cue and contextual conditioning. It was conducted in three phases: first fear conditioning (based on a study by Marschner et al. 2008), followed by extinction learning (24 hours later) and subsequent drug or placebo administration. The third phase (7-8 days after conditioning) tested for spontaneous recovery and reinstatement of the cue and contextual conditioned fear memories.

The paradigm in Study B consisted of context dependent differential cue conditioning and extinction learning. The study was conducted in two phases (separated by 24 hours) and is a modification of the paradigm used before by Kalisch et al. (2006). The

first phase consisted of fear conditioning and extinction learning, followed by administration of L-DOPA or placebo. In the second phase (24 hours later), the context-dependent renewal of fear could be tested.

As the dependent measurements, subjective rating values, psychophysiological parameters (SCR) and neuronal correlates (fMRI) were analyzed.

These studies were thought to reveal treatment group differences in the return of fear. Importantly, as the drug was administered after learning and presumably washed out before the recall test, any potential effects can only result from the L-DOPA effects on the consolidation phase of the extinction memory. The studies thus tested if enhanced dopaminergic transmission in the consolidation of extinction memories led to reduced return of fear (i.e., a stronger extinction memory recall) in humans.

3. Methods

The following chapter contains a short description of the theoretical background of the methods used within this doctoral thesis, as well as a summary of the methods used in the studies.

3.1 SCR

The SCR is a useful non-invasive measure of the response of the autonomic nervous system to an arousing stimulus. It consists of a phasic change in the galvanic conductivity of the skin.

The human skin is innervated by the autonomic nervous system, which can be divided into the sympathetic and parasympathetic branches. In arousing situations, activation of sympathetic neurons elicits bodily responses. Sympathetic postganglionic neuronal projections regulate the activity of the eccrine sweat glands, which mainly account for galvanically measurable skin responses (Lykken & Venables 1971).

Measurement of this response is easily achieved with two non-polarising Ag/AgCl (3M, Poland) electrodes placed on the palmar side of the hand or the plantar side of the foot. Electrodes can for instance be placed on the index and the middle finger or on the thenar and the hypothenar (or both on the hypothenar). If a constant voltage is applied across these electrodes (limited to 0.5V), changes in the current flow can be measured, which are linear to the changes in the resistance of the skin. Each sweat gland can be seen as one resistor arranged in parallel; hence the sum of each conductivity (reciprocal resistance) defines the conductivity between the electrodes. Thus, the SCR is measured in µSiemens. Activity of the eccrine sweat glands produces mainly water with a low concentration on ions. Thus, activity of these glands reduces resistance, which is measured as an linear increase in conductivity between the electrodes (Lykken & Venables 1971).

An exemplary timecourse of a single SCR is displayed in fig M1.

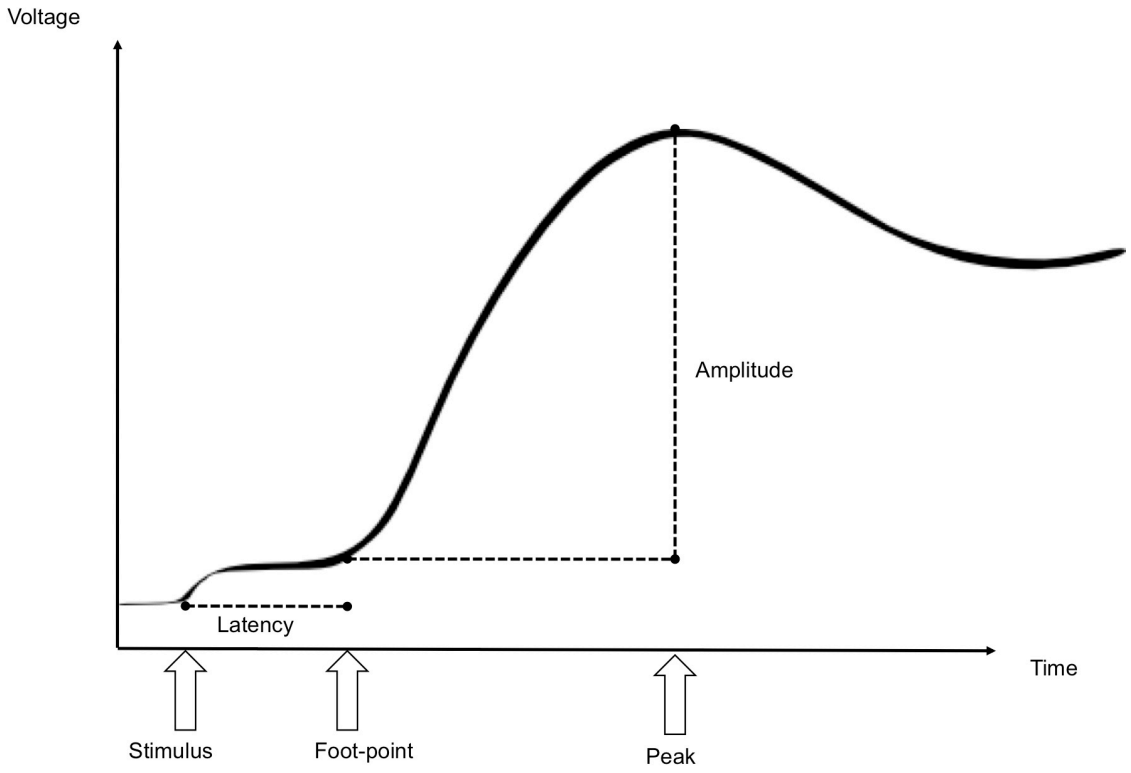

Figure M1. Exemplary timecourse of a SCR

The SCR here is defined as the first response setting on after a latency of 1 to 4 s to the stimulus onset (the latency in Study B was 1 to 3.5 s, due to the timing of the experiment). The response is manually scored as the amplitude from the foot point to the peak. These scored values have to be logarithmised, to obtain a normal distribution for statistical analyses. Moreover, in the present studies, they were range-corrected for the maximum response in a given subject and experimental phase, in order to control for individual differences in activity of the sweat glands, position of the electrodes or room temperature.

SCR is a common measurement in studies of fear conditioning, in order to detect autonomic reactions as an aversive response to the CS.

3.2 fMRI

3.2.1 fMRI and BOLD

Changes in the regional blood flow in the brain are known to be related to neural activity. Functional magnetic resonance imaging is able to detect these changes in the brain, hence measuring neuronal activity indirectly.

The technique of magnetic resonance tomography (MRT) or imaging (MRI) is based on the spin orientation of hydrogen protons. In a normal environment, the orientations of these spins are distributed randomly. In a strong magnetic field (such as inside an MRT scanner) the majority (99,9979% at 3 Tesla at 20°C) of the proton spins are oriented along the magnetic field lines (Tipler & Mosca 2009). Excitation of all spins is achieved through short electromagnetic pulses at the protons' resonance frequency and results in a sum magnetization vector that is no longer oriented in parallel to the external field. After excitation, the proton spins relax back to the former orientation. This relaxation can be described as a precession of the magnetization vector around the external magnetic field vector, with a decreasing perpendicular magnetization component (spin-spin relaxation, with time constant T2) and an increasing parallel component (spin-lattice relaxation, with time constant T1) (Skoog & Leary 1996). The changes of resulting magnetic vector fields in the relaxation processes can be measured as voltage changes in appropriate coils antennae. This is the basis of the MRI signal.

Different tissues differ in their T1 values. Hence, T1-weighted imaging can be used in structural tomography in order to distinguish different brain tissues, for example. T2 relaxation times depend on interactions between spins which lead to successive dephasing of the individual spins and hence loss of perpendicular magnetization. In the case of fMRI, physiological effects on T2 relaxation (T2*) are exploited (Weishaupt 2009). The so-called blood-oxygenation-level-dependent (BOLD) contrast is based on the divergent magnetic properties of oxygenated and deoxygenated haemoglobin. Deoxygenated haemoglobin is paramagnetic, because of the unpaired Fe (II) electron-pair in the porphyrin in the heme body (Williams 2007). These paramagnetic molecules locally enhance the magnetic field, while at the same time reducing its homogeneity. Protons that diffuse through a heterogeneous external field show enhanced T2 (T2*) relaxation and thus generate less MRI signal. The proportion of

oxygenated and deoxygenated haemoglobin is dependent on neuronal activity. Increased neuronal activity is coupled to increased blood flow for the supply of energy and oxygen. This is accompanied by an increase of oxygenated haemoglobin (or "overspill"), as the enhanced supply of fresh haemoglobin outweighs the actual consumption of oxygen. The concomitant relative decrease of deoxygenated haemoglobin results in less T2* relaxation and, hence, a stronger MRI signal. Thus, neuronal activation can be indirectly measured as an increase of blood oxygenation. Due to the vascular nature of the effect, changes in the BOLD signal to a specific stimulus or cognitive process are comparably slow (in the order of seconds) and follow a haemodynamic response function (HRF). This is important, considering analyses of the obtained fMRI datasets.

3.2.2 fMRI data analysis

The evaluated MRI data in this thesis are fMRI (T2*) datasets. These are three-dimensional volumes of BOLD contrasted images acquired repeatedly throughout the experiment with a temporal resolution of 2 – 3 seconds. The whole time series of these volumes thus consists of four dimensions, three spatial and one temporal.

The obtained MRI data series first has to be pre-processed. After this, it can be statistically analyzed. Preprocessing and analyses in this thesis were performed using the software package Statistical Parametric Mapping 8 (SPM8, Wellcome Trust Centre for Neuroimaging, London). A detailed description of the procedures can be found in Friston (2007).

3.2.2.1 Pre-processing

Pre-processing begins with "realignment", a linear transformation of volumes in order for them to spatially match the position of the first acquired volume. This corrects for subjects' head motion between scans (volumes). Subsequent "unwarping" is a non-linear transformation that takes into account interactions between head motion and inhomogeneities in the magnetic field due to the different magnetic effects of the head's various tissues and air-filled spaces. In order for different brains to be compared, that is, to be treated in a group-statistical analysis, individual brains are

then normalised using non-linear transformation into the same dimensions. This allows for indicating the spatial position of a voxel in the standardized x,y,z, (x=left/right; y= anterior/posterior; z=dorsal/ventral) coordinate system of the Montreal Neurological Institute (NMI).

Normalization in both studies was done to a template created with the "Diffeomorphic Anatomical Registration using Exponentiated Lie algebra"-tool (DARTEL) (Ashburner 2007). For this template, subject's structural (T1-weightend) images are segmented into grey matter, white matter and cerebro-spinal fluid. The segmented grey and white matter images of each subject are then registered onto the intensity averages of the grey and white matter images of the whole group (templates) using non-linear transformations. A time-invariant velocity field parameterises these non-linear deformations of each subject's images. This procedure reoccurs several times, and after each registration step, the template is iteratively updated.

The DARTEL template is then normalised onto a reference brain image in stereotactic space defined by the NMI. The individual normalization parameters obtained through the creation of the DARTEL template and the normalization to the NMI space were then applied to subjects' fMRI (T2*-weighted) images. As a last step, images are then spatially smoothed with a Gaussian kernel in order to improve signal to noise and to take into account inter-individual differences in brain anatomy and function.

3.2.2.2 Single-subject analysis

The pre-processed data is first analyzed on the single-subject level ("first level") using a general lineal model (GLM), that is, multiple regression, approach.

The GLM tries to explain the experimentally observed variance in the fMRI signal time course (Y) in a given voxel by a linear combination of regressors x plus noise (error) k:

$Y = beta_1 * x_1 + beta_2 * x_2 + \ldots + beta_n * x_n + k$

Each regressor is a predictor of experimentally induced variance (e.g., the time course of an experimental condition or stimulus presentation) and is convolved with the HRF before being entered into the GLM, in order to better predict the typical BOLD time

course. The GLM includes regressors for high-pass filtering, which filter out low frequency signal components due to physiological processes such as breathing or heartbeat. Temporal autocorrelations (dependence) between subsequent volumes that result from the sluggish nature of the haemodynamic response and from physiological processes are estimated from the error variance and corrected. The resulting regression parameter estimates or "betas" express the size of the contribution of an experimental factor (a condition, a stimulus) to the signal in that voxel. Beta estimation is performed voxel-wise. Beta estimates can be linearly combined to compare experimental conditions (e.g., condition 1 – condition 2), yielding voxel-wise "contrast estimates" ($beta_1$ - $beta_2$).

3.2.2.3 Group statistics

Like first level analysis, group-level statistics uses the GLM, but there is no HRF-convolution. In the random-effects group statistics ("second level"), beta or contrast estimates in every voxel resulting from the first-level analysis are analyzed for group effects. For instance, if comparing activation to condition 1 between two groups (verum and placebo) using a two-sample t-test, the individual $beta_1$ estimates in a given voxel constitute Y and group assignment is modelled as two regressors x_1 (with values 1 for all subjects in the verum group and 0 for all subjects in the placebo group) and x_2 (with values 0 for all subjects in the verum group and 1 for all subjects in the placebo group). The resulting voxel-wise t-values can be seen as a measure of the effect size in that voxel. Factorial tests are analogue to analyses of variance (ANOVA). Other than simple t-tests, they also permit to correct for violations of the sphericity assumption (independence of the error variance between conditions or factor levels, homogeneity of the error variance between conditions or factor levels).

3.2.2.4 Caveats of fMRI analysis

The application of a GLM to analyze fMRI datasets assumes that the HRF is constant over time and comparable in every brain region. Studies reported different onsets of the HRF function in different brain regions (Handwerker et al. 2004). Nevertheless, the BOLD contrast remained stable in studies comparing the amplitude in one region in

different sessions (Neumann et al. 2003) and over a long time (Menz et al. 2006). In addition, individual haemodynamic responses measured with BOLD contrast to fearful faces were stable in different examinations separated in time (Manuck et al. 2007) and within long sessions (Johnstone et al. 2005).

Another problem in fMRI statistics is the large number of voxels in which comparisons are computed. With some ten thousand voxels analysed, even an alpha threshold of p=0.001 will produce many false positives. Bonferroni correction, on the other hand, is often too conservative, as it neglects the dependence of neighbouring voxels. A more realistic correction for multiple comparisons used in SPM is the family wise error (FWE) method that follows Gaussian random field theory. This can be combined with an anatomical a-priori hypothesis about the expected location of an effect ("small volume correction", SVC), which limits the number of voxels in the comparison to a defined anatomical region of interest (ROI).

3.3 Methods Study A

3.3.1.1 Subjects

45 healthy, right-handed male volunteers were recruited for this study, 3 subjects were excluded, before the intake of medication, due to abuse of illegal drugs (N=2) or their own will (N=1). One participant was excluded on day 8, due to massive movement in the scanner. The remaining 41 (placebo N=19; verum N=21) healthy, right-handed male volunteers were 25-41 (mean= 28.37 +/- 3.3(SD), no differences between groups (p>0.1) years of age. Written informed consent was obtained from all participants in accordance with the requirements of the local Ethics Committee of the Medical Board in Hamburg and the federal institute for pharmaceutical and medical products in Germany (BfArM). Subjects reported no past or present psychiatric or neurological diseases or any other disease affecting major organs. None of the subjects reported taking regular medication, or prescription-free medication, at any timepoint of the experiment. Abuse of illegal drugs was tested using an urine drug

screen (Diagnostik Nord, Schwerin, Germany) that included the common classes of illegal drugs (THC, Cocaine, Phenylethylamines (Methylenedioxy- /Met-/Amphetamine), Extasy, Opiates and prescriptive medication as Benzodiazepines and Opioids (Buprenorphine and Methadone).

Trait anxiety was assessed before each experimental day using the State-Trait Anxiety Inventory (Spielberger et al. 1970, Laux et al. 1981). Trait anxiety scores ranged from 21 to 56 (mean 32.93 ± 6.19) and were not different between groups at any experimental day ($p>0.3$). These values did not deviate from a German normal population (Laux et al. 1981).

3.3.1.2 Randomization

Beforehand, a third person randomly assigned subjects to the groups receiving either placebo (Mannitol) or 150 mg L-DOPA (with 37.5 mg Benserazide). This person never obtained any experimental data nor had any contact with the subjects.

3.3.1.3 Experimental design

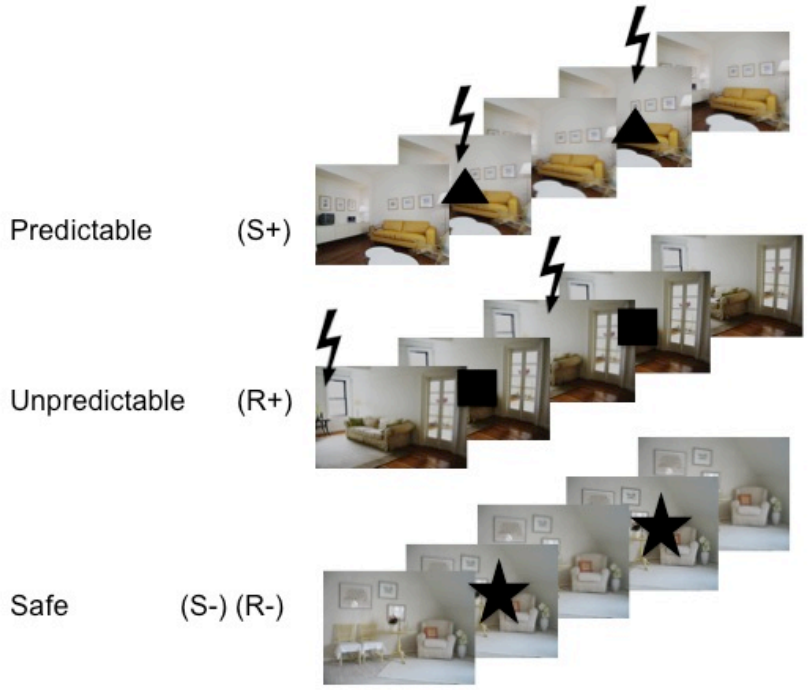

Figure M2. Experimental design of day 1 in Study A.

The experiments on day 1 and day 2 took place in a psychophysiological laboratory, where the visual stimulus material was presented on a computer screen (24''; 1920x1200 pixel), with blinds on the left and right side. On day 8, the experiment was conducted inside the MR scanner, with the visual stimuli being projected onto a screen at the back of the magnet's bore. Subjects could see the screen via a mirror mounted over their heads.

3.3.1.3.1 Day 1 (Conditioning)

Three background pictures of similar but easily distinguishable rooms were used as experimental contexts (context CS). Three geometric symbols (a triangle, a circle, a star) served as cue CS. The US was an electric stimulus consisting of a train of 3 square-wave pulses of 2 ms length, delivered through a surface electrode with platinum pin (Clyde's Polo Kit Supplies, Bexley, UK) on the right dorsal hand. Stimuli were applied using a Digitimer DS7A electrical stimulator (Digitimer, Welwyn Garden

City, UK). US intensity was individually adjusted before the experiment to the maximum tolerable pain (intensity range 1.4 – 43.0 (9.09 +/- 8.3) mA). The intensities were not different between groups ($p > 0.8$). Participants were asked to rate the unpleasantness of the US between 0 (“I feel nothing”) to 10 (“maximally unpleasant”) (rating = 3-10; mean = 7.46 +/- 1.3). There were no differences in rating between groups ($p>0.3$).

One trial lasted 45 s during which the corresponding background picture (context CS) was continuously present on the screen. The corresponding symbol (discrete CS) was presented twice for 5 s each in two time windows (13-15 s and 31-35 s after the onset of the context CS). In the predictable condition, the discrete CS (S+) was always paired with a US 4.8 s after CS onset. This made S+ the best predictor of the US. In the unpredictable condition, one, two, or three US (mean two) were randomly administered in the time periods where only the context was present. Hence, the context itself (R+) was the best US predictor. To avoid that subjects would identify the discrete CS as a safety signal, two US were applied together with the discrete CS in the unpredictable condition. In the safe condition, no US occurred. Hence, the corresponding room (R-) and the corresponding symbol (S-) could be used as control CS-. Each trial was followed by a 6–8 (mean 7s) s inter-stimulus-interval, consisting of a black screen with a fixation cross. There were altogether 27 trials, 9 in each condition in pseudo-randomised order. The entire experiment lasted 30 min.

Participants were not informed about these contingencies or the learning element in this experiment. In each participant, the combinations of rooms and symbols were randomly assigned beforehand and were consistent throughout all experimental days.

Before the experiment, participants were familiarised with the rating scales (see below) and stimulus material. For this purpose, each room and room symbol combination was presented once without US delivery in a habituation phase.

At the end of the experiment, participants were asked about their awareness of contingencies between symbols, rooms and shocks in a semi-structured questionnaire. Results of this questionnaire classified the groups into aware, semi-aware (aware of the safe vs. the two shock conditions) and non-aware. Awareness was not different between groups ($p>0.5$).

3.3.1.3.2 Day 2 (Extinction)

Participants returned 24 hours later. The US electrode was placed on the forearm again, without adjusting the shock intensity. 6 trials in each condition were presented in pseudo-randomised order again, without administering any shock in any condition. The entire experiment lasted 15 min.

Directly after the experiment, participants received either placebo or 150 mg L-DOPA in a double-blind manner. Subjects stayed under medical observation for at least 60 min after drug intake.

3.3.1.3.3 Day 8 (Test)

Participants returned 7 to 8 days after experimental day 1. They were placed in the fMRI scanner and equipped with the US electrode on the right dorsal hand and response keys. The shock intensity was not adjusted. The first half (spontaneous recovery test) consisted of the presentation of 6 trials in pseudo-randomised order in each condition, without any US. This was followed by 3 unsignalled US while a grey screen was present. After 2 min of rest, the second half (reinstatement test) began, consisting of the presentation of 6 trials in pseudo-randomised order in each condition, without any US again.

3.3.2 Ratings

Participants were intermittently asked to give explicit ratings for each symbol and room on a computerised Visual Analogue Scale (VAS, 0-100), using the left and right arrow keys (day 1 and day 2) or a button-response box (day 8) with their right hand. Each rating value had to be confirmed by key press. These ratings consisted of a question about the level of CS-evoked stress/fear/tension (0 = no stress/fear/tension, 100 = high stress/fear/tension).

On day 1, ratings were given after the habituation phase and every 9th trial (3 in each condition), resulting in a total of 4 ratings of stress/fear/tension.

On day 2, ratings occurred at the beginning and after every 9th trial (3 in each condition), resulting in a total of 3 ratings.

On day 8, ratings occurred at the beginning and after every 9th trial (3 in each condition), as well as directly at the beginning of the reinstatement test phase resulting in a total of 8 ratings of stress/fear/tension.

Ratings that were not confirmed were not included into the analysis. Ratings of zero in all trials on one of the VAS lead to exclusion of one subject (day 1: placebo N = 3, verum N=2; day 2: placebo N = 0, verum N=5; day 8: placebo N = 2, verum N=2) from the rating analyses on that day.

3.3.3 SCR

SCRs were recoded with a Biopac MP-100 (Biopac Systems Inc, Goleta, California, USA) with AcqKnowledge 4 software on day 1 and day 2 and with a CED2502-SA skin conductance unit with Spike 2 software (Cambridge Electronic Design, Cambridge, UK) on day 8.

Self-adhesive Ag/AgCl (3M, Poland) – electrodes were placed on the palmar side of the hand on the distal and proximal hypothenar. Skin conductance was downsampled to 10 Hz and responses were manually scored off-line as described in 3.1. Amplitudes were logarithmised and divided through the maximum response on that day.

Artefacts in the SCR recordings led to exclusion this individual data (day1: verum N=1; day 2: placebo N=2, verum N=4; day 8: placebo N=4, verum N= 6).

3.3.4 Statistical analysis of behavioural data

Repeated-measures analysis of variance (ANOVA) was performed in PASW 17.0 (SPSS Industries), separately for each day.

The 2x3x2 ANOVA used the within-subject factors stimulus (symbol/room) (2) and condition (predictable/unpredictable/safe) (3) and the between-subject factor group (placebo, verum) (2).

The addition of time (first and second half of experiment, or alternatively first, second and third of experiment) (2 or 3) as a third within-subject factor was optional (2x3x2x2 ANOVA).

Planned simple comparisons with one-sample one-sided t-tests examined if responses (to symbols and rooms) were higher in the predictable and unpredictable condition as compared to the safe condition. Here, cue conditioned fear was defined as higher responses to the symbol in the predictable condition in comparison to the symbol in the safe condition (S+ > S-). Contextual conditioned fear was indicated through higher responses to the room in the unpredictable condition as compared to the room in the safe condition (R+ > R-).

If the ANOVA yielded a significant interaction with the factor group, planned two-sample t-tests were used to evaluate potential group differences before drug intake (day 1 and 2 (two sided)) or, one day 8, the a-priori hypothesis of group effects (one-sided). The latter hypothesis was that the L-DOPA group shows decreased responding in the recall of cued (S+ > S-) and contextual (R+ > R-) fear on day 8 in comparison to the placebo group. The significance level was set to $p=0.05$ and results between $p=0.05$ and $p<0.1$ were reported as a trend. Greenhouse-Geisser correction for sphericity violation was used, if appropriate.

3.3.5 fMRI (day 8)

fMRI data in this study was obtained with a 3 Tesla Siemens scanner (MAGNETOM trio, Siemens Germany) using a 32-channel head coil. 34 continuous axial slices (2 mm thick) were acquired using a T2*-sensitive gradient echo-planar imaging (EPI) sequence (repetition time, 2.23 s; echo time, 30 ms; field of view, 220 x 220 mm, adjusted to the position of the individual temporal lobe). Task presentation and recording of behavioural responses were performed with Presentation® (NeuroBehavioral Systems, Albany California, USA). High-resolution T1-weighted structural images were also acquired after the experimental session.

Data series were pre-processed with SPM8 as described before and normalised onto a cohort-specific DARTEL template in the MNI space. Normalised data series were spatially smoothed with a 6 mm isotropic Gaussian kernel (full width half maximum,

FWHM). For statistical first-level analysis, a GLM was used with the following regressors :

One regressor per room type (unpredictable, predictable, safe) which modelled each room presentation (trial) of 45 sec as a continuous block using a “box car” function (on during presentation, otherwise off); one regressor per symbol type which modelled each onset of a symbol as an event using a “stick” or delta function (zero everywhere except at onset, with an integral of one over the entire real line). These regressors were built separately for the phase of spontaneous recovery (before the reinstatement shocks) and for the post-reinstatement phase. In addition, we defined six explanatory variables that represented interactions of these main effect regressors with time. These were created by multiplying each main effect regressor with a linear decaying function (predictable room $_{\text{linear decreasing}}$, unpredictable room (R+)$_{\text{linear decreasing}}$, safe room (R-)$_{\text{linear decreasing}}$, predictable symbol (S+)$_{\text{linear decreasing}}$, unpredictable symbol $_{\text{linear decreasing}}$, safe symbol (S-)$_{\text{linear decreasing}}$), thus modeling CS-evoked responses that decrease over trials. The main effect regressors are therefore called “categorical” regressors, while the linear decreasing regressors are called “parametric”.

Additional nuisance regressors were included to factor out experimental effects of no interest: event-type regressors modelled each onset of the ITI; each onset of a rating; and each reinstatement shock. A block-type regressor modelled the 2 min rest period after the reinstatement shocks. Each regressor was convolved with the HRF.

The parameter estimates (betas) of the regressors of interest were analysed in separate second-level analyses for rooms and symbols (due to different scaling of block- and event-type regressors) and for the two phases. For each of these analyses, a “full factorial” test was chosen which involved the factors: condition (3) and group (2).

Definition of ROIs

Correction for multiple comparisons was limited to small volumes, centreed on coordinates reported in previous studies investigating conditioned fear or extinction recall with human fMRI. Significant results of these studies are displayed in table M1 and relatively consistent comprise posterior hippocampus (see chapter 1.4 Neural systems mediating fear extinction) and dmPFC for fear recall and anterior hippocampus and vmPFC (see chapter chapter 1.4 Neural systems mediating fear extinction) for extinction recall. If necessary, coordinates were transformed to MNI space (using the tal2mni Matlab® function *http://imaging.mrc-*

cbu.cam.ac.uk/imaging/MniTalairach) and averaged. X-coordinates of the medial PFC activation was set 0, to avoid multiple testing of the same overlapping (left/right) voxels in two lateralized boxes. Small volumes were spheres with radii of 6 mm for the two bilateral hippocampus ROIs and boxes of 20x16x16 mm for medial cortical regions in the PFC (as in prior studies (Kalisch et al. 2009, Raczka et al. 2011)).

Due to the strong theoretical interest in the amygdala (see chapter chapter 1.4 Neural systems mediating fear extinction), an anatomical mask was used as an additional ROI for activations related with the recall of fear. The amygdala masks were probability masks taken from the “Harvard-Oxford cortical and subcortical structural atlases” provided by the Harvard Center for Morphometric Analysis (http://www.cma.mgh.harvard.edu) with a probability threshold at 0.70.

Table M1 Coordinates (MNI) reported in previous studies of fear and extinction recall

Publication	**Extinction recall (MNI)**				**Fear recall (MNI)**				**Type of test**
	Region	x	y	z	Region	x	y	z	
Phelps (2004)	vmPFC	0	36	-7	not investigated				
Kalisch (2006)	L vmPFC	-2	42	-22	R post HC	-38	-32	-12	reinst./ren
	L ant HC	-24	-12	-32					
	L ant HC	-26	-18	-26					
	aver. L ant HC	-25	-15	-29					
Milad (2007)	R vmPFC	6	26	-12	not reported				
	R vmPFC	2	36	-7					
	aver. R vmPFC	4	31	-9,5					
	L ant HC	-30	-22	-19					
	R ant HC	29	-20	-18					
Kalisch (2009)	not investigated				L post HC	-34	-32	-16	reinst./ren /DCS
					R DMPFC	2	46	34	
					L DMPFC	-2	46	34	
					L DMPFC	-2	48	28	
					aver. L DMPFC	-2	47	31	
Milad (2009)	L vmPFC	-10	45	-11	L DMPFC	-2	37	22	spont. recov./ PTSD
	R vmPFC	2	47	-12					
	R ant HC	32	-8	-33					
Spoormaker (2010)	R vmPFC	8	36	-6	DMPFC	n. g.			spont. recov.
Spoormaker (2011)	not significant				not significant				
Resulting ROI centre									
	vmPFC	0	40	-12	dmPFC	0	43	29	
	ant HC	29	-16	-25	post HC	36	-32	-14	

Abbrevations: R=right; L=left; aver.=average; n.g.=not given;

spont.recov.=spontaneous recovery; reinst/ren. =reinstatement / renewal;

3.4 Methods Study B

While Study A had investigated the spontaneous recovery and reinstatement of fear, this study was designated to test how L-DOPA modulates the renewal of cued fear. The paradigm was a modification of a paradigm previously established in our group by Kalisch et al. (2006).

3.4.1 Subjects

40 right-handed healthy male volunteers (placebo N=20; verum N=20) participated in this study. One subject had to be excluded during the experiment on the second day, due to alcohol intake before the experiment (verum N=1).

The remaining 39 volunteers were 25-42 (29.26 +/- 4.1), no differences between groups ($p>0.1$)) years of age. Written informed consent was obtained from all participants in accordance with the requirements of the local Ethics Committee of the Medical Board in Hamburg and the federal institute for pharmaceutical and medical products in Germany (BfArM). Subjects had no past or present psychiatric or neurological diseases or any other disease affecting major organs. Abuse of illegal drugs was tested using an urine drug screen (Diagnostik Nord, Schwerin, Germany) that included the common classes of illegal drugs (THC, Cocaine, Phenylethylamines (Methylenedioxy- /Met- /Amphetamine), Extasy, Opiates and prescriptive medication as Benzodiazepines and Opioids (Buprenorphine and Methadone).

Trait anxiety was assessed before the experiment using the State-Trait Anxiety Inventory (Spielberger et al. 1970, Laux et al. 1981). Trait anxiety scores ranged from 25 to 52 (mean 33.34 ± 5.9). These values did not deviate from a German normal population (Laux et al. 1981).

3.4.2 Randomization

Beforehand, a third person randomly assigned subjects to the groups receiving either placebo (Vitamin E) or 150 mg L-DOPA (with 37.5 mg Benserazide). This person never obtained any experimental data nor had any contact with the subjects.

3.4.3 Experimental design

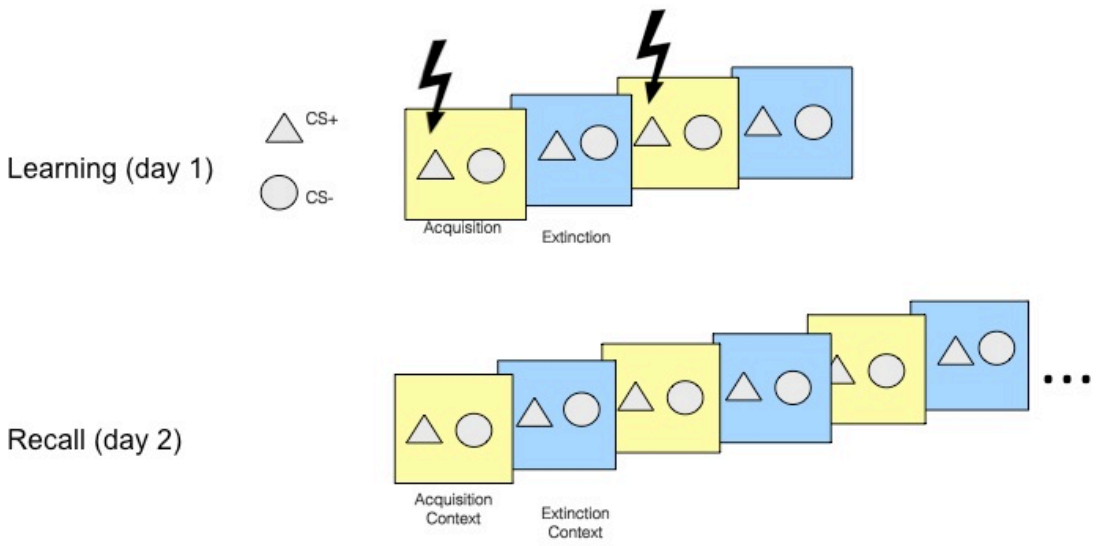

Figure M3. Experimental design of day 1 and 2 in Study B

3.4.3.1 Day 1 (Conditioning + Extinction)

Participants were not informed about any contingencies, or the learning element in this experiment. Instead, subjects were told that this was an experiment that tested how some people can maintain attention to a cognitive task despite strong occasional

distraction (by aversive electric shocks). Throughout the experiment, the participants were thus asked to indicate the presented symbol (triangle or circle) by pressing the corresponding key on a button box, as fast and accurately as they could.

The US was an electric stimulus consisting of a train of 3 square-wave pulses of 2 ms length, delivered through a surface electrode with platinum pin (Clyde's Polo Kit Supplies, Bexley, UK) using a Digitimer DS7A electrical stimulator (Digitimer, Welwyn Garden City, UK). The US was applied to the right dorsal hand and intensity was individually adjusted before the experiment to each maximum tolerable pain (intensity = 2-76 mA; mean = 12,72 mA +/-12.4). There were no differences in chosen intensity between groups ($p>0.2$). In addition, participants were asked to rate the unpleasantness of the US between 0 ("I feel nothing") to 10 ("maximally unpleasant") (rating = 7-10; mean 8.46 +/- 1). There were no differences in ratings between groups ($p>0.9$).

Day 1 consisted of two fear conditioning acquisition phases and two extinction phases in an ABAB design. During acquisition, the two geometric symbols (duration of 3 s) were repeatedly presented in pseudo-randomised order while the background was kept constant (yellow or blue). One symbol (randomly assigned beforehand) served as a conditioned stimulus (CS+) and was paired in 50% of the presentations with a painful electric stimulus (US) 2.5 s after the onset of the CS+. The other symbol served as a control stimulus (CS-) and was never paired with the US. The inter-stimulus-interval was jittered between 2.5 - 5 seconds (mean of 3.6 sec).

During extinction, both CS were again presented in the same amount and duration as in the acquisition phases, but without pairing the CS+ with the US and using the alternative background colour (blue or yellow). The change of the background colour was used to create different contexts for the acquisition and extinction phases.

Each phase consisted of 24 CS presentations (12 CS+ and 12 CS-) and the whole experiment lasted 35 min.

Before the experiment, participants were familiarised with all stimuli (all symbols and background colours without any shocks) and the ratings (see below). Assignment of symbols to the CS+ or CS- and the background colour to the acquisition or extinction phase was counterbalanced across participants and groups.

Directly after the experiment, participants received either placebo or 150 mg L-DOPA in a double-blind manner. Subjects stayed under medical observation for at least 60 min after drug intake.

3.4.3.2 Day 2 (Test)

On day 2, the electrode was placed on the dorsal right hand and the intensity of the UCS was adjusted again. This effectively corresponded to a reinstatement procedure and was done to achieve maximal return of fear (intensity = 4-80 mA; mean = 14.96 +/- 12.9). There were no differences between groups ($p>0.2$). Again, participants were asked to rate the unpleasantness of the US between 0 (“I feel nothing”) to 10 (“maximally unpleasant”) (rating = 7-10; mean = 8.31 +/- 0.9). There were no differences in rating between groups ($p>0.7$).

In the experiment, the total number of CS presentations was equal to that on the first day, but no US was administered. Furthermore, each presentation of the acquisition context and extinction contexts was shorter and incorporated only 4 CS presentations (two CS+ and two CS- in pseudo-randomised order) in a ABAB… design, with a total of twelve acquisition and twelve extinction context phases. It was expected that the presentation of the CS+ in the acquisition context would lead to renewal of fear.

The CS duration was the same as on day one. The inter-stimulus-interval was jittered between 2.5 - 5 seconds (mean of 5.2 sec). The whole experiment lasted 45 min.

3.4.4 Ratings

Participants were intermittently asked to give explicit ratings about each CS on a Visual Analogue Scale (VAS, 0-100). These ratings consisted of two categories, one for the level of CS-evoked stress/fear/tension (0 = no stress/fear/tension, 100 = high stress/fear/tension) and one for the level of US expectancy induced by a CS (0 = no expectancy, 100 = high expectancy).

On day 1, ratings were given after every 8th trial (four CS+ and four CS- presentations), resulting in three CS+ and three CS- ratings, respectively, per each

phase. These ratings consisted of one question for the level CS-evoked stress/fear/tension (0 = no stress/fear/tension, 100 = high stress/fear/tension) and one for the level of US expectancy induced by a CS (0 = no expectancy, 100 = high expectancy). At the end of the experiment on day 1, subjects rated the contingency of the CS and the US on the VAS for each CS and context.

On day 2, the same ratings occurred after every 4th trial (two CS+ and two CS- presentations), resulting in twelve CS+ and twelve CS- ratings for stress/fear/tension and US expectancy in each context (24 CS+ and 24 CS- ratings in total).

Ratings of zero in all trials on one of the VAS lead to exclusion of one subject (day 1 and day 2 : verum N=1) from the rating analyses on that day.

3.4.5 SCR

SCRs on both days were recorded with a CED2502-SA skin conductance unit with Spike 2 software (Cambridge Electronic Design, Cambridge, UK) at 100 Hz. Self-adhesive Ag/AgCl – electrodes (3M, Poland) were placed on the palmar side of the hand on the distal and proximal hypothenar. SCRs were manually scored off-line as described in 3.1. Amplitudes were logarithmised and divided through the maximum response on that day.

Artefacts in the recordings through fMRI-scanning led to exclusion of 10 subjects (placebo N =6; verum N= 4) in the SCR analysis of day 1 and exclusion of 6 subjects (placebo N =3; verum N= 3) on day 2. Analyses of the SCR data with the same subjects excluded (placebo N =7; verum N= 6) on day 1 and day 2 did not change significant effects (data not shown).

3.4.6 Statistical analysis of behavioural data

Repeated-measures analysis of variance (ANOVA) was performed in PASW 17.0 (SPSS Industries) separately for each day.

The 2x2x2 ANOVA used the within-subject factors stimulus (CS+/CS-) (2) and context (acquisition/extinction) (3) and the between-subject factor group (placebo, verum) (2).

The addition of time (first and second half of experiment) (2) as a third within-subject factor was optional (2x2x2x2 ANOVA).

On both days, planned simple comparisons with one-sample, one-sided t-tests examined if responses were higher to the CS+ as compared to the CS- in the acquisition context, indicating successful fear conditioning (day 1) and renewal if fear (day 2), respectively. Furthermore, it was tested if the differential response (CS+>CS-) is higher in the acquisition context as compared to the extinction context ((CS+>CS-)A > (CS+>CS-)E), indicating successful extinction (day 1) and extinction recall (day 2), respectively.

If the ANOVA yielded a significant interaction with the factor group, planned two-sample t-tests were used to evaluate potential group differences before drug intake (day 1, two-sided) or the a-priori hypothesis of group effects on day 2 (one-sided). The latter hypothesis was that the L-DOPA group shows decreased differential responding (CS+>CS-) in the renewal ((CS+>CS-)A > (CS+>CS-)E) on day 2 in comparison to the placebo group. The significance level was set to $p=0.05$ and results between $p=0.05$ and $p<0.1$ were reported as a trend. Greenhouse-Geisser correction for sphericity violation was used, if appropriate.

3.4.7 fMRI (day 2)

On both days, fMRI data in this study was obtained with 3 Tesla Siemens scanner (MAGNETOM trio, Siemens Germany) using a 12-channel head coil. 34 continuous axial slices (2 mm thick) were acquired using a T2*-sensitive gradient echo-planar imaging (EPI) sequence (repetition time, 2.23 s; echo time, 30 ms; field of view, 220 x 220 mm, adjusted to the position of the individual temporal lobe). Task presentation and recording of behavioural responses were performed with Presentation® (NeuroBehavioral Systems, Albany California, USA). Stimulus material was projected to the participant via a 45° mirror placed atop the head coil. High- resolution T1-weighted structural images were also acquired after the experimental session.

Data series were pre-processed with SPM8 as described before and normalised onto a cohort-specific DARTEL template in the MNI space. Normalised data series were spatially smoothed with a 6 mm isotropic Gaussian kernel (full width half maximum, FWHM).

Only day 2 data are reported here, as they are critical for the testing the treatment effect. For statistical first-level analysis on day 2, a GLM was used analysing the first half of the experiment with the following regressors:

Each CS (CS+ context A, CS- context A, CS+ context E, CS- context E) was modelled as an event of the CS onset, using a "stick" or delta function (zero everywhere except at onset, with an integral of one over the entire real line). In addition, we defined four explanatory variables that represented interactions of the main effect regressors with time. These parametric modulations were created by multiplying each main effect regressor with a linear decaying function (CS+ context $A_{\text{linear decrasing}}$, CS- context $A_{\text{linear decrasing}}$, CS + context $E_{\text{linear decrasing}}$, CS- context $E_{\text{linear decrasing}}$, thus modeling CS-evoked responses that decrease over trials. Additional nuisance regressors were included to factor out experimental effects of no interest: One regressor per context type (extinction context, acquisition) which modelled each context duration as a continuous block using a "box car" function (on during presentation, otherwise off) and each onset of a rating and each response as event-type regressors. Each regressor was convolved with the HRF.

The parameter estimates (betas) of the regressors of interest were analysed in a second-level analyses in a "full factorial" test, which involved the factors: stimulus (2), context (2) and group (2).

Definition of ROIs

The same small volume correction, that was used in Study A, was conducted in Study B. Table M2 shows the centre of the ROIs.

Table M2. Centre of the ROIs (MNI)

Resulting ROI centres:	vmPFC	0	40	-12	dmPFC	0	43	29
	ant HC	29	-16	-25	post HC	36	-32	-14

4. Results

4.1 Results Study A

4.1.1 Day 1

Subjects were fear conditioned on day 1 in conditions of different predictability. Each of the three conditions consisted of a room (context) presentation with an intermittently appearing symbol (cue). In the predictable condition, the appearance of the symbol predicted the US (S+). In the unpredictable condition, the appearance of the room predicted the US (R+). The safe condition was never paired with a shock (S- and R-) and was used as a baseline. Marschner and collegues (2008) used a similar paradigm before and revealed psychophysiological and neural correlates of cued fear and context conditioning.

The analysis of day 1 tested if coherence existed between the manipulation of cued and contextual fear conditioning and the dependent variables, SCR and ratings of fear/distress (Hypothesis 1A). Furthermore, this analysis tested if groups already differed before the intake of either placebo or L-DOPA (Hypothesis 1B).

4.1.1.1 SCR

The 2x3x2 ANOVA (placebo N=19, verum N=20) revealed a trend-wise significant result for the main effect of stimulus (room/ symbol) ($F(1,1)=3.43$; $p=0.072$) and a significant result for the main effect of condition (predictable/ unpredictable/ safe) ($F(1,2)=12.10$; $p<0.001$). No group main effects or interactions were observed. Averages of SCRs to symbols and rooms in the different conditions are illustrated in figures R1 and R2.

Simple comparisons indicated significantly higher responding in conditions that were associated with the US (predictable condition > safe condition ($F(1,1)=22.61$; $p<0.001$) and unpredictable condition > safe condition ($F(1,1)=4.47$; $p=0.041$)).

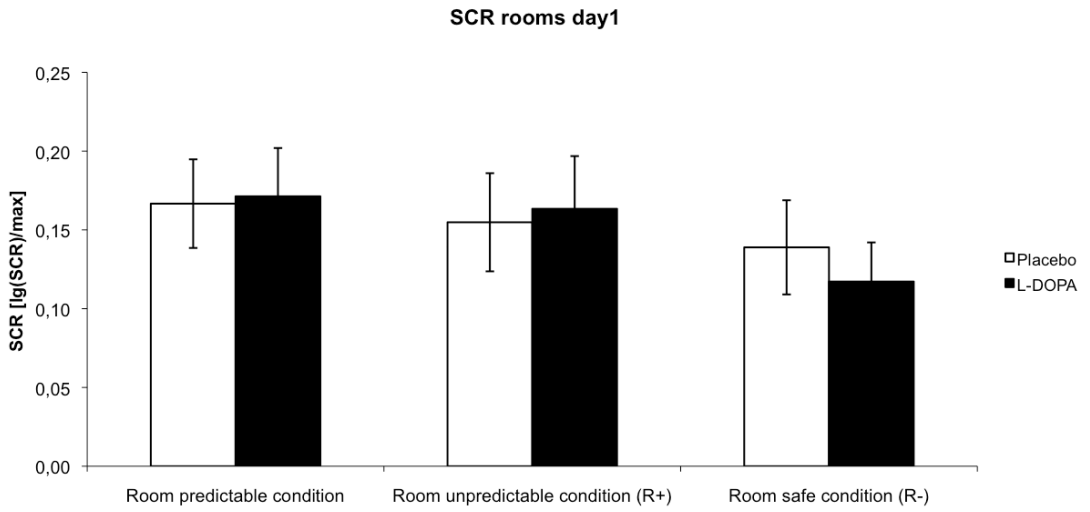

Figure R1. Average of the SCR for rooms in each condition during fear conditioning acquisition on day 1. Error bars indicate the standard error of the mean (SEM). lg= logarithmical ; max = highest individual SCR on that day

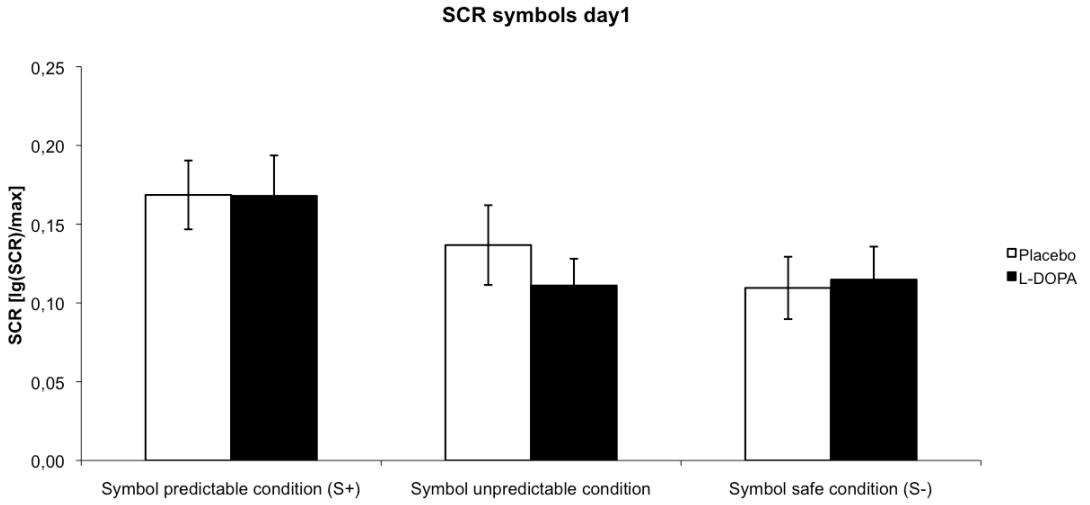

Figure R2. Average of the SCR for symbols in each condition during fear conditioning acquisition on day 1. Error bars indicate the SEM.

Paired t-tests (one-sided) yielded significant results for the effects of cue conditioning (S+ >S- ($T(1,39) = 4.61$; $p<0.001$)) and, of context conditioning (R+>R- ($T(1,39) = 1.84$; $p=0.037$)).

Averages of cue and context conditioning indices during day 1 are illustrated in figure R3.

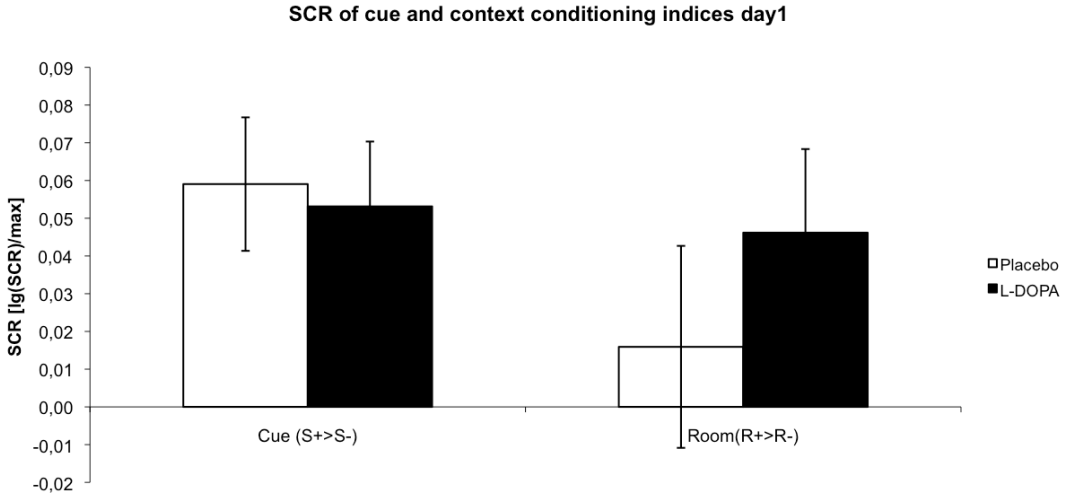

Figure R3. Average of the differential score of cue (S+>S-) and context (R+>R-) SCRs during acquisition of fear conditioning on day 1. Error bars indicate the SEM.

A repeated measurements 2x3x2x2 ANOVA with the additional factor time (first half/second half) extended the results of the first ANOVA, with significant main effects of stimulus ($F(1,1)=5.00$; $p=0.031$) and time ($F(1,1)=28.81$; $p<0.001$). Furthermore, trend-wise interaction effects between stimulus and time ($F(1,1) =2.68$; $p=0.063$) and stimulus, condition and time ($F(1,1.9) =2.98$; $p=0.061$) emerged.

The interaction of stimulus, condition and time is further qualified through a significant increase (first half vs second half) in the differentiation of the symbol and room in the predictable condition in comparison to the safe condition ($F(1,1)=3.80$; $p=0.041$).

In addition, paired t-tests (one-sided) of the indices for cued and contextual conditioning in the first half on day 1 yielded significant results for the effect of cue conditioning (S+ >S- ($T(1,38)=2.60$; $p=0.007$)) only. Whereas paired t-tests (one-sided) of the indices for cued and contextual conditioning in the second half on day 1 yielded significant results for the effect of cue conditioning (S+ >S- ($T(1,38)=4.70$; $p<0.001$)) and, of context conditioning (R+>R- ($T(1,38) = 2.03$; $p=0.025$)).

Taken together, analyses of the SCRs indicated the expected psychophysiological responses to cue and context conditioning that are increasing with time. The increase reflects learning of the best predictors of the US (S+ and R+) in the different conditions. Furthermore, there were no significant differences between the pharmacological treatment groups on day 1.

4.1.1.2 Rating of fear/distress

The 2x3x2 ANOVA (placebo N=16, verum N=19) revealed a significant result for the main effect of condition ($F(1,2)=86.96$; $p<0.001$) and the interaction of stimulus and group revealed a trend-wise significant result ($F(1,1)=3.82$; $p=0.059$). No main effects of the factor group were observed. Averages of the fear/distress ratings to symbols and rooms in the different conditions are illustrated in figures R4 and R5.

Simple comparisons indicated significantly higher responding in conditions that were associated with the US (predictable condition > safe condition ($F(1,1)=93.98$; $p<0.001$) and unpredictable condition > safe condition ($F(1,1)=98.28$; $p<0.001$)).

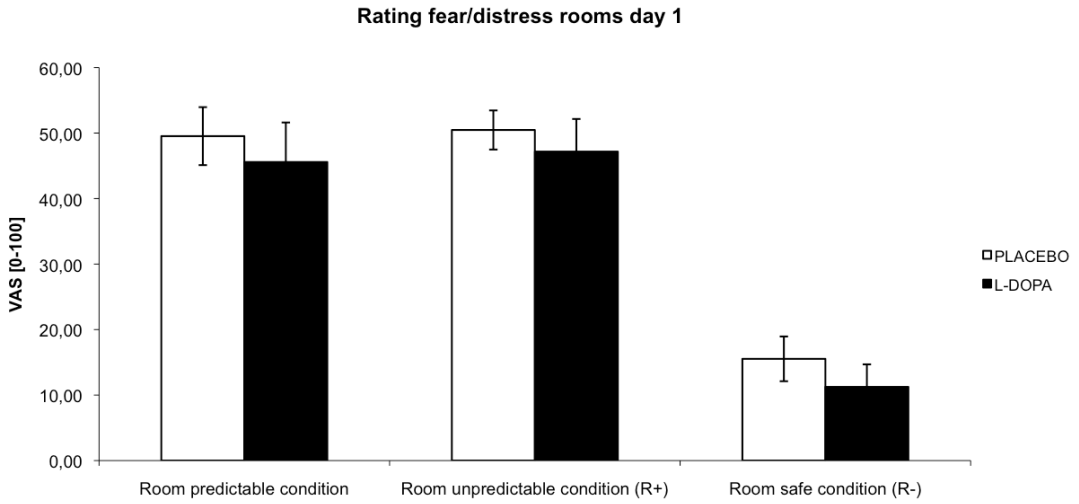

Figure R4. Average of the fear/distress rating for rooms in each condition during fear conditioning acquisition on day 1. Error bars indicate the SEM.

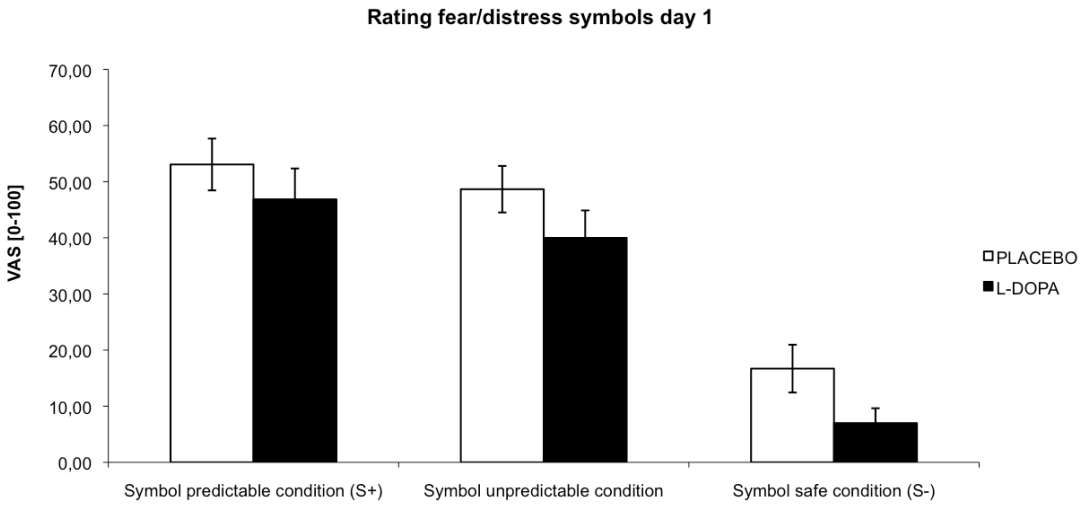

Figure R5. Average of the fear/distress rating for symbols in each condition during fear conditioning acquisition on day 1. Error bars indicate the SEM.

Paired t-tests (one-sided) yielded a significant effect of cue conditioning (S+ >S-) ($T(1,37)=8.88$; $p<0.001$) and context conditioning ($T(1,38)=9.87$; $p<0.001$), see figure R6.

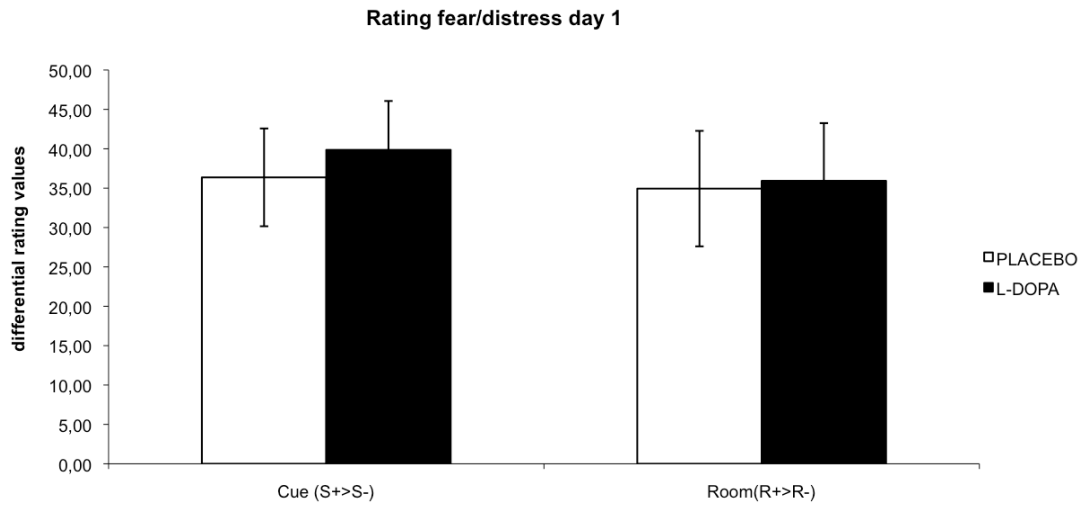

Figure R6. Average of the differential score of cue (S+>S-) and context (R+>R-) ratings of fear/distress during acquisition of fear conditioning on day 1. Error bars indicate the SEM.

A repeated measurements 2x3x2x2 ANOVA (with the factor time (2)) extended the results, with a significant main effect of time ($F(1,1)=61.13$; $p<0.001$) as well as interaction effects between condition and time ($F(1,1.9) =46.33$; $p<0.001$) and

between stimulus, condition and time ($F(1,1.4) = 4.85$; $p=0.021$). In addition, the above interaction effect between stimulus and group was no longer significant ($p>0.2$).

These results were further qualified through a significant increase in the predictable ($F(1,1)=67.11$; $p<0.001$) and unpredictable condition ($F(1,1)=87.84$; $p<0.001$) compared to the safe condition. The interaction between stimulus, condition and time was due to the significant increasing difference between the ratings to the symbol and the room in the predictable condition as compared to the safe condition ($F(1,1)=5.30$; $p=0.028$). This suggests that learning may have taken place at different pace between conditions.

Paired t-test (one-sided) of the second half on day 1 revealed significant results for the indices of cue ($T(1,35)=10.15$; $p<0.001$) and context conditioning ($T(1,35)=11.86$; $p<0.001$). Furthermore, these indices were not different between groups (two-sided unpaired t-test; $p>0.6$)

4.1.1.3 Summary

Results indicated successful cue conditioning as well as a context conditioning in SCRs and ratings of fear/distress. Both dependent measurements increased over time as an effect of learning. The null hypothesis 1A can be rejected. Moreover, no differences between groups on day 1 were observed, thus the null hypothesis 1B does not have to be rejected.

4.1.2 Day 2

In the experiment on day 2, the US was absent in every condition. Consequently subjects learned extinction, indicated as a decrease of conditioned responding over time.

The analysis of day 2 tested if coherence existed between extinction learning and the dependent variables, SCR and ratings of fear/distress (Hypothesis 2A). Furthermore, these analyses tested if groups already differed before the intake of either placebo or L-DOPA (Hypothesis 2B).

4.1.2.1 SCR

The 2x3x2 ANOVA (placebo N =17; verum N=17) revealed a significant result for the main effect of stimulus ($F(1,1)=5.13$; $p=0.031$) and a significant interaction effect between stimulus and condition ($F(1,1.8)=3.74$; $p=0.033$). No group main effects or interactions were observed. See figures R7 and R8.

Simple comparisons of the conditions that were associated with the US (predictable condition and unpredictable condition) compared with the safe condition revealed no significant results ($p>0.4$). The interaction between stimulus and condition was further qualified through higher responses to the stimulus (S+) compared to the room in the predictable condition as compared to the safe condition ($F(1,1)=9.20$; $p=0.005$). Paired t-test (one-sided) of cue conditioning (S+>S-) ($T(1,35)=1.92$; $p=0.032$) revealed a significant result, whereas the t-test for the index of context conditioning (R+>R-) was not significant ($p>0.9$).

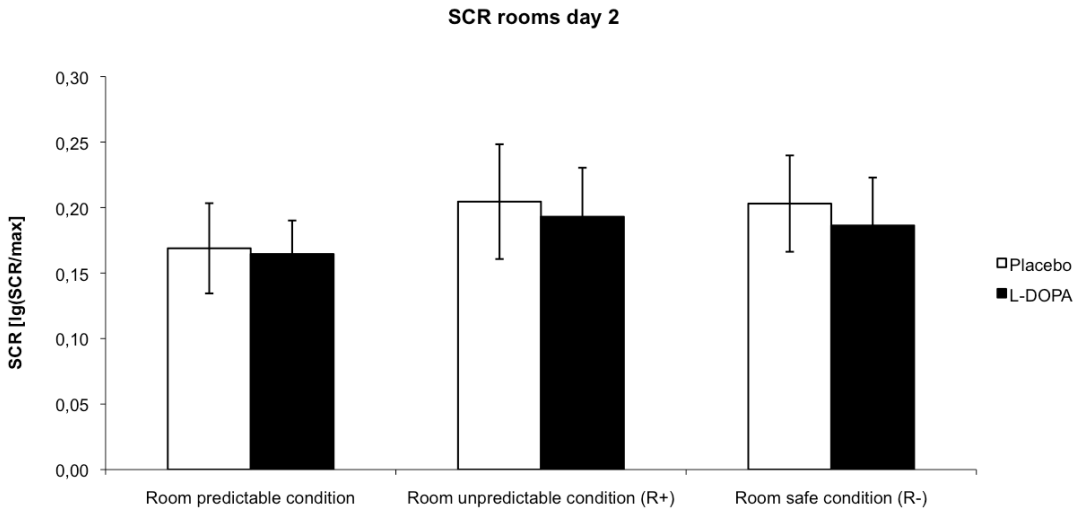

Figure R7. Average of the SCR for rooms in each condition during extinction learning on day 2. Error bars indicate the SEM

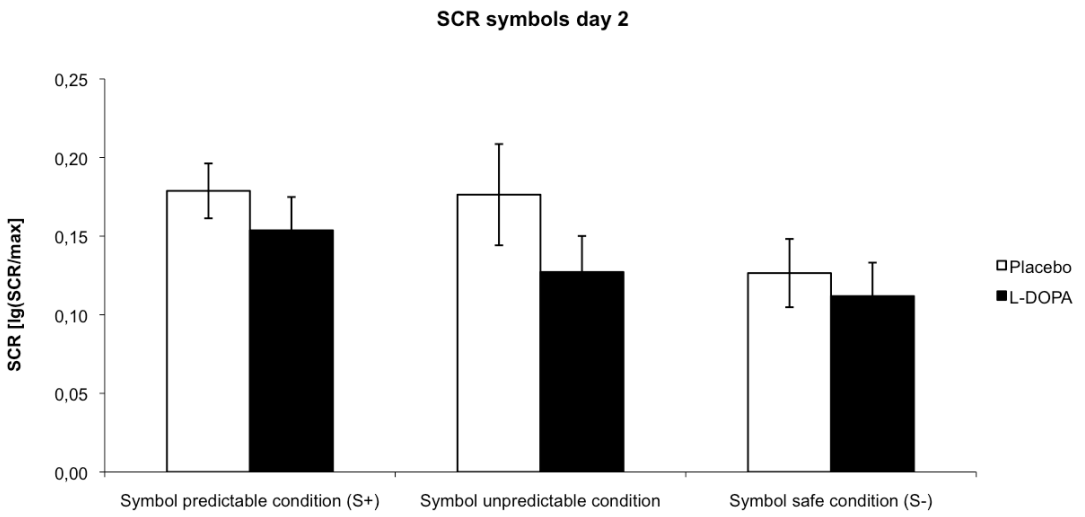

Figure R8. Average of the SCRs for symbols in each condition during extinction learning on day 2. Error bars indicate the SEM.

A repeated measurements 2x3x2x2 ANOVA (with the factor time (2) (first half/second half)) extended the results, with a significant main effect of time ($F(1,1)=51.85$; $p<0.001$) and a trend-wise interaction between stimulus and time ($F(1,1)=3.79$; $p=0.060$).

To analyze successful recall of cued and contextual fear, post-hoc paired t-tests (one-sided) of average values during the first half of extinction learning were conducted. Results indicated recall of cued fear (S+>S-) ($T(1,33)=3.91$; $p<0.001$). Results for recall of contextual fear (R+>R-) were not significant ($p>0.5$). All those effects were absent during the second half ($p>0.3$).

Cue and context conditioning indices during extinction learning are illustrated as a timecourse in figure R9 and R10, respectively.

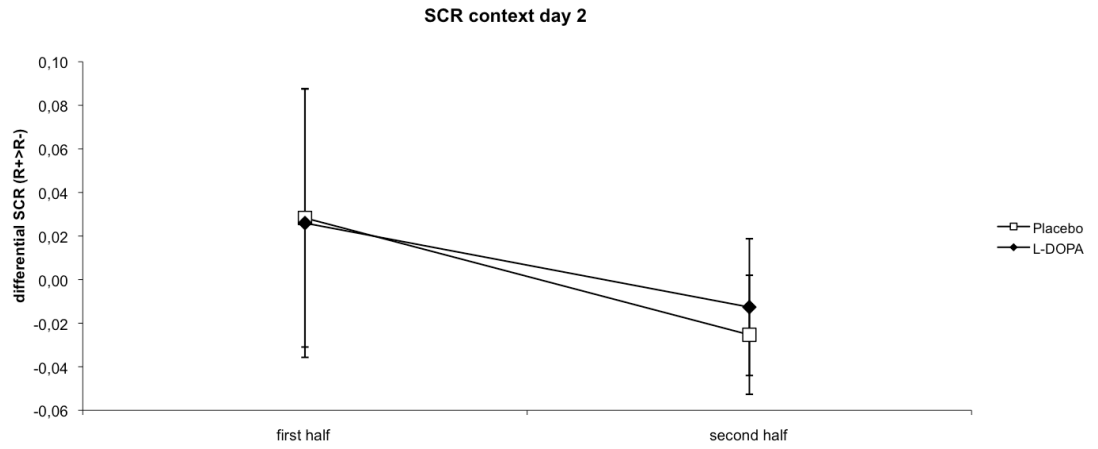

Figure R9. Averages of the differential SCRs of context conditioning(R+>R-) during extinction learning on day 2. Error bars indicate the SEM.

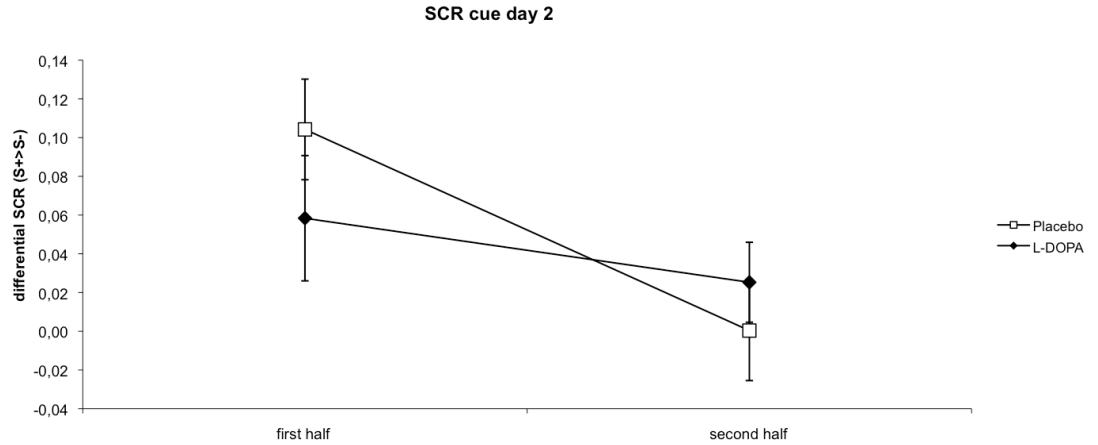

Figure R10. Averages of the differential SCRs of cue conditioning (S+>S-) during extinction learning on day 2. Error bars indicate the SEM.

In sum, analyses of the SCR on day 2 indicated successful extinction learning. Furthermore there were no pre-existing differences between groups.

4.1.2.2 Ratings of fear/distress

The 2x3x2 ANOVA (placebo N =19; verum N=16) revealed a significant result for the main effect of condition ($F(1,1.7) =42.70$; $p<0.001$) and a significant interaction effect between stimulus and condition ($F(1,1.8)=6.86$; $p=0.003$). No main effect or interaction of the factor group was observed.

Simple comparisons indicated significant higher responding in conditions that were associated with the US (predictable condition > safe condition ($F(1,1)=49.75$; $p<0.001$) and unpredictable condition > safe condition ($F(1,1)=56.42$; $p<0.001$)). The interaction between stimulus and condition was qualified through a significant difference between rooms and symbols comparing the unpredictable to the safe condition ($F(1,1)=13.45$; $p=0.001$)

Paired t-tests (2-sided) yielded a significant effect of cue conditioning (S+>S-) ($T(1,35)=6.23$; $p<0.001$) and context conditioning (R+>R-) ($T(1,34) =7.68$; $p<0.001$).

Day 2 consisted of only 3 ratings, hence only these 3 time points can be used in the ANOVA with the additional between subject factor time. Furthermore, the values in this ANOVA are single trials, which means that subjects with one missing value in one trial (due to missing confirmation of the rated value) were excluded in the whole ANOVA. Thus, the group sizes in this ANOVA are very low and not balanced between groups (subjects that responded correctly in every trial: placebo N=10, verum N=5).

Repeated measurements 2x3x3x2 ANOVA (with the factor time (3)) (placebo N =10; verum N=5) extended the results of the first ANOVA, with a significant main effect of time ($F(1,1.2)=12.74$; $p=0.002$) and stimulus as a trend ($F(1,1)=3.154$; $p=0.099$). In addition, analyses revealed a significant interaction between condition and time ($F(1,2.1)=12.90$; $p<0.001$) and a significant interaction between stimulus, condition and time ($F(1,2.7)=3.36$; $p=0.032$). Furthermore, the interaction between stimulus, condition, time and group yielded a trend-wise result ($F(1,2.8)=2.89$; $p=0.064$). No main effect of the factor group was observed.

The interaction of stimulus and time was further qualified through a significant decrease (1^{st} rating vs 3^{rd} rating) in the both conditions that were associated with the shock, as compared to the safe condition (predictable condition vs safe condition ($F(1,1)=19.92$; $p=0.001$); unpredictable condition vs safe condition ($F(1,1)=19.23$; $p=0.001$)).

The interaction between all between subject variables and the factor group was not further analysed, due to the unbalanced group sizes in the ANOVA.

Average rating values of fear/distress in the extinction are illustrated in figures R11 and R12.

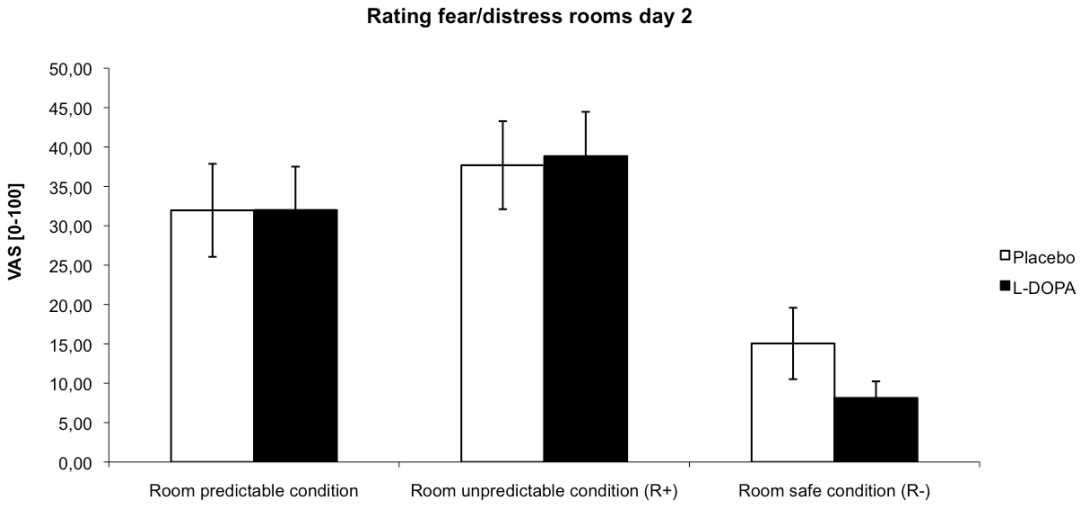

Figure R11. Average of the fear/distress rating for the rooms in each condition during extinction learning on day 2. Error bars indicate the SEM.

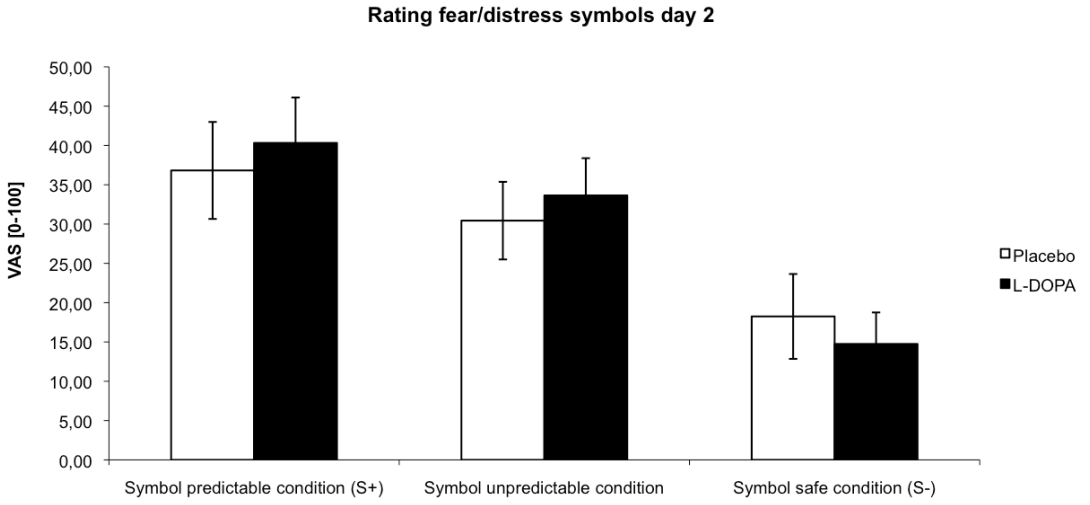

Figure R12. Average of the fear/distress rating for the symbols in each condition during extinction learning on day 2. Error bars indicate the SEM.

To analyze successful recall of cued and contextual fear, post-hoc paired t-tests (one-sided) of average values during the first rating of the experiment (before extinction learning) were conducted. Results indicated significant recall of cued fear (S+>S-) ($T(1,26) = 5.27$; $p<0.001$) and contextual fear (R+>R-) ($T(1,29) = 8.27$; $p<0.001$).

Cue and context conditioning indices during extinction learning are illustrated in the line graphs of figure R13 and R14, respectively.

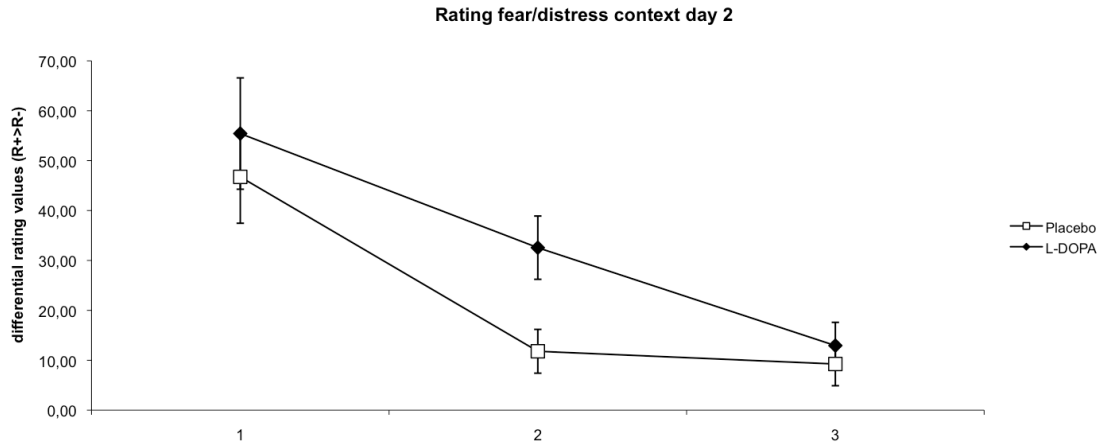

Figure R13. Fear/distress ratings for the differential score of context (R+>R-) conditioning during extinction learning on day 2. The first data point represents the rating before the extinction learning. Error bars indicate the SEM.

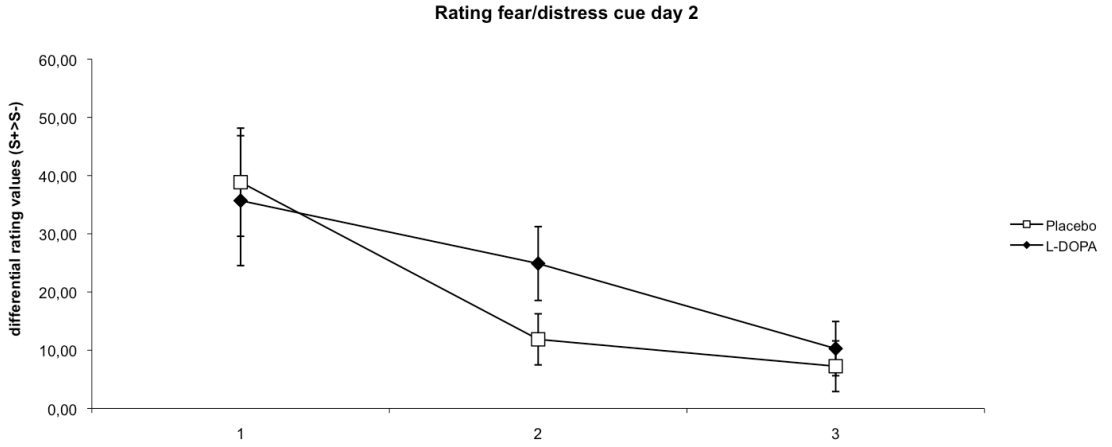

Figure R14. Fear/distress ratings for the differential score of cue (S+>S-) conditioning during extinction learning on day 2. The first data point represents the rating before the extinction learning. Error bars indicate the SEM.

4.1.2.3 Summary

Results of the analyses indicated successful extinction learning in measurement of SCRs and rating of fear/distress. The null hypothesis 2A can be rejected.

Furthermore, no differences between groups were observed before the intake of drug, thus the null hypothesis 2B does not have to be rejected.

4.1.3 Day 8

The experiment on day 8 consisted of two phases: first, a phase of spontaneous recovery that was subsequently followed by reinstatement shocks and a phase of post-reinstatement fear recall. Analyses were separated for these two phases.

Statistical analyses tested if coherence existed between the dependent variables and the manipulation of spontaneous recovery and reinstatement, respectively (Hypothesis 3A and 4A). Reinstatement was indicated through the comparison of return of fear in the post-reinstatement phase relative to the phase of spontaneous recovery ((R+ > R-) post-reinstatement > (R+ > R-) spontaneous recovery). Furthermore, these analyses tested if the intake of L-DOPA on day 2 led to different responses in spontaneous recovery (Hypothesis 3B) or reinstatement of fear (Hypothesis 4B).

Because day 8 took place in the MR scanner and both skin conductance and ratings on day 8 were recorded with a different apparative set-up than on days 1 and 2, we refrained from any comparison of SCRs between days (esp. day 8 vs. day 2), which could otherwise serve as a test for return of fear (Vervliet et al. 2012).

The hypothesised effect of the pharmacological treatment was enhanced recall of extinction memory, that is, decreased return of fear during spontaneous recovery and reinstatement in the L-DOPA group.

4.1.3.1 Spontaneous Recovery

4.1.3.1.1 SCR

The 2x3x2 ANOVA (placebo N =15; verum N=15) revealed a significant main effect of condition ($F(1,1.9)=5.217$; $p=0.009$). No main effect of the factor group or any interactions were observed.

Simple comparisons indicated significantly higher responding the unpredictable condition as compared to the safe condition ($F(1,1)=8.530$; $p<0.007$) and, as a trend, higher responding in the predictable condition as compared to the safe condition ($F(1,1)=3.159$; $p=0.086$).

The average SCRs in the phase of spontaneous recovery are illustrated in figures R15 and R16.

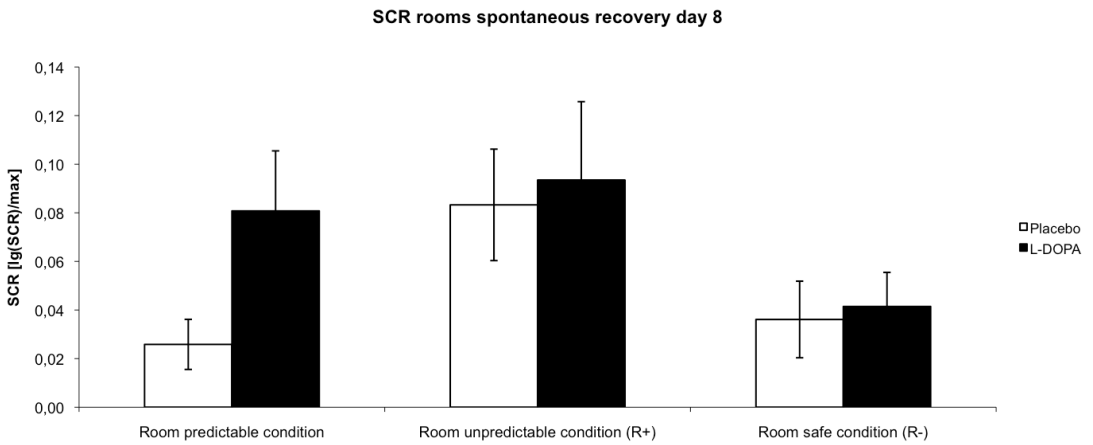

Figure R15. Average of the SCRs for rooms in each condition during spontaneous recovery on day 8. Error bars indicate the SEM.

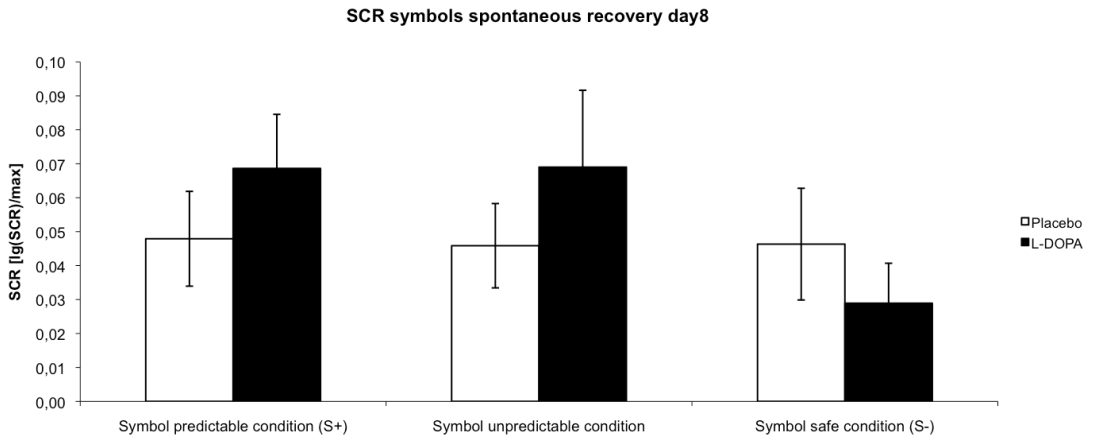

Figure R16. Average of the SCRs for symbols in each condition during spontaneous recovery on day 8. Error bars indicate the SEM.

Paired t-tests (one-sided) yielded a significant result for context conditioning (R+>R-(T(1,29) = 2.70; p=0.006)), and a trend for the effect of cue conditioning (S+>S-(T(1,29)=1.68; p=0.052)).

Figure R17 displays the indices of cued and contextual conditioned fear during the spontaneous recovery phase.

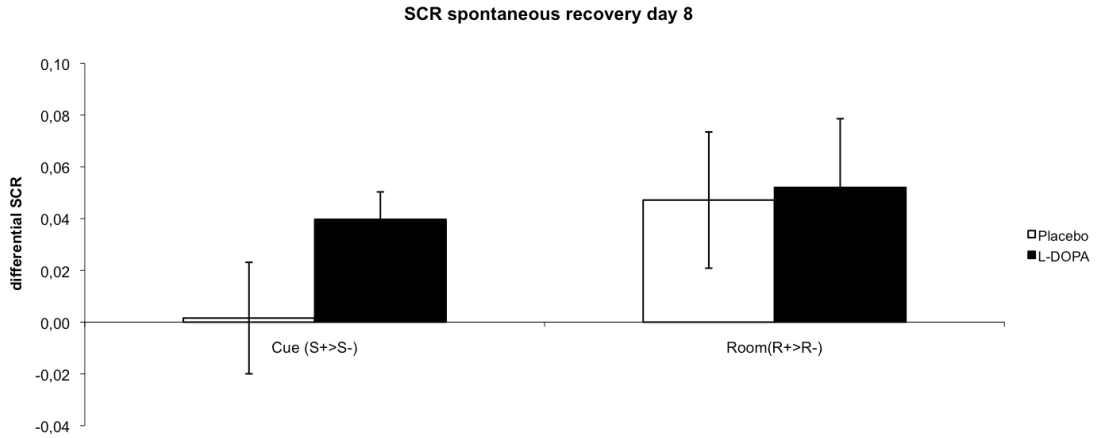

Figure R17. Average of the differential score of cue (S+>S-) and context (R+>R-) SCRs during the phase of spontaneous recovery on day 8. Error bars indicate the SEM.

4.1.3.1.2 Ratings of fear/distress

The 2x3x2 ANOVA (placebo N=17; verum N=19) revealed a significant main effect of condition ($F(1,1.1)=17.90$; $p>0.001$). No main effect of the factor group or any interactions were observed.

Simple comparisons indicated significantly higher responding in conditions that were paired with the US on day 1 (predictable condition > safe condition ($F(1,1)=18.81$; $p<0.001$) and unpredictable condition > safe condition ($F(1,1)=19.47$; $p<0.001$)).

These results are illustrated in figure R18 and R19, respectively.

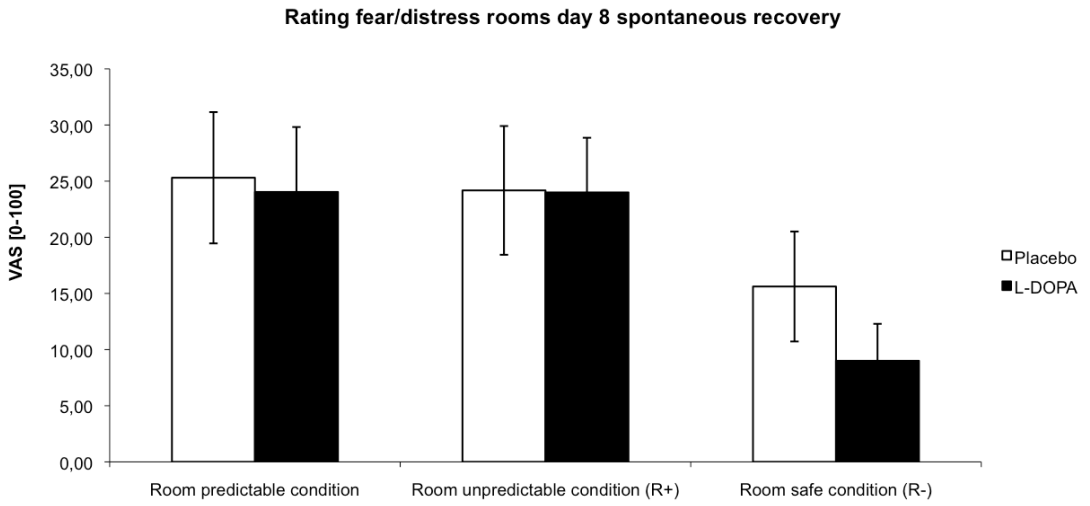

Figure R18. Average of the ratings of fear/distress for rooms in each condition during spontaneous recovery on day 8. Error bars indicate the SEM.

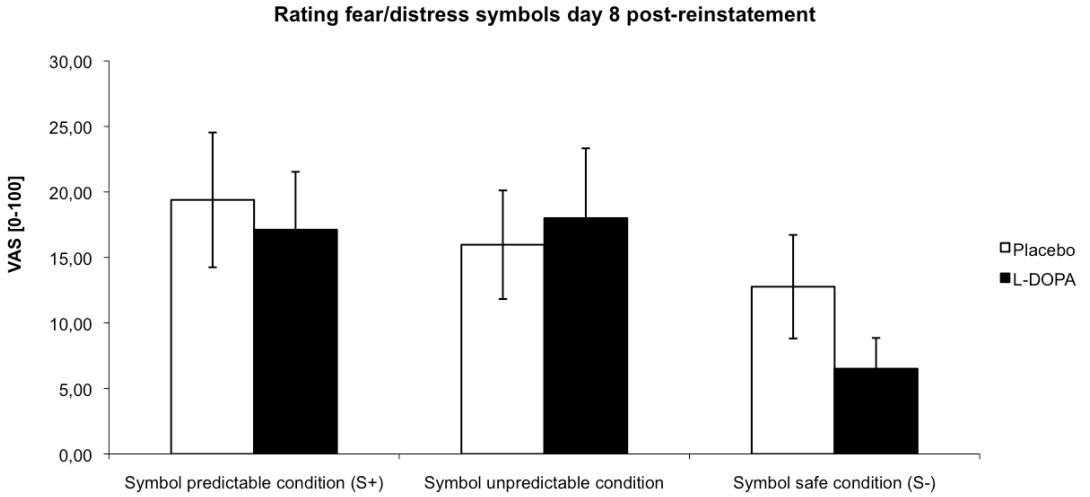

Figure R19. Average of the ratings of fear/distress for symbols in each condition during spontaneous recovery on day 8. Error bars indicate the SEM.

In addition, significant effects for spontaneous recovery of contextual (R+>R-) ($T(1,36)=4.88$; $p<0.001$) and cued (S+>S-) ($T(1,35)=4.01$; $p<0.001$) fear were observed.

The indices of cued and contextual fear recall during spontaneous recovery were displayed in figure R20.

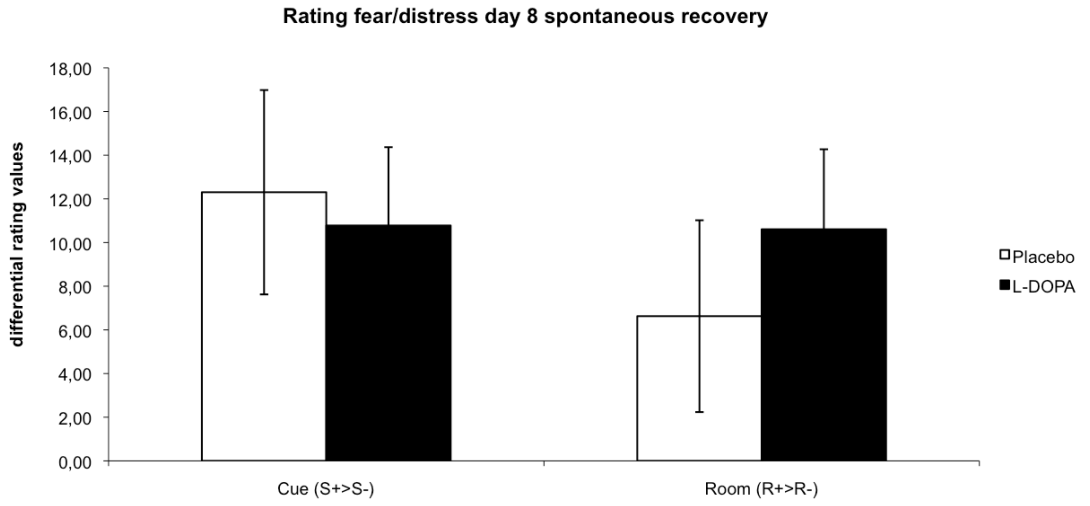

Figure R20. Average of the differential score of cue (S+>S-) and context (R+>R-) ratings of fear/distress during spontaneous recovery on day 8. Error bars indicate the SEM.

4.1.3.1.3 Summary

Analyses of the SCR and ratings of fear/distress revealed spontaneous recovery of conditioned fear responses. Unexpectedly, no hypothesised interactions with the factor group were observed.

4.1.3.2 Post-reinstatement

4.1.3.2.1 SCR

The 2x3x2 ANOVA (placebo N=15; verum N=15) of the SCR in the phase after the reinstatement shocks revealed no significant effects. See figures R21 and R22.

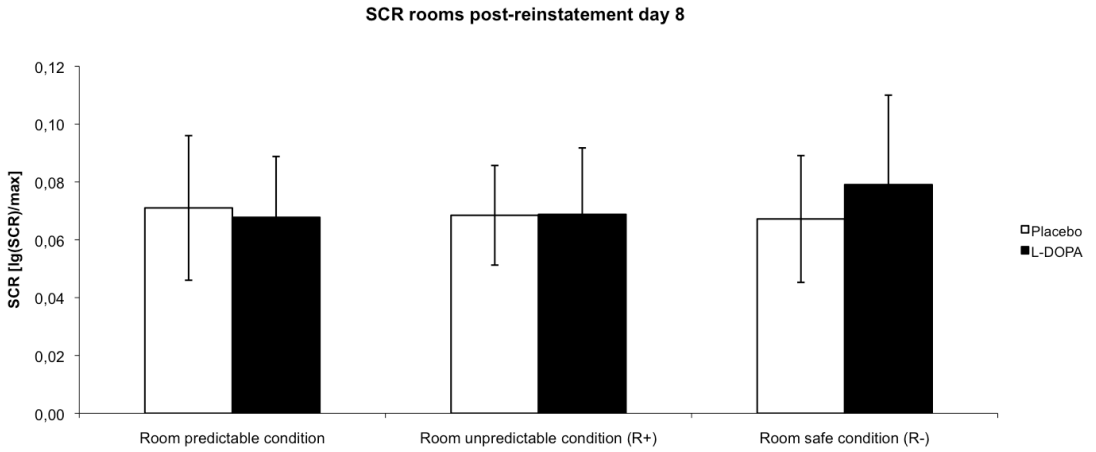

Figure R21. Average of the ratings of the SCR for each room in each condition after reinstatement on day 8. Error bars indicate the SEM.

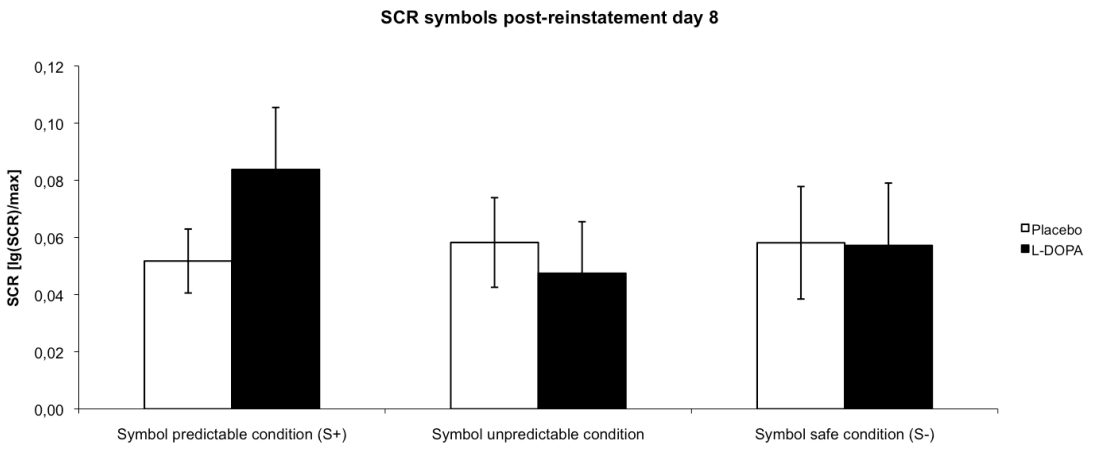

Figure R22. Average of the ratings of the SCR for each symbol in each condition after reinstatement on day 8. Error bars indicate the SEM.

Average values of the a priori indices of reinstatement were negative, indicating no effect of reinstatement. Paired t-tests of the indices of cued and contextual fear were not significant (p>0.4). Post-hoc tests (paired t-test, two-sided) only revealed a higher value for the room in the safe condition (R-) during the reinstatement phase compared to the spontaneous recovery phase, as a trend (t(1,29)=1.837; p=0.076).

The trend-like reinstatement in the safe condition together with the lack of a main effect of conditions after reinstatement might indicate an unspecific effect of reinstatement in all conditions.

4.1.3.2.2 Ratings of fear/distress

The 2x3x2 ANOVA (placebo N =17; verum N=20) of the rating values after the reinstatement revealed a significant result for the main effect of condition (F(1,1.3)=11.67; p=0.001). No interaction or main effect of the factor group was observed. See figures R23 and R24.

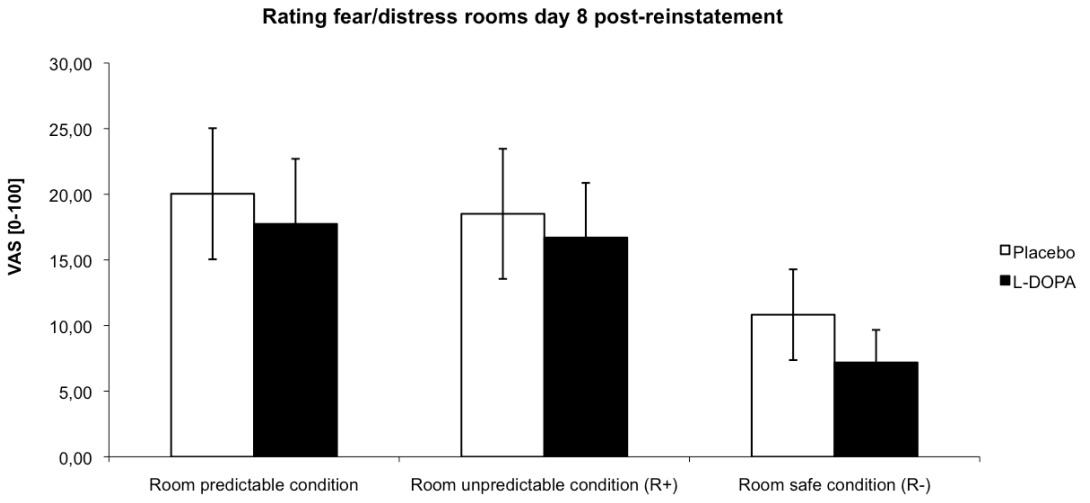

Figure R23. Average of the ratings of fear/distress for rooms in each condition after reinstatement on day 8. Error bars indicate the SEM.

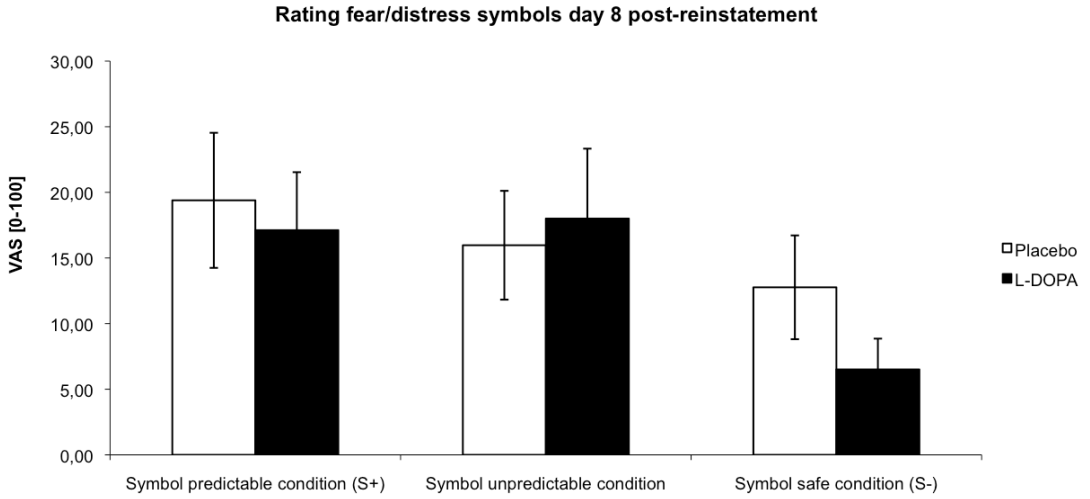

Figure R24. Average of the ratings of fear/distress for rooms in each condition after reinstatement on day 8. Error bars indicate the SEM.

The average values of the planned indices for reinstatement were negative, analogue to the SCR. Simple comparisons indicated significantly higher responding in conditions that were paired with the US on day 1 (predictable condition > safe condition ($F(1,1)=12.40$; $p=0.001$) and unpredictable condition > safe condition ($F(1,1)=13.14$; $p=0.001$)). In addition, a significant effect for the recall of contextual (R+ > R-) ($T(1,36) = 3.13$; $p=0.003$) and cued (S+ > S-) ($T(1,36) = 4.28$; $p<0.001$) fear was observed (see figure R25).

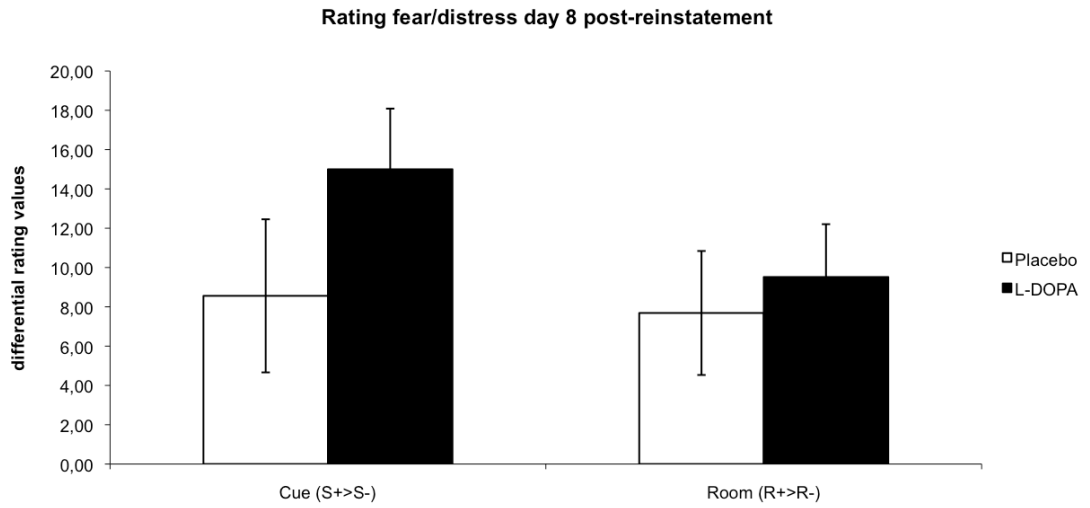

Figure R25 average of the differential score of cue (S+>S-) and context (R+>R-) ratings of fear/distress after reinstatement on day 8. Error bars indicate the SEM.

The results of the ANOVA indicated fear responses after reinstatement.

4.1.3.3 Summary and discussion

In sum, the analyses of spontaneous recovery revealed fear responses in the conditions that had been associated with a shock on day 1. The null hypothesis 3A can be rejected. Planned indices of reinstatement were negative or not suitable in this experiment. SCRs suggested an unspecific effect of reinstatement in all conditions whereas the ratings of fear/distress revealed a main effect of condition in the phase of reinstatement that was however not more pronounced than during spontaneous recovery. Therefore, the null hypothesis 4A cannot be rejected. More importantly, no interactions of the factor group were observed, unexpectedly. The null hypotheses 3B and 4B cannot be rejected.

The spontaneous recovery of fear responses on day 8 was unexpectedly weak. Comparing the rating values of the spontaneous recovery phase on day 8 to the last rating on day 2, revealed that there was no significant difference ($p>0.4$). This could be due to a good extinction learning on day 2 and a strong extinction memory recall. This strong extinction memory would also explain the lack of behavioural reinstatement effects. The differences between treatment groups may thus be diminished trough a “floor-effect” of low fear responses.

4.1.4 fMRI (day 8)

The analysis of the imaging data focussed on the critical question whether L-DOPA administered after extinction learning on day 2 changed neural indices of return of fear a week later.

The over-arching hypothesis was that L-DOPA enhances cued and contextual extinction memory recall and hence reduces neural correlates of return of fear in spontaneous recovery and after reinstatement shocks.

Consequently, Hypothesis 5B was that neural indices of cue and contextual conditioned fear in the phase of spontaneous recovery were larger in the placebo than

in the L-DOPA group (R+>R- in placebo>verum and S+>S- in placebo>verum). Hypothesis 6B was that the placebo group showed higher reinstatement of fear compared to the L-DOPA group. As the specific behavioural indices of reinstatement (comparison of fear after vs. before reinstatement shocks) had not given any significant results, the latter analysis was restricted to cued and contextual fear during the post-reinstatement phase (R+>R- in placebo>verum and S+>S- in placebo>verum).

4.1.4.1 Spontaneous recovery

4.1.4.1.1 Cued fear (S+>S-)

The contrast of cued fear in the categorical estimates revealed higher heamodynoamic responses for the placebo group as compared to L-DOPA group, in the left posterior hippocampus, extending into the collateral sulcus, the right amygdala and, as a strong trend, in the right dmPFC (see Table R2). In addition higher contrast estimates were observed in the right insula (MNI x,y,z coordinates=56;-8;0; T(1,114)=3.83; Z=3.71; p<0.001(uncorrected)) at a threshold of p=0.001 uncorrected. The group comparisons of the parametrically decreasing regressors revealed no differences in the ROIs.

Table R1. Activation during the phase of spontaneous recovery, comparing the categorical index of cue conditioned fear between groups (S+>S- and placebo>verum) in the ROIs.

Region	X	Y	Z	T	Z	P(SVC)	P(uncorrected)
R dmPFC	10	46	26	3.54	3.44	0.051	<0.001
L post HC	-36	-32	-14	3.42	3.33	0.017	<0.001
R amygdala	20	0	-24	3.29	3.16	0.043	=0.001

Activations in the dmPFC, left posterior hippocampus and right amygdala in the contrast for cue fear conditioning in the placebo group as compared to the L-DOPA group are displayed in figures R26 and R27.

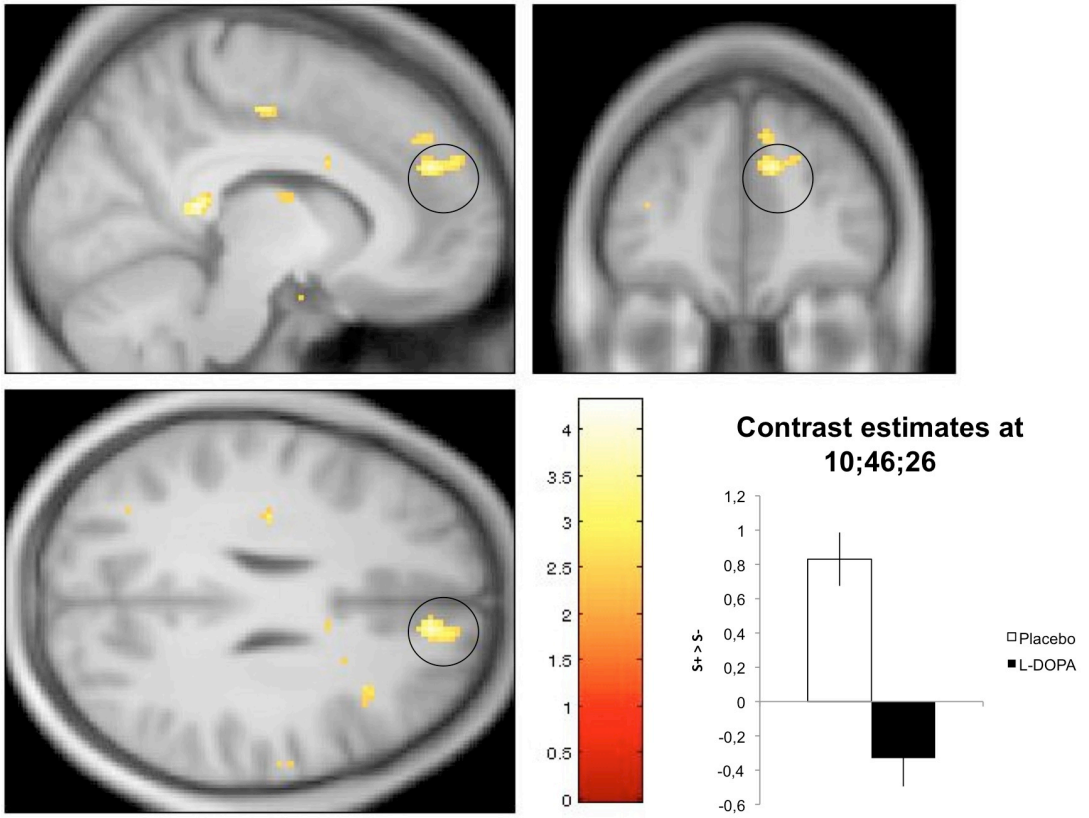

Figure R26. Significant voxels in the categorical contrast of S+>S- in placebo>verum at x=10; y=46; y=26. The resulting T-map is overlaid on a mean structural image, normalised into the NMI space. The bottom bar graphs illustrate the average contrast estimates in the peak voxel (Error bars indicate the SEM). The display threshold is set to p<0.01 uncorrected and the colour bar indicates T-scores.

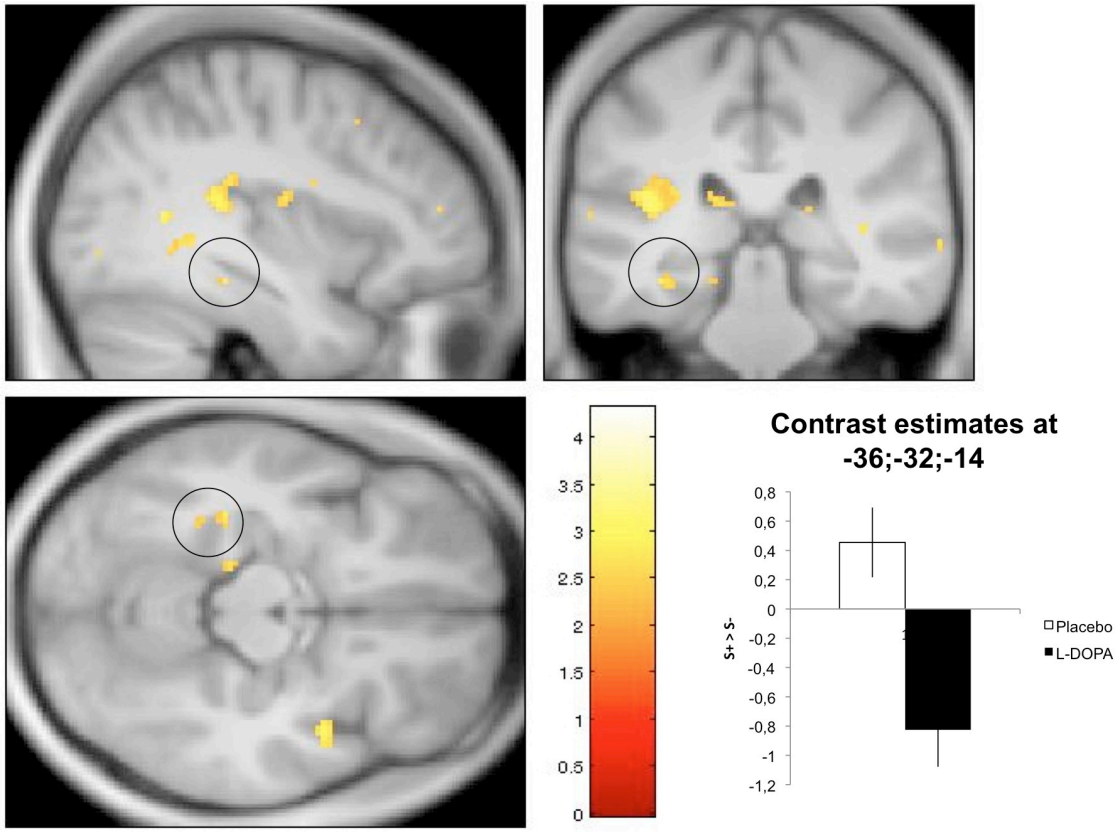

Figure R27. Significant voxels in the categorical contrast of S+>S- in placebo>verum at x=-36; y=-32; y=-14. The resulting T-map is overlaid on a mean structural image, normalised into the NMI space. The bottom bar graphs illustrate the average contrast estimates in the peak voxel (Error bars indicate the SEM). The display threshold is set to p<0.01 uncorrected and the colour bar indicates T-scores.

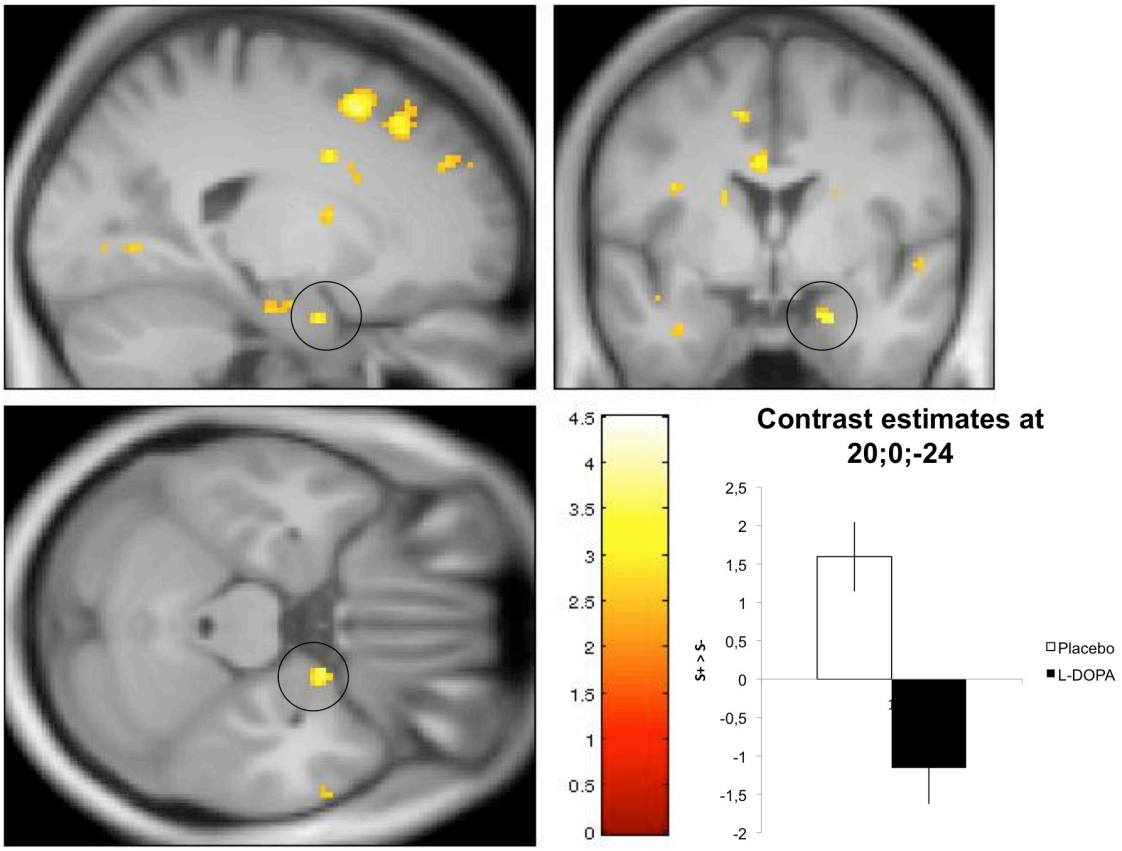

Figure R28. Significant voxels in the categorical contrast of S+>S- in placebo>verum at x=-20; y=0; y=-24. The resulting T-map is overlaid on a mean structural image, normalised into the NMI space. The bottom bar graphs illustrate the average contrast estimates in the peak voxel (Error bars indicate the SEM). The display threshold is set to p<0.01 uncorrected and the colour bar indicates T-scores.

4.1.4.1.2 Contextual fear (R+>R-)

The contrast of contextual fear revealed no significant activation in the ROIs in the categorical or parametric estimates. However, this contrast at a threshold of p=0.001(uncorrected) revealed higher heamodynamic responses for the placebo group as compared to the L-DOPA group in the left ventral forebrain, dorsal to the amygdala (-18;-8;-10;T(1,114)=3.41;Z=3.32;p<0.001(uncorrected)).

4.1.4.2 After Reinstatement

4.1.4.2.1 Cued fear (S+>S-)

The contrast of cued fear revealed no significant categorical heamodynoamic response differences in the ROIs between the placebo group and the L-DOPA group. The same contrast of parametrically decreasing responses revealed trend-wise higher contrast estimates in the right anterior hippocampus for the placebo group. That is, responses in this hippocampal region decayed more quickly in the placebo than in the L-DOPA group.

Table R2. Activation after reinstatement comparing the linearly decreasing responses in the contrast of cued fear between groups (S+ > S- in placebo>verum) in the ROIs.

Region	X	Y	Z	T	Z	P(SVC)	P(uncorr)
L ant HC	28	-16	-24	2.66	3.62	0.082	0.004

4.1.4.2.2 Contextual fear (R+>R-)

The contrasts of contextual fear revealed, in the categorical estimates, trend-wise higher heamodynoamic responses for the placebo group as compared to the L-DOPA group in right posterior hippocampus extending into the collateral sulcus (see Table R3). In addition, at a threshold of p=0.001 (uncorrected), higher contrast estimates were observed in the ACC (16;40;10; T(1,114)=3.39; Z=3.30; p<0.001), in the bilateral ventral forebrain, dorsal to the amygdala (-16;-8;-12; T(1,114)=3.74; Z=3.62; p<0.001 and -18;-10;-10; T(1,114)=3.34; Z=3.26; p=0.001) and the right nucleus accumbens (10;14;-4; T(1,114)=3.28; Z=3.20; p=0.001) .

Table R3. Activation after reinstatement comparing the categorical index of contextual fear between groups (R+>R- and placebo>verum) in the ROIs.

Region	X	Y	Z	T	Z	P(SVC)	P(uncorr)
R post HC	36	-32	-18	2.82	2.76	0.055	0.003

The same contrast of parametrically decreasing responses revealed higher estimates for the placebo group in comparison to the L-DOPA group in the left vmPFC. In addition, at a threshold of p=0.001 uncorrected, higher estimates in the right vmPFC (outside the ROI volume) was observed (8;30;-24; T(1,114)=3.65; Z=3.55; p<0.001). The effect in the left vmPFC was mainly driven by a decrease over time in the

responses to the R+ (room in the unpredictable condition) in the placebo group that was abolished in the L-DOPA group. Conversely, in this group the responses to the R- (room in the safe condition) showed an increasing pattern (see figure R28).

Table R4. Activation after reinstatement comparing the linearly decreasing responses in the contrast of contextual fear between groups (R+>R- and placebo>verum) in the ROIs.

Region	X	Y	Z	T	Z	P(SVC)	P(uncorr)
L vmPFC	-2	44	-20	3.44	3.35	0.048	<0.001

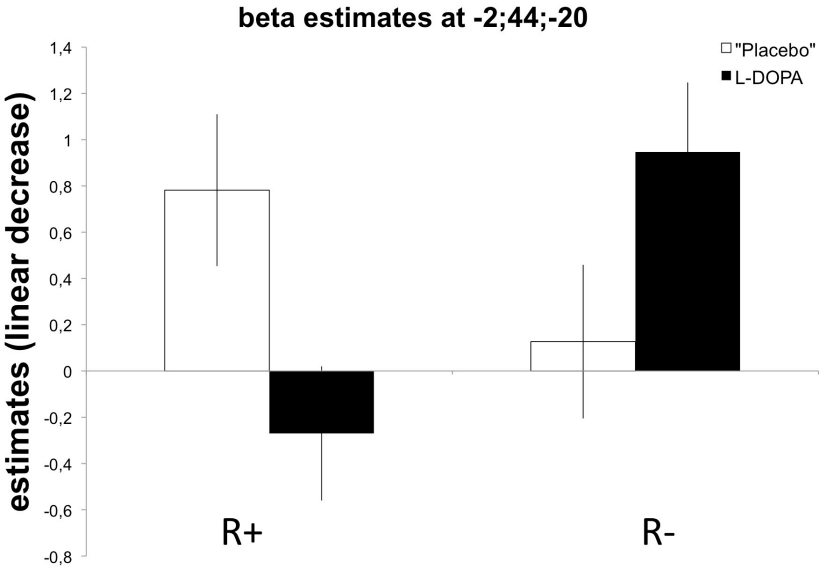

Figure R29. Average contrast estimates in the peak voxel of the left vmPFC in the linear decrasing responses at x=-2; y=44; y=-20. Error bars indicate the SEM.

4.1.4.3 Summary and discussion fMRI

Analyses of the ROIs revealed differences between groups on day 8 in the contrasts for return of contextual and cued fear. The placebo group showed higher heamodynamic responses in the posterior hippocampus and the dmPFC (as a trend) during spontaneous recovery of cued fear. In the phase after reinstatement, the index of contextual fear revealed trend-wise higher contrast estimates in the posterior hippocampus for the placebo group. In addition, after reinstatement, the placebo group had higher contrast estimates for decreasing responses in the vmPFC in the

contrast of contextual fear and in the anterior hippocampus (as a trend) in the contrast of cued fear. In sum, the placebo group showed higher neural responses in brain regions that were associated with fear recall during spontaneous recovery and after reinstatement. In addition, regions that have been associated with recall of extinction in previous studies reacted to R+ presentation after reinstatement with a decreasing response in the placebo group. This might reflect a gradual inhibition of these fear-inhibitory areas in response to the reinstatement, which was not observed in the L-DOPA group.

4.2 Results Study B

4.2.1 Day 1

During the experiment on day 1, subjects were fear conditioned to a geometric figure and subsequently learned extinction in a different context, indicated through the change of background colour. Analyses of the dependent variables were thought to reflect this task performance. Kalisch and colleagues (2006) reported successful conditioning and extinction, that is, context discrimination based on SCRs.

Ratings of US expectancy have been found to reflect associative learning processes (Iberico et al. 2008 , Vansteenwegen et al. 2008). Ratings of fear and distress were thought to reflect the emotional valence of the stimuli. A similar fear/distress rating scale was used in our group before and had shown effects of fear conditioning and extinction learning (Raczka et al. 2011).

The analyses tested if coherence existed between the manipulation of fear conditioning and extinction learning and the dependent variables, SCR and both rating scales (Hypothesis 1A). Successful fear conditioning was defined as higher differential (CS+ > CS-) responses in the context of acquisition (context A) and successful extinction learning was defined as higher differential (CS+ > CS-) responses in context A than in the context of extinction (context E): (CS+ >CS-)A > (CS+>CS-)E (in accordance with Kalisch et al. 2006)). Furthermore, it was tested if both groups already differed before the intake of either placebo or verum (Hypothesis 1B).

4.2.1.1 Contingency ratings (stimuli and context)

Participants were asked to rate the contingency of each geometrical figure and the background colours with the US at the end of the experiment on day 1. Paired t-test (one-sided) revealed that the subjects rated contingencies higher for the CS+ in comparison to the CS- ($T(1,37)=11.44$; $p<0.001$) and for the context A in comparison to the context E ($T(1,37)=9.30$; $p<0.001$). Comparisons between the groups (unpaired, two-sided t-test) revealed no differences (CS+ ($T(1,36)=0.544$; $p=0.590$), CS- ($T(1,36)=-1.515$; $p=0.139$), context A ($T(1,36=0.930$; $p=0.359$) or context E ($T(1,33.8)=0.865$; $p=0.393$)).

As illustrated in figure R30, participants exhibited context-dependent differential SCRs on day 1. More specifically, the differential responses were elevated in context A (A1-A6) and decreased in context E (E1-E6).

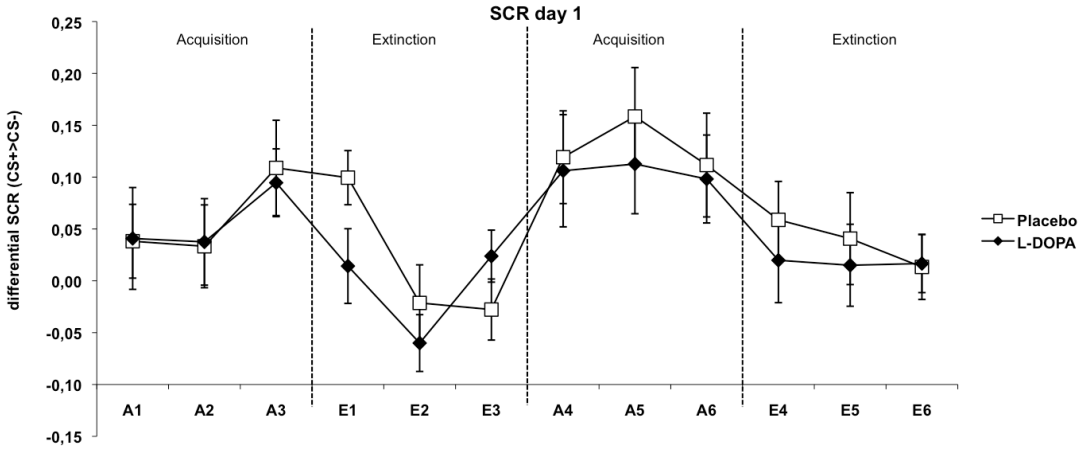

Figure R30. Block-wise (A=Acquisition block / E=Extinction block) average (Block = 8 CS presentation) of differential SCR (CS+>CS-) on day 1. Error bars indicate the SEM.

The same pattern can be observed in the graphs showing the US expectancy ratings (figure R31) and the ratings of fear/distress (figure R32).

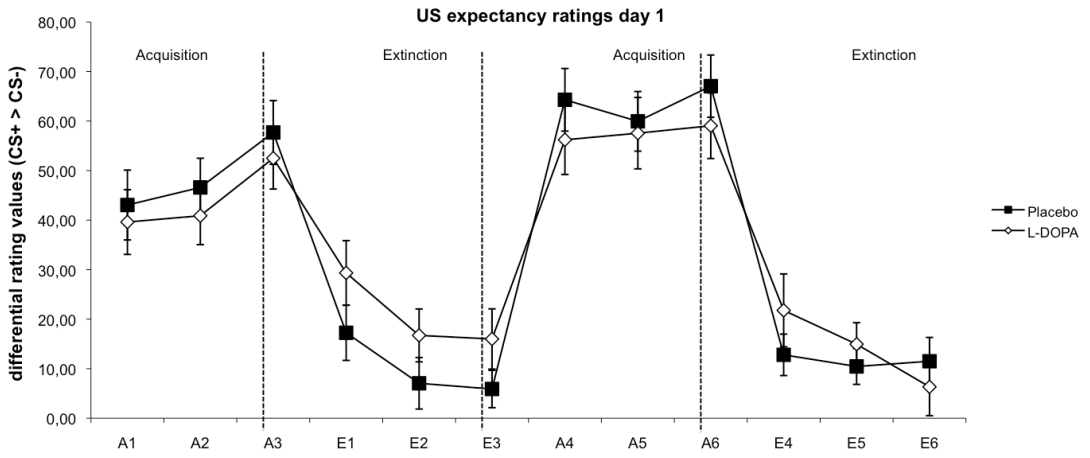

Figure R31. Single differential rating trials of US expectancy (CS+>CS-) on day 1. Error bars indicate the SEM.

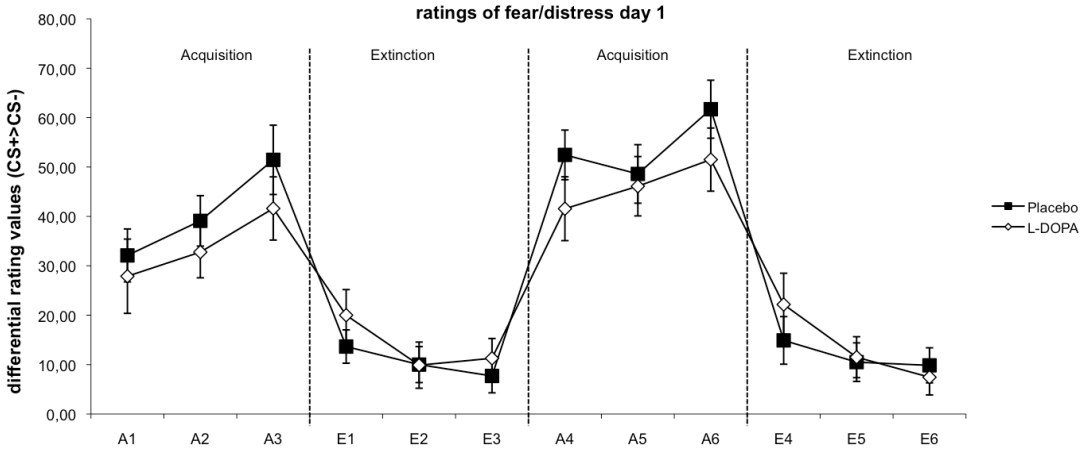

Figure R32. Single differential rating trials of fear/distress (CS+>CS-) on day 1. Error bars indicate the SEM.

4.2.1.2 SCR

The 2x2x2 ANOVA of the SCR revealed significant main effects of stimulus ($F(1,1)= 27.266$; $p <0.001$) and context ($F(1,1)= 25.683$; $p <0.001$) as well as a significant stimulus by context interaction ($F(1,1)=10.592$; $p =0.003$). No group main effects or interactions were observed.

Paired t-tests (one-sided) indicated that SCRs were significantly higher for the CS+ as compared to the CS- in context A ($T(1,29)=4.821$; $p<0.001$), whereas this was only a trend in context E ($T(1,29)=1.47$; $p=0.077$). As expected, differential responses (CS+>CS-) were significantly higher in the acquisition context as compared to the extinction context ((CS+ > CS-)A > (CS+ > CS-)E) ($T(1,29)=3.321$; $p=0.003$) (see figure R33).

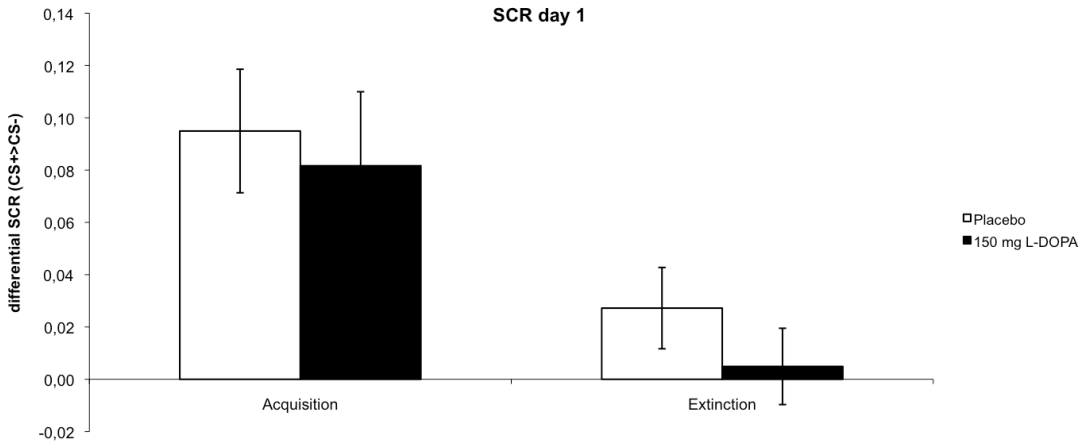

Figure R33. Mean differential SCR (CS+>CS-) in both contexts on day 1. Error bars indicate the SEM.

Repeated measurements 2x2x2x2 ANOVA (with the factor time (first half /second half) (2)) extended the results, with a main effect of time ($F(1,1)=8.083$; $p=0.008$), as well as a trend-like stimulus by time interaction ($F(1,1)=3.962$; $p=0.057$). Post-hoc paired t-test (two-sided) revealed that only the CS- decreased significantly in the second phase in the context A ($T(1,28)=4.42$; $p<0.001$) and in the context E ($T(1,28)=2.29$; $p=0.030$) in comparison to the first phase.

These results indicate successful differential context-dependent fear conditioning and extinction and replicate the findings of Kalisch et al. (2006). Importantly, the groups were not different in their SCRs before the intake of drug (see figures R29 and R32).

4.2.1.3 US expectancy ratings

The 2x2x2 repeated-measures ANOVA of US expectancy ratings revealed significant main effects of stimulus ($F(1,1)=189.237$; $p<0.001$) and context ($F(1,1)=63.942$;

p<0.001) as well as a stimulus by context interaction (F(1,1)=109.659; p<0.001). The interaction between context and group was trend-wise significant (F(1,1)=3.194; p=0.082), however, no main effect of the factor group was observed.

Paired t-test (one-sided) indicated that the US expectancy ratings were significantly higher for the CS+ as compared to the CS- in context A (T(1,37)=15.91; p<0.001) and in context E (T(1,37)=5.02; p<0.001). Differential responses (CS+>CS-) were significantly higher in the context A as compared to the context E ((CS+ > CS-)A > (CS+ > CS-)E) (T(1,37)=10.35; p<0.001).

To further qualify the interaction between group and context post-hoc unpaired t-tests (two-sided) between groups were conducted. They revealed no differences in rating values of any CS, in differential (CS+ >CS-) ratings in one of the contexts or in differential ratings in context A in comparison to context E ((CS+ > CS-)A > (CS+ > CS-)E) (p>0.1).

Averages of the differential ratings (CS+ > CS-) for the US expectancy in both contexts are displayed in figure R34.

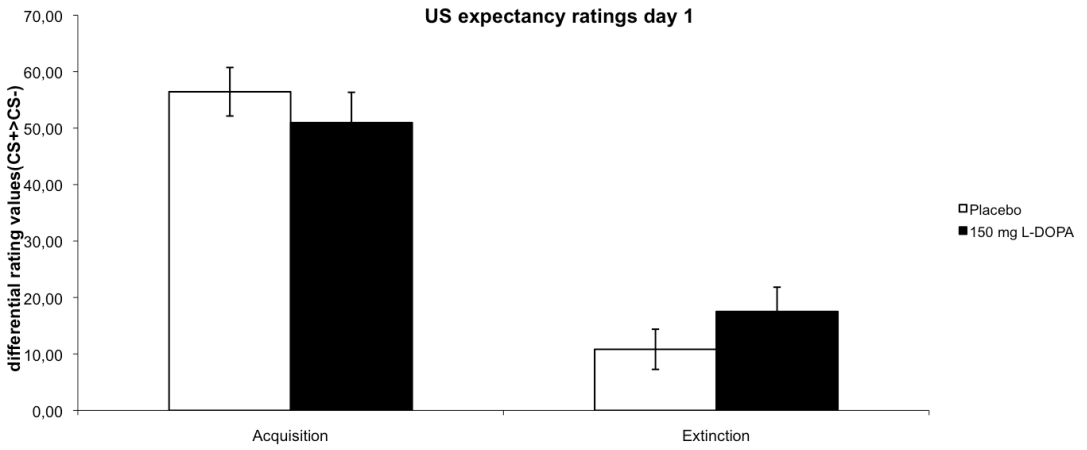

Figure R34. Mean differential US expectancy ratings (CS+>CS-) in both contexts on day 1. Error bars indicate the SEM.

Repeated measurements 2x2x2x2 ANOVA (with the factor time (2) (first half /second half)) extended the results with a main effect of time (F(1,1)=12.931; p=0.001) as well as significant interactions of stimulus by time (F(1,1)=7.088; p=0.012), context by time (F(1,1)=39.683; p<0.001) and stimulus by context by time (F(1,1)=14.792; p<0.001). Again, there was a trend-wise significant context by group interaction (F(1,1)=3.194; p=0.082) but no main effect of the factor group or other interactions with this factor.

Post-hoc paired t-tests (two-sided) indicated that the US expectancy ratings significantly increased over time (first half < second half) for the CS+ in context A ($T(1,37)=3.08$; $p<0.001$) and significantly decreased for the CS- in context A ($T(1,37)=2.53$; $p=0.016$) and for both CS in context E (CS+ ($T(1,37)=5.65$; $p<0.001$); CS- ($T(1,37)=4.57$; $p<0.001$). In addition, the differential responding (CS+>CS-) increased (first half < second half) only in context A significantly ($T(1,37)=4.35$; $p<0.001$) (context E $p>0.4$). As expected, the increase (first half < second half) in differential responding in context A in comparison to the context E ((CS+ > CS-)A > (CS+ > CS-)E) was significant as well ((CS+ > CS-)A > (CS+ > CS-)E) ($T(1,37)=3.83$; $p<0.001$).

Analyses of the US expectancy ratings were in accordance with the contingency of the different stimuli and contexts. Higher differences between CS+ and CS- in the context of fear conditioning (A) than in the extinction context (E) (see figure R34) indicate successful fear conditioning and extinction.

4.2.1.4 Ratings of fear/distress

The 2x2x2 repeated-measures ANOVA of the ratings of fear/distress revealed significant main effects of the factors stimulus ($F(1,1)=150.786$; $p<0.001$) and context ($F(1,1)=82.294$; $p<0.001$) and a significant stimulus by context interaction ($F(1,1)=81.943$; $p<0.001$). No group main effects or interactions were observed.

Paired t-test (one-sided) indicated that the ratings of fear/distress were significantly higher for the CS+ as compared to the CS- in context A ($T(1,37)=13.24$; $p<0.001$) and in context E ($T(1,37)=5.32$; $p<0.001$). Differential responses (CS+>CS-) were significantly higher in the acquisition context as compared to the extinction context ((CS+ > CS-)A > (CS+ > CS-)E) ($T(1,29)=9.01$; $p<0.001$).

Averages of the mean differential ratings (CS+ > CS-) for fear/distress ratings in both contexts are displayed in figure R35.

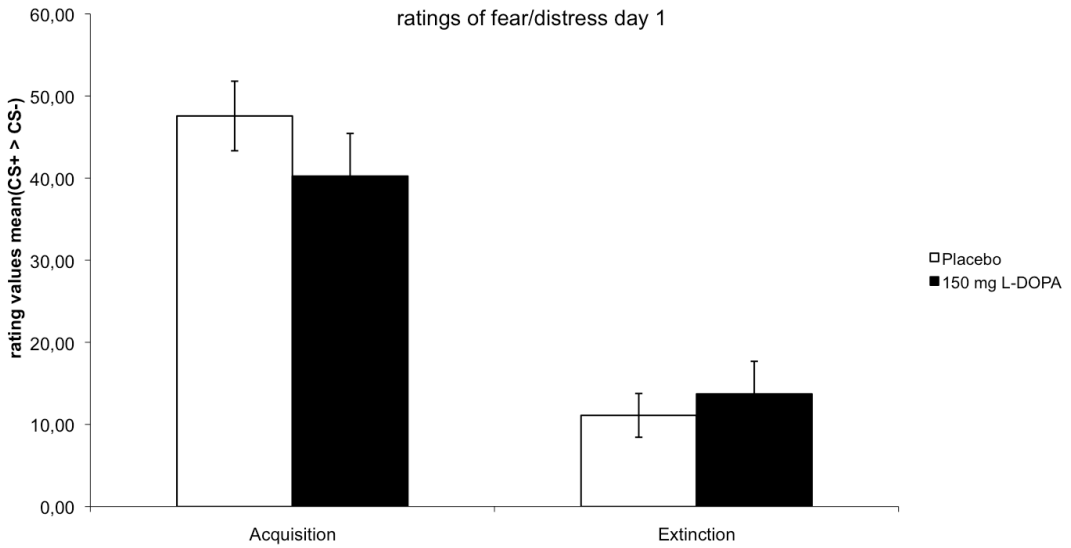

Figure R35. Mean differential ratings of fear/distress in the both contexts on day1. Error bars indicate the SEM.

Repeated measurements 2x2x2x2 ANOVA (with the factor time (2) (first half/second half)) yielded, in addition to these results, a significant main effect of time ($F(1,1)=5.681$; $p=0.023$) as well as interactions of stimulus by time ($F(1,1)=9.955$; $p=0.003$), context by time ($F(1,1)=38.500$; $p<0.001$), and stimulus by context by time ($F(1,1)=15.084$; $p<0.001$).

Post-hoc paired t-tests (two-sided) indicated that the US expectancy ratings significantly increased over time (first half < second half) for the CS+ in context A ($T(1,37)=3.84$; $p<0.001$) and decreased trend-wise for the CS- in context A ($T(1,37)=1.85$; $p=0.072$) and significantly for both CS in context E (CS+ ($T(1,37)=4.12$; $p<0.001$); CS- ($T(1,37)=4.96$; $p<0.001$). In addition, the differential responding (CS+>CS-) increased (first half < second half) only in context A significantly ($T(1,37)=4.30$; $p<0.001$) (context E $p>0.7$). As expected the increase (first half < second half) in differential responding in context A in comparison to the context E ((CS+ > CS-)A > (CS+ > CS-)E) was significant as well ((CS+ > CS-)A > (CS+ > CS-)E) ($T(1,37)=3.94$; $p<0.001$).

These results indicate successful emotional valence learning (fear/distress) of fear conditioning and extinction on day 1 and followed the results of the US expectancy ratings. Furthermore they suggest the absence of pre-existing group differences before the intake of study medication.

4.2.1.5 Reaction times

The 2x2x2 repeated-measures ANOVA revealed significant main effects of the factors stimulus ($F(1,1)=4.676$; $p=0.037$) and context ($F(1,1)=11.705$; $p=0.002$). No group main effects or interactions were observed.

Repeated measurements 2x2x2x2 ANOVA (with the factor time (2) (first half /second half)) extended the results with a significant main effect of time ($F(1,1) =7.410$ $p=0.010$) as well as a significant context by time interaction ($F(1,1)=4.899$ $p=0.033$).

In contrast to previous studies (Kalisch et al. 2006), the RT to the CS+ was faster as compared to the CS-. Moreover, RTs in the extinction context were slower in general, with reduced differences between CS+ and CS-.

4.2.1.6 Summary and discussion day 1

The null hypothesis 1A can be rejected. A significant effect of fear conditioning and extinction learning was observed as assessed by SCRs, US expectancy ratings and ratings of fear/distress. Thus, the behavioural manipulation of context dependent differential fear conditioning and extinction learning was successful. In contrast, the analyses of RTs failed to confirm previous studies, which suggests that this measure may not be a reliable indicator of fear responding.

Moreover, the null hypothesis 1B does not have to be rejected. There were no significant differences in the response of the pharmacological treatment groups before administration of the drug.

4.2.2 Day 2

Analyses of day 2 investigated the recall of conditioned fear and extinction memories. Analogously to the acquisition of conditioned fear, high SCRs are observed in the recall of fear and decreased SCRs in the recall of extinction memory (LaBar & Phelps 2005, Milad et al. 2005, Vansteenwegen et al. 2005).

The analyses of the ratings of fear/distress and US expectancy were thought to reflect the learned associations of day 1. Thus, higher differential values can be interpreted as indicating the recall of conditioned fear (predicted to prevail in the acquisition context A) and decreased values the recall of extinction memory (predicted to prevail in the extinction context E). The induction of recall of fear by presenting the CS+ in context A corresponds to a renewal manipulation and thus constitutes one critical test for the context-dependent return of fear, which may be a mechanism underlying relapse in anxiety treatment.

The statistical analyses tested if coherence existed between the contextual manipulation of fear and extinction recall and the dependent variables, SCRs and both rating scales (Hypothesis 2A). Successful fear recall was defined as higher CS+ than CS- responses. Successful renewal in particular was defined as higher differential responding in the acquisition context compared to the extinction context ((CS+ >CS-)A > (CS+>CS-)E); in accordance with Kalisch et al. (2006)). Conversely, the relatively attenuated differential responses in the extinction context then would show successful extinction memory recall in this context. In addition, analyses tested if the intake of L-DOPA on day 1 led to different responses in the renewal on day 2 (Hypothesis 2B). The hypothesised effect of the pharmacological treatment was a decreased renewal of the conditioned fear memory (i.e., an increased recall of the extinction memory in the acquisition context) in the L-DOPA group.

Figures 36 to 38 illustrate the time courses of differential (CS+ >CS-) responses in SCRs and in both types of ratings on day 2. Visual inspection of the inherently noisy SCR time courses (figure R36) does not permit clear conclusions, while US expectancy (figure R37) and fear/distress (figure R38) ratings already show a clear renewal effect, with differential responses that are elevated in context A (A1-A12) and relatively decreased in context E (E1-E12). The global response level, as well as the renewal effect, appears to decline over time and, importantly, both appear to be less pronounced in the L-DOPA group. This would suggest fear recall-attenuating (extinction recall-enhancing) effects of L-DOPA treatment after extinction learning on day 1.

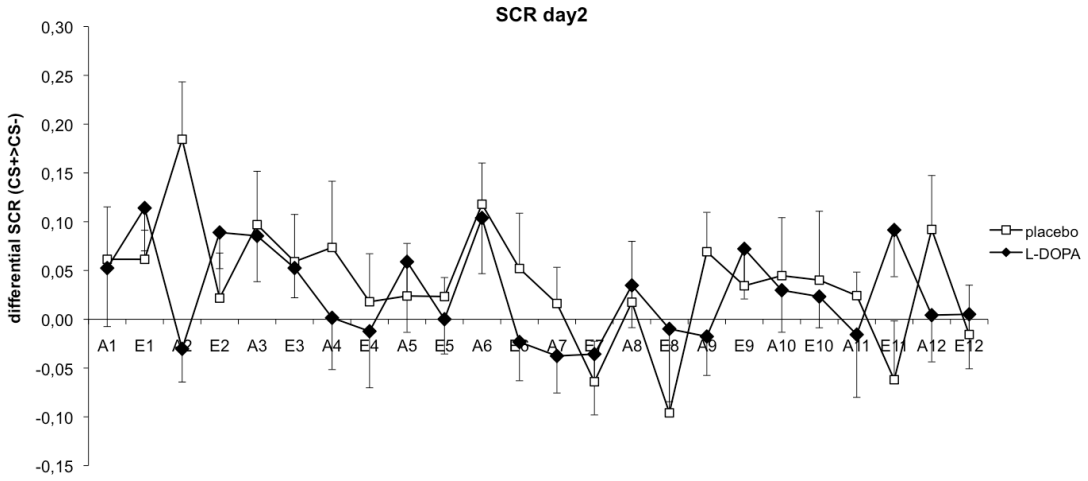

Figure R36. Blockwise (A= context A/ E= context E) average (Block=2 CS presentations) for the differential SCR (CS+>CS-) on day 2. Error bars indicate the SEM (in order to visualise results clearly, only one direction is illustrated)

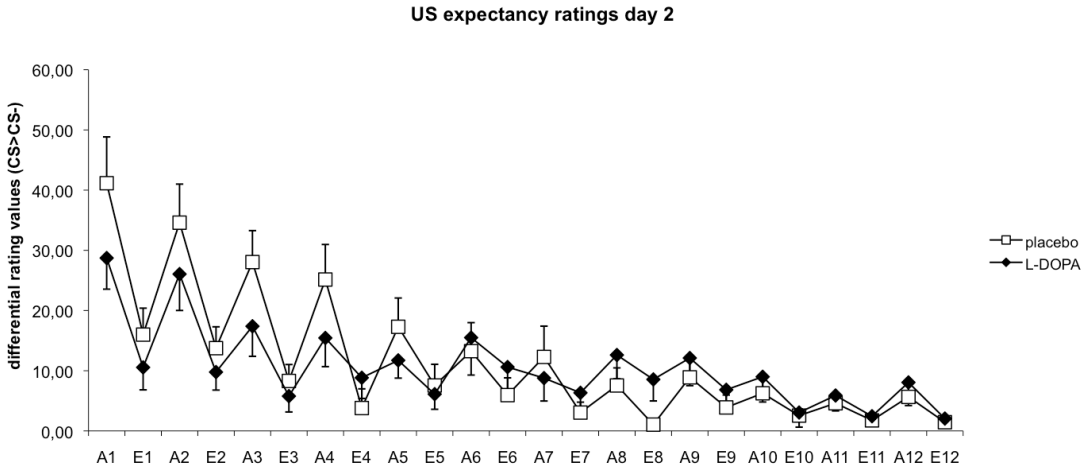

Figure R37. Differential US expectancy ratings (CS+ and CS-) on day 2. Error bars indicate the SEM (in order to visualise results clearly, only one direction is illustrated)

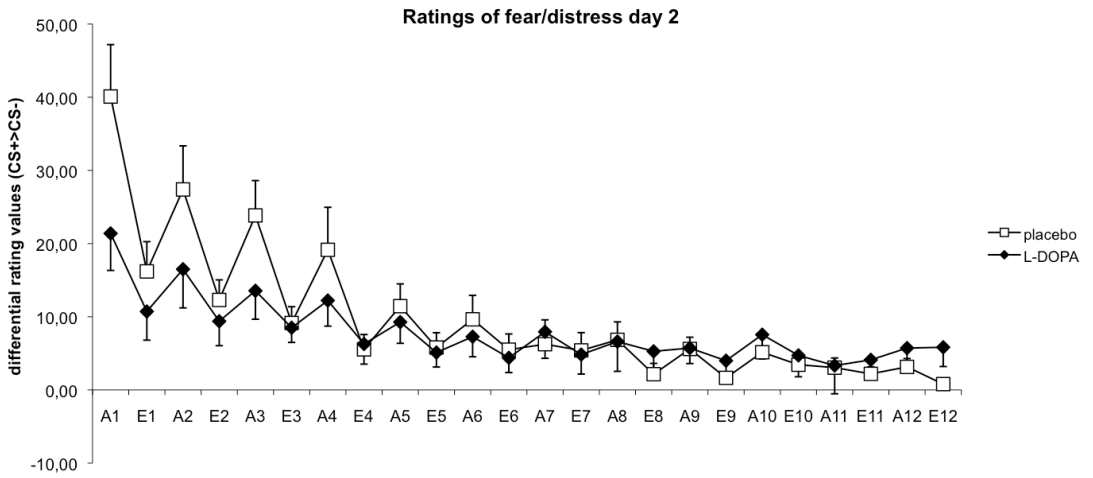

Figure R38. Differential ratings of fear/distress (CS+ > CS-) on day 2. Error bars indicate the SEM (in order to visualise results clearly, only one direction is illustrated)

4.2.2.1 SCR

The 2x2x2 ANOVA revealed significant main effects of stimulus (F(1,1)=10.012; p=0.003) and context (F(1,1)=9,872; p=0.004) as well as a trend for a stimulus by context interaction (F(1,1)=3,11; p=0.088). Importantly, there was a significant stimulus by context by group interaction (F(1,1)=5,232; p=0.029).

Planned comparisons indicated that SCRs were higher for the CS+ as compared to the CS- (F(1,1)=9.872; p=0.004) and higher in context A as compared to the context E (F(1,1)=10.012; p=0.003). There was only a trend for higher differential responses (CS+>CS-) in context A as compared to context E ((CS+ > CS-)A > (CS+ > CS-)E)) (F(1,1)=3.111; p=0.088) (see fig R39).

Paired t-test (one-sided) indicated that SCRs were significantly higher for the CS+ as compared to the CS- in context A (T(1,32)=3.27; p=0.002) and trend-wise in context E (T(1,32)=1.53; p=0.068). Differential responses (CS+>CS-) were significantly higher in the context A as compared to the context E ((CS+ > CS-)A > (CS+ > CS-)E) (T(1,32)=1.72; p=0.046).

The three-way interaction effect (stimulus, context and group) was statistically further qualified (unpaired, one-sided t-tests, unequal variance) through higher SCR in the placebo group to the CS+ in context A (T(1,30.9)=1.81; p=0.041) and differential responding (CS+>CS-) in context A as a trend (T(1,26.3)=1.70; p=0.51) compared to

the L-DOPA group. As indicated through the effect of interaction, the differential responding in context A as compared to context E was significantly higher in the placebo group relative to the L-DOPA group ((CS+>CS-)A>(CS+>CS-)E in placebo>verum) T(1,29.5)=2.307; p=0.014). These results indicate, that the renewal effect in the placebo groups is clearly abolished in the L-DOPA group.

The direction of the effect between groups is displayed through the bar graph figure R38, illustrating the averages of differential responding (CS+>CS-) in the both contexts. No main effect of the factor group was observed.

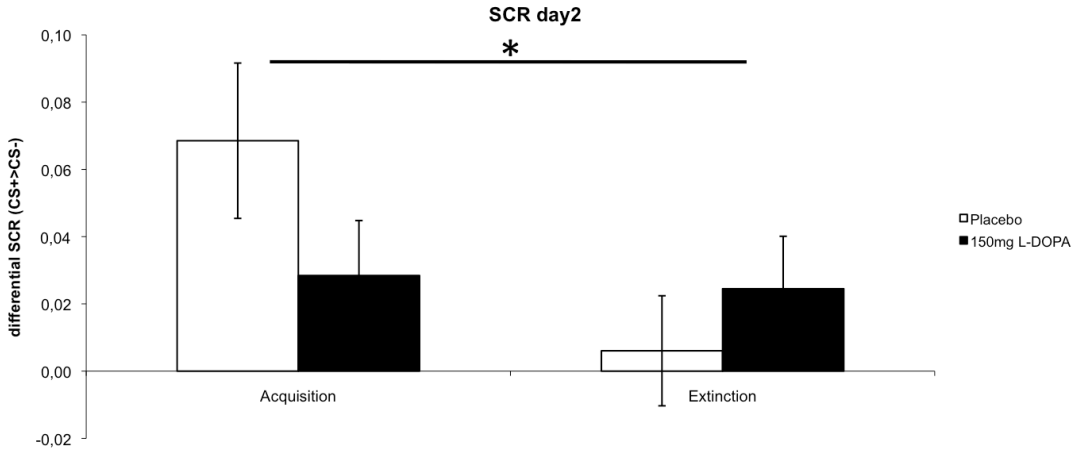

Figure R39 mean differential SCRs (CS+>CS-) in the context A and E on day2. Error bars indicate the SEM. * = significant effect between treatment groups.

Post-hoc analyses of the successful recall of conditioned fear and extinction memory separated by group were intended to further qualify the effect of pharmacological treatment. Comparisons in the placebo group indicated higher SCRs to the CS+ as compared to the CS- (F(1,1)=5.474; p=0.033), higher SCRs in context A as compared to the context E (F(1,1)=10.664; p=0.005) and higher differential responses (CS+>CS-) in context A as compared to context E((CS+ > CS-)A > (CS+ > CS-)E)) (F(1,1)=6.653; p=0.020). These results indicate the expected renewal of fear, as described above. In contrast, comparisons in the verum group indicated that, SCR were higher for the CS+ as compared to the CS- (F(1,1)=4.652; p=0.048), but yielded no differentiation of contexts.

Repeated measurements 2x2x2x2 ANOVA (with the factor time (first half /second half) (2)) extended the results with a significant main effect of time (F(1,1)=7.895;p =0.009) as well as the interaction effect of stimulus and time (F(1,1)=13.947 p=0.001).

Post-hoc paired t-test (two-sided) indicated that SCR significantly decreased (first half >second half) for the CS+ in context A ($T(1,32)=3.04$; $p=0.003$) and context E ($T(1,32)=2.46$; $p=0.0019$), whereas this was not significant for the CS- in both contexts ($p>0.1$).

In sum, analyses revealed differences between the groups in the SCRs. The L-DOPA group showed significantly decreased differential responding in the context A as compared to the context E. The recall of fear memory seemed furthermore only successful in the placebo group, indicated through significant renewal of fear in a separated analysis.

4.2.2.2 US-expectancy ratings

The 2x2x2 repeated-measures ANOVA of the US expectancy ratings yielded significant main effects of the factors stimulus ($F(1,1) =58.451$; $p<0.001$) and context ($F(1,1)=26.229$; $p<0.001$), as well as the significant effect of their interaction ($F(1,1)=26.200$; $p<0.001$). No interaction or main effect of group was observed.

Paired t-test (one-sided) indicated significantly higher US expectancy ratings for the CS+ as compared to the CS- in context A ($T(1,37)=7.52$; $p>0.001$) and context E ($T(1,37)=5.46$; $p>0.001$). As expected, differential responses were significantly higher in the context A as compared to context E ((CS+ > CS-)A > (CS+ > CS-)E) ($T(1,37)=5.18$; $p>0.001$).

Averages of the mean differential ratings (CS+ > CS-) for the US expectancy ratings in both contexts are displayed in figure R40.

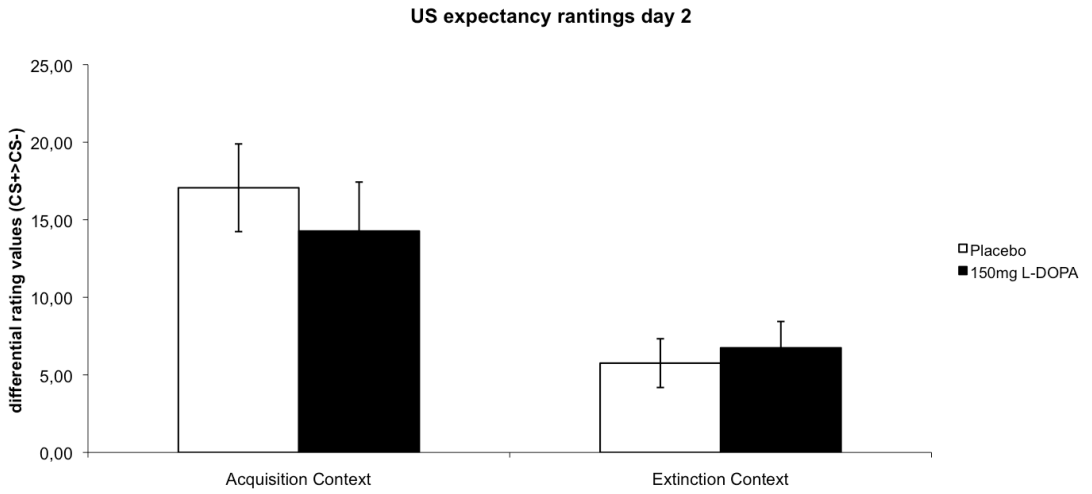

Figure R40. Mean differences of the US expectancy ratings for each CS+ and the CS- in context A and E on day2. Error bars indicate the SEM.

Repeated measurements 2x2x2x2 ANOVA (with the factor time (first half/ second half) (2)) extended the results with a significant main effect of time ($F(1,1) = 119.637$ $p<0.001$), as well as interaction effects of stimulus and time ($F(1,1)=52.819$ $p<0.001$), context and time ($F(1,1)=9.828$ $p=0.001$) and stimulus, context and time ($F(1,1)=13.417$; $p<0.001$).

More importantly, a significant interaction effect between stimulus, time and group ($F(1,1)=5.316$ $p=0.027$) was observed. No main effect of the factor group was observed.

The timecourse of differential (CS+ > CS-) US expectancy ratings on day 2 (see figure R 37) suggests group differences in renewal were restricted to early trials.

Post-hoc paired t-test (two-sided) indicated that the US expectancy ratings significantly decreased over time (first half < second half) for the CS in each context ((CS+ context A $T(1,37)=11.41$; $p<0.001$) (CS- context A $T(1,37)=7.20$; $p<0.001$) (CS+ context E $T(1,37)=7.70$; $p<0.001$) (CS- context E $T(1,37)=4.92$; $p<0.001$)). Furthermore, the differential responding (CS+>CS-) decreased (first half > second half) in both contexts significantly (context A ($T(1,37)=6.33$; $p<0.001$) context E ($T(1,37)=3.98$; $p<0.001$)). As expected, the decrease (first half > second half) in differential responding in context A in comparison to the context E ((CS+ > CS-)A > (CS+ > CS-)E) was significant as well ((CS+ > CS-)A > (CS+ > CS-)E) ($T(1,37)=3.71$; $p=0.003$).

The three-way interaction effect between stimulus, time and group was statistically further qualified through post-hoc unpaired t-test (one-sided, unequal variance). Tests revealed, that the differential responding (CS+>CS-) was trend-wise higher in context A in the placebo group ($T(1,35.7)=1.37$; $p=0.090$). In addition, the decrease (first half> second half) was significantly higher only for the CS+ in context A for the placebo group as compared to the L-DOPA group ($T(1,34.7)=2.14$; $p=0.020$).

These results indicated a significant effect of renewal for the predictive values of the US. More important, results indicate that the L-DOPA group showed decreased renewal of US expectancy for the CS+ in context A in the first half on day 2 in comparison to placebo.

4.2.2.3 Ratings – fear/distress

The 2x2x2 repeated-measures ANOVA of ratings of fear/distress yielded significant results for the main effects of the factors stimulus ($F(1,1) =48.257$; $p<0.001$), context ($F(1,1)=28.343$; $p<0.001$) and their interaction ($F(1,1)=14.126$; $p<0.001$). Again, no interaction or main effect of the factor group was observed.

Paired t-test (one-sided) indicated significantly higher ratings of fear/distress for the CS+ as compared to the CS- in context A ($T(1,37)=6.63$; $p>0.001$) and context E ($T(1,37)=5.50$; $p>0.001$). As expected, differential responses were significantly higher in the context A as compared to context E((CS+ > CS-)A > (CS+ > CS-)E) ($T(1,37)=3.76$; $p=0.001$).

Averages of the mean differential ratings (CS+ > CS-) for the rating of fear/distress in both contexts are displayed in figure R41.

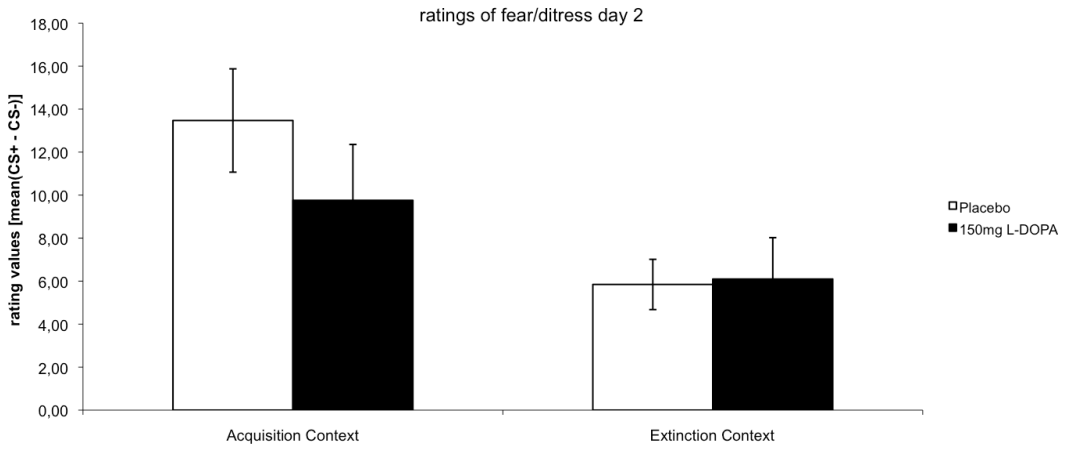

Figure R41. Mean differences of the ratings for fear/distress for each CS+ and the CS- in context A and E on day2. Error bars indicate the SEM.

Repeated measurements 2x2x2x2 ANOVA (with the factor time (first half/ second half) (2)) extended the results of with a significant main effect of time ($F(1,1) = 99.897$ $p<0.001$), as well as interaction effects of stimulus and time ($F(1,1)=45.494$ $p<0.001$), context and time ($F(1,1)=13.337$ $p<0.001$) and stimulus, context and time ($F(1,1)=13.088$ $p=0.001$). Furthermore, a significant interaction effect between stimulus, time and group ($F(1,1) = 7.596$ $p=0.009$) was observed, analogue to the US expectancy ratings. No main effect of the factor group was observed.

The timecourse of differential (CS+ > CS-) ratings of fear/distress on day 2 (see figure R 38) suggests group differences in renewal were restricted to early trials.

Post-hoc paired t-tests (two-sided) indicated that the US expectancy ratings significantly decreased over time (first half < second half) for the CS in each context ((CS+ context A $T(1,37)=10.30$; $p<0.001$) (CS- context A $T(1,37)=5.54$; $p<0.001$) (CS+ context E $T(1,37)=7.36$; $p<0.001$) (CS- context E $T(1,37)=4.79$; $p<0.001$)). In addition, the differential responding (CS+>CS-) decreased (first half > second half) in both contexts significantly (context A ($T(1,37)=5.78$; $p<0.001$) context E ($T(1,37)=4.19$; $p<0.001$)). As expected the decrease (first half > second half) in differential responding in context A in comparison to the context E ((CS+ > CS-)A > (CS+ > CS-)E) was significant as well ((CS+ > CS-)A > (CS+ > CS-)E) ($T(1,37)=3.65$; $p=0.001$).

The three-way interaction effect between stimulus, time and group was statistically further qualified through post-hoc unpaired t-test (two-sided unequal variance). Tests revealed a significantly higher differential rating in context A in the first half in the

placebo group as compared to the L-DOPA group (T(1,35.1)=1.816; p=0.039). Furthermore the decrease (first half >second half) for the CS+ in context A was significantly higher for the placebo group as compared to the L-DOPA group (T(1,33.8)=2.07; p=0.023).

The analyses yielded renewal of the negative emotional value. More important, results indicated that the L-DOPA group showed decreased renewal of rated fear and distress for the CS+ in context A in the first half on day 2 in comparison to placebo.

4.2.2.4 RT

The 2x2x2 repeated-measures ANOVA revealed significant main effects of the factors stimulus (F(1,1)=7.734; p=0.008) and context (F(1,1)=6.627; p=0.014). No group main effects or interactions were observed.

Repeated measurements 2x2x2x2 ANOVA (with the factor time (first half/second half) (2)) extended the results with a significant main effect of time (F(1,1)=6.712; p=0.014).

In contrast to previous studies, the RT to the CS+ was faster as compared to the CS- (analogue to day 1). Moreover, RTs in the extinction context were slower in general, with reduced differences between CS+ and CS-. Importantly, the pharmacological treatment had no influence on RTs.

4.2.2.5 Summary and discussion day 2

The null hypothesis 2A can be rejected. Significant recall of fear conditioning (renewal) and extinction memory as measured by SCR, US expectancy ratings and ratings of fear/distress were observed.

Also, the null hypothesis 2B can be rejected. There are significant differences in the pharmacological treatment groups in renewal.

The behavioural measurements showed a successful renewal of fear in the placebo group, as reported by Kalisch et al. (2006) (for the SCR). The treatment with L-DOPA

abolished these fear responses measured as SCR, thus led to prevention of renewal. In particular, the responses in the L-DOPA group were equally decreased in both contexts, speaking for a strong recall of extinction. This was accompanied in this group with a decreased rated US expectancy and fear/distress in the context of fear recall in the first half if day 2.

The analysis of the behavioural data suggested, that the L-DOPA group showed a strong extinction memory recall, preventing the return of fear as renewal.

4.2.2.6 fMRI

Given that behavioural renewal effects were mainly restricted to early trials, the group analysis on day 2 examined group differences in the first half only. The analysis of focussed on the critical stimulus by context by group interaction effect (Hypothesis 5A) that had reached significance in the SCR analysis, showing fear renewal ((CS+>CS)A> (CS+>CS-)E) in the L-DOPA group that was attenuated in the placebo group.

The analysis of the categorical regressors revealed higher heamodynamic responses in left ventral mPFC, anterior hippocampus, and as a trend, in the dmPFC in the L-DOPA group (see table R5 and figure R40). Furthermore, at the threshold of p=0.001 (uncorrected) there was activation in the right anterior hippocampus (24;24;6; T(1,148)=3.67; p<0.001(uncorrected)) and the right putamen (20;-8;-20; T(1,148)=3.36; p<0.001(uncorrected)) in the same contrast.

Table R5. Significant interaction effects in the ROIs ((CS+>CS-)A > (CS+>CS-)E) in verum>placebo).

Region	X	Y	Z	T	Z	P(SVC)	P(uncorr)
L vmPFC	-8	43	-20	3.64	3.65	0.041	<0.001
L ant HC	-30	-14	-22	3.25	3.19	0.028	0.001
dmPFC	-6	50	24	3.55	3.47	0.053	<0.001

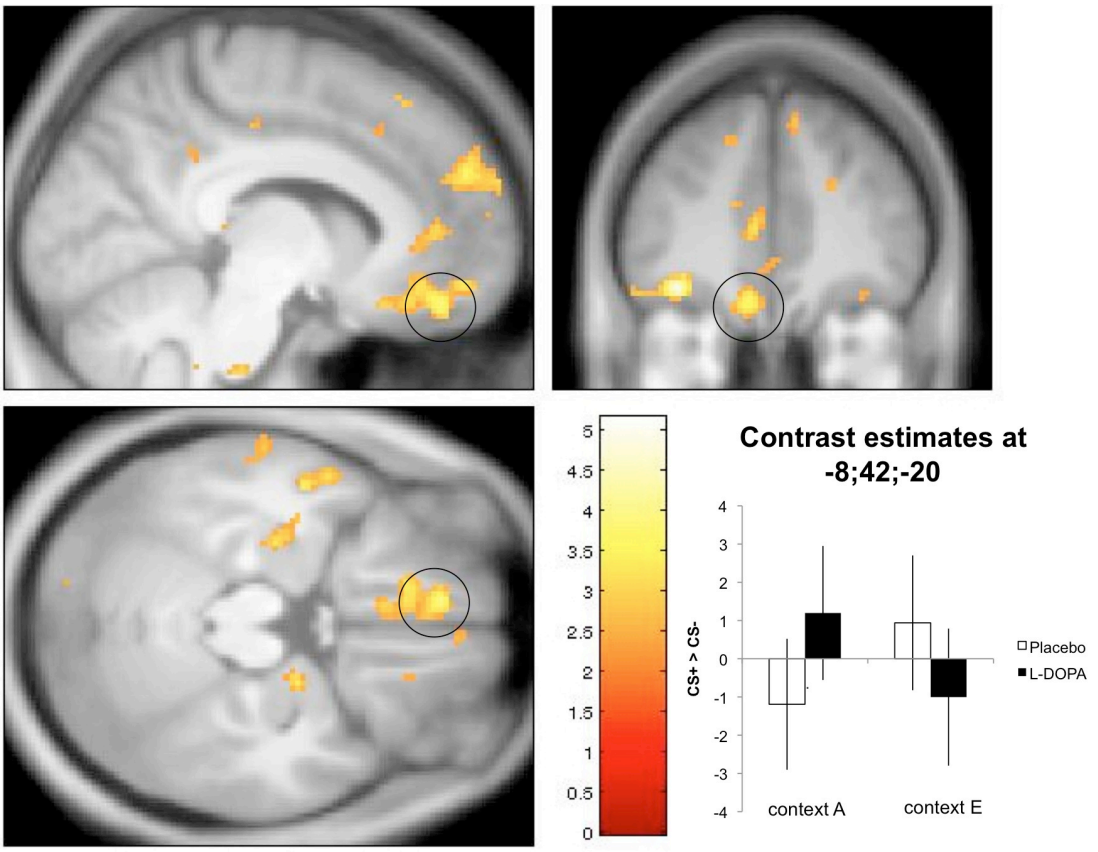

Fig R42. Significant voxels in the categorical contrast (CS+>CS-)A > (CS+>CS-)E) in verum>placebo at x=-8; y=42; y=-20. The resulting T-map is overlaid on a mean structural image, normalised into the NMI space. The bottom bar graphs illustrate the average contrast estimates in the peak voxel (Error bars indicate the SEM). The display threshold is set to p<0.01 uncorrected and the colour bar indicates T-scores.

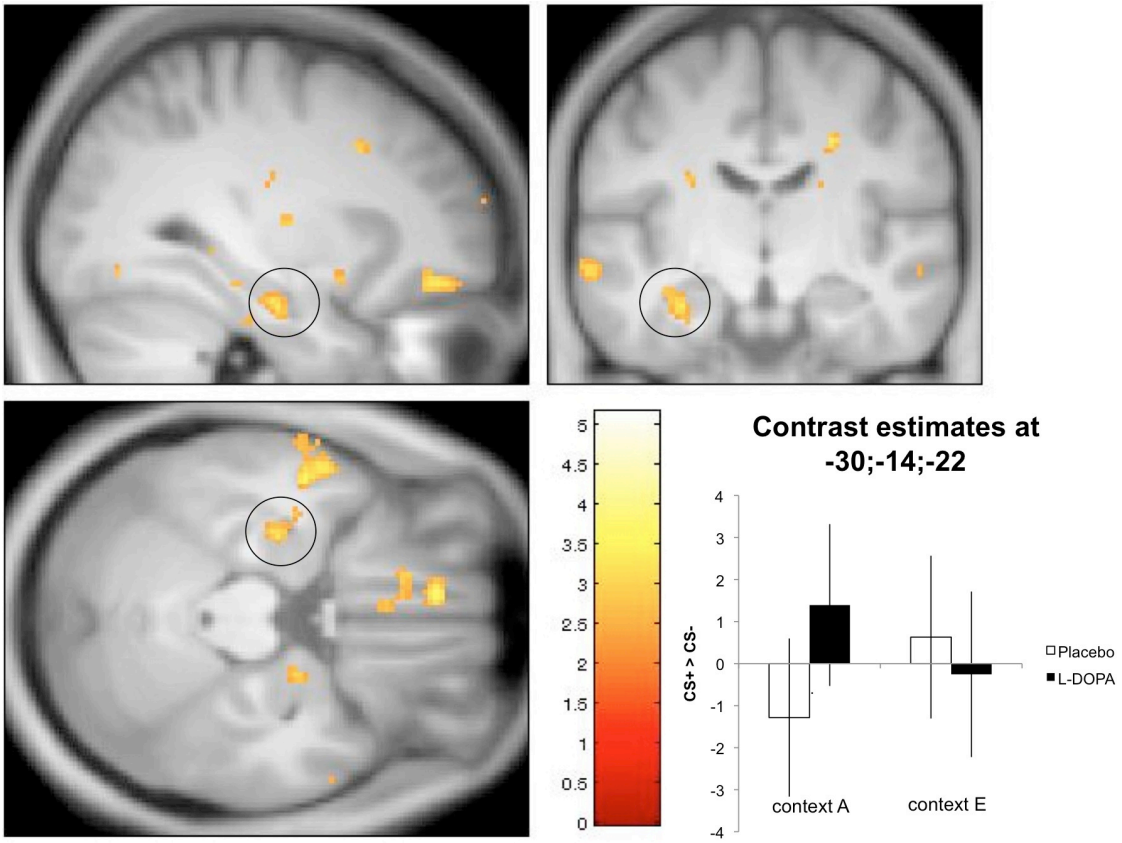

Fig R43. Significant voxels in the categorical contrast (CS+>CS-)A > (CS+>CS-)E) in verum>placebo at x=-30; y=-14; y=-22. The resulting T-map is overlaid on a mean structural image, normalised into the NMI space. The bottom bar graphs illustrate the average contrast estimates in the peak voxel (Error bars indicate the SEM). The display threshold is set to p<0.01 uncorrected and the colour bar indicates T-scores.

The inverts contrast placebo>verum revealed no significant activations inside the ROIs.

The same contrast in parametrically decreasing responses revealed a group difference in the right posterior hippocampus (see table R6 and figure R42).

Table R6. Significant interaction effects comparing the linear decreasing responses in the ROIs ((CS+>CS-)A > (CS+>CS-)E) in placebo>verum).

Region	X	Y	Z	T	Z	P(SVC)	P(uncorr)
R post HC	36	-28	-14	3.35	3.28	0.021	0.001

The difference between groups was mainly driven by the strong increase for the CS- in the context A in the placebo (negative estimates), whereas the responses decreased in the L-DOPA group (positive estimates) (see figure R44).

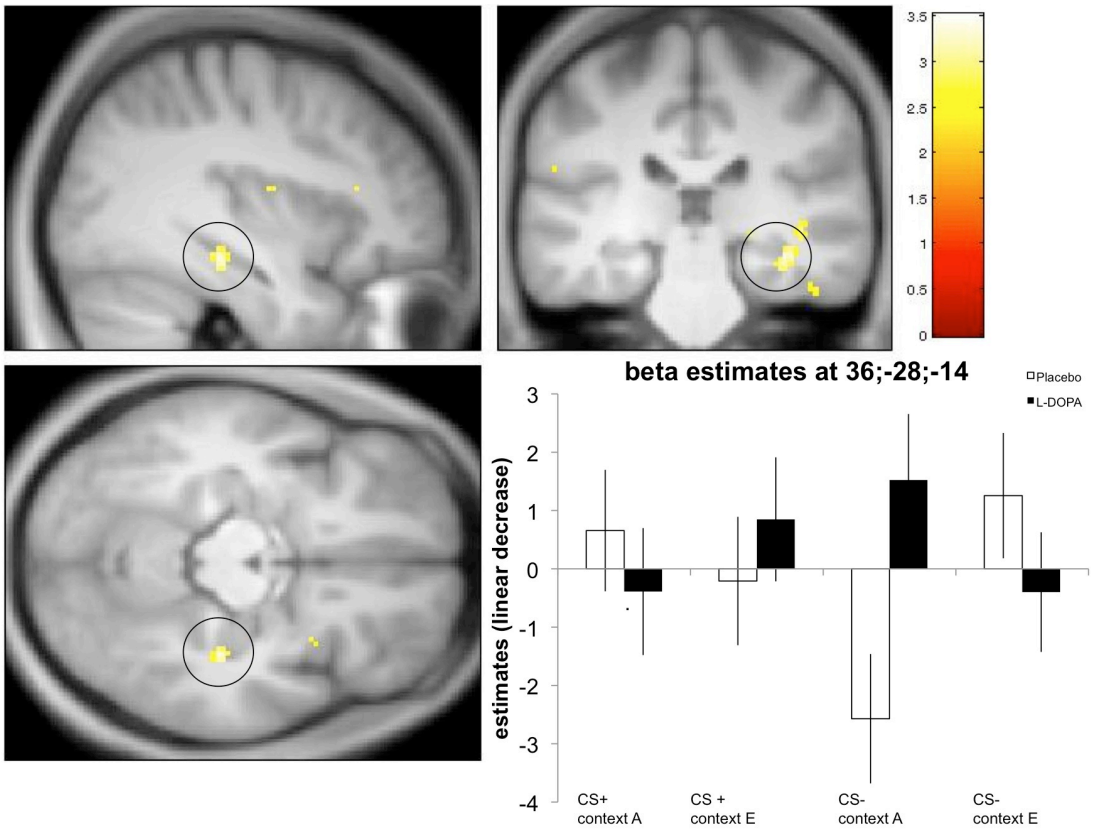

Fig R44. Significant voxels in the parametric contrast (CS+>CS-)A > (CS+>CS-)E) in placebo >verumat x=36; y=-28; y=-14. The resulting T-map is overlaid on a mean structural image, normalised into the NMI space. The bottom bar graphs illustrate the average contrast estimates in the peak voxel (Error bars indicate the SEM). The display threshold is set to p<0.01 uncorrected and the colour bar indicates T-scores.

4.2.2.7 fMRI summary and discussion

The analyses of the ROIs during renewal of fear revealed differences between treatment groups. Thus, the null hypothesis 5B can be rejected. The heamodynamic responses in the L-DOPA group were higher in brain regions associated with the recall of extinction, namely the vmPFC and the anterior hippocampus. The placebo group showed the typical, previously reported pattern of high CS+ responses in the context E, reflecting the context dependent recall of extinction (Kalisch et al. 2006). The relative deactivation of the vmPFC and ant HC in the context A is reminiscent of animal (Garcia et al. 1999) and human data (Phelps et al. 2004). That relates vmPFC inhibition to facilitation of fear recall. The group difference was in part due to a disinhibition of the vmPFC in context A in the L-DOPA group. Moreover, only the

placebo group showed increasing responses to the CS+ in the context of extinction in the posterior hippocampus in the placebo group. This perhaps reflected the return of fear as renewal that was no longer apparent in the L-DOPA group.

In sum, as a neural correlate of the decreased behaviourally measured fear in the contrast of renewal, the L-DOPA group recruited brain regions of extinction recall. Thus, this absence of renewal might be related to the disinhibition of the vmPFC-hippocampal network. In parallel, the placebo group showed increasing responses in brain regions of fear recall in the context of renewal, which was abolished in the L-DOPA group.

5. General discussion

The two studies reported here revealed converging results for an augmented recall of extinction memory through the administration of L-DOPA in the consolidation phase of extinction learning.

Study A provided neural evidence of group differences in the spontaneous recovery and reinstatement of cued and contextual fear in the sense of less return of fear in L-DOPA subjects. No behavioural differences between groups were observed. This may be explained by a floor effect resulting from the strong extinction on day 2 and the consequently generally low fear recall on day 8. Nevertheless, fMRI analyses indicated reduced heamodynamic responses in fear related brain regions in the L-DOPA group.

In Spontaneous Recovery, the L-DOPA group showed lower contrast estimates for the index of cued fear in the dmPFC and, as a trend, in the posterior hippocampus. The index of contextual fear after reinstatement revealed reduced heamodynamic responses in the posterior hippocampus in the L-DOPA group. Furthermore, the L-DOPA group showed increasing responses in vmPFC, a brain region associated with extinction memory recall, after reinstatement.

Study B tested renewal of cued fear as an additional pathway to return of fear. The L-DOPA group showed significantly decreased psychophysiological responding and decreased subjective fear/distress and expectancy of the US during renewal. Neural correlates of reduced renewal in the L-DOPA group included higher extinction recall-related contrast estimates in the vmPFC and the anterior hippocampus.

The brain regions showing effects of L-DOPA in contrasts of fear recall trough renewal, reinstatement and spontaneous recovery in both studies are majorly the vmPFC and the hippocampus. These brain regions are in accordance with the animal literature and existing human fMRI studies of fear conditioning and extinction.

The vmPFC is known (and already mentioned in the chapter 1.4 Neural systems mediating fear extinction) as an important structure of extinction memory recall. In animals, Milad and Quirck (2002) could show that neurons in the IL (corresponding to the vmPFC in humans) are only activated in the recall of extinction and that

stimulation of these neurons reduces conditioned fear responses behaviourally. Kalisch and co-worker (2006) could translate the finding into humans, showing that the vmPFC is activated in the recall of extinction memory. In recent studies, the diminished activity in the vmPFC could be shown to explain individual failure of reducing fear conditioned responses, for example in PTSD patients as compared to controls (Milad et al. 2009, for review Milad & Quirk 2012). The findings in Study B follow this concept and revealed reduced heamodynamic responses in the vmPFC in the placebo group that showed return of fear behaviourally. In addition, in Study A, the placebo group showed decreasing activity in the vmPFC after reinstatement.

The hippocampus has important role in the contextual gating of fear and extinction memories (as already described in the chapter 1.4 Neural systems mediating fear extinction). The involvement of the hippocampus in extinction memory recall (Corcoran & Maren 2001, Ji & Maren 2007) could be related to the context-specific encoding (Corcoran et al. 2005, Ji & Maren 2007) in animals. A recent review by Maren (2011) conceptualises the different contextual encoding in the hippocampus, which leads to either expression of fear or extinction, to different connections with prefrontal and amygdaloid regions. Interestingly, renewal of fear lead to enhanced c-FOS expression in the BLA and the ventral hippocampus (Orsini et al. 2011). Extinction recall lead to c-FOS expression in the IL, the intercalated nuclei in the amygdala (which exerts inhibitory projections to the BLA) and the dentate gyrus, a dorsal region in the hippocampus (Knapska & Maren 2009).

The hippocampus is also found to mediate contextual dependent extinction recall in humans (LaBar & Phelps 2005, Kalisch et al. 2006, Milad et al. 2007, Milad et al. 2009). Interestingly, these studies reveal activity in the anterior part of the hippocampus during extinction recall, whereas the posterior part of the hippocampus could be shown during the recall of fear in two studies by Kalisch et al. (2006), (2009). In accordance with these findings, higher contrast estimates in the anterior hippocampus in the L-DOPA group were associated with diminished conditioned fear responses. Importantly, the anterior hippocampus is thought to gate the contextual dependent extinction memory, and the L-DOPA group exerted neuronal activity of the anterior hippocampus in the context of fear (renewal). It seems that the extinction memory recall in the L-DOPA group lost its contextual dependence and transferred to the conditioning context. In line with this, the L-DOPA group showed reduced activity in the posterior hippocampus as compared to the placebo group during spontaneous recovery of cued (as a trend) and reinstatement of contextual fear.

These findings suggest, that the effect of L-DOPA is related to modulatory activation of the vmPFC and the anterior hippocampus. Both regions has been found to have correlating activity during the recall of extinction (Kalisch et al. 2006), (Milad et al. 2007).

Enhanced extinction in the presented studies was likely due to the augmented, majorly dopaminergic, transmission in the nigro-striatal pathway (Rodríguez et al. 2007) during the consolidation phase of the extinction memories. Both brain regions that have been found to differ between groups, the vmPFC and the hippocampus, are dopaminergically innervated (Swanson 1982 , Oades & Halliday 1987).

Furthermore, both regions were found to be modulated by dopamine during memory consolidation: The extinction of conditioned fear induces dopamine release in the prefrontal cortex in rats (Hugues et al. 2007) and disruption of dopaminergic transmission in that region impair contextual long-term extinction (Espejo 2003). Pharmacological blockade of dopaminergic D4 receptors in the medial prefrontal cortex in the rat impairs only the recall of extinction memories, when administered before extinction learning (Pfeiffer & Fendt 2006). Similarly, blockade of dopaminergic D2 receptors in the IL before extinction learning impairs only extinction memory recall and extinction related neural activity in infralimbic neurons (Mueller et al. 2010). A recent study administered methylphenidate in animals after extinction and showed enhanced extinction memory recall (Abraham et al. 2012). Methylphenidate was shown to increase extracellular dopamine majorly in prefrontal areas (Berridge et al. 2006).

The consolidation of memories, besides fear and extinction, in the hippocampus is known to be dopamine dependent (as described in chapter 1.7 Dopamine). Frey and co-workers (1990) could show in hippocampal slices that LTP induction is associated with dopamine releases, but more importantly, that dopaminergic blockade impaired long-term LTP. Furthermore, electrophysiological studies implied that stimulation of the nucleus accumbens modulated LTP induction in the hippocampus (López et al. 2008), and this mechanism was dopamine dependent (Kudolo et al. 2010). This is in accordance with human neuroimgaing studies that revealed striatal activity during reward prediction to be coupled with higher hippocampal activity during memory retrieval at a later timepoint (Wittmann et al. 2005). These findings of midbrain-hippocampal signalling could be in line with the proposed influence of prediction error based signalling on extinction memory consolidation (Orsini et al. 2011). Orsini and Maren (2011) suggest that prediction error coding to omission of the shock during

extinction influence extinction memory consolidation. They review the study of Huh et al. (2009) that reported increased phosphorylation of an important kinase in learning (extracellular signal-regulated kinase) in the hippocampus to the omission of predicted footshocks in animals. In addition, they review the study of Holtzman-Assif et al. (2010) that found dopaminergic signalling in the nucleus accumbens during the extinction to be learning necessary for the recall of extinction memories.

The strength of the presented findings in humans is further underlined by a series of studies by Dr. Fabio Morellini from the Centre of Molecular Neurobiology Hamburg (ZMNH), conducted in the context of a collaboration that is part of the Hamburger Landesexzellenzinitiative ("neurodapt!" consortium). Dr. Morellini tested the effects of post-extinction L-DOPA administration (relative to saline) on the spontaneous recovery and reinstatement of context conditioning. These experiments showed a dose-dependent enhancement of extinction memory recall even when tested 30 days after extinction and prevented the return of fear after reinstatement. This suggests the effects of L-DOPA may be species-independent and long-term.

This doctoral thesis therefore is an example of translational research, bridging the animal and human level and generating a clear hypothesis for future clinical work: Investigation of dopaminergic agonists as possible augmentative treatment strategies in exposure-based therapy of anxiety disorders. Studies may transfer the present results, as shown in clinical trials with DCS (Ressler et al. 2004, Davis et al. 2006) or hydrocortisol (Soravia et al. 2006, de Quervain et al. 2011).

Limitations of these studies arise from the selected study cohort. The results were obtained in young healthy males who were mostly University students. Consequently, the applicability of the results to the general population and in particular to women has to determined. The latter is especially important as women have a statistically higher disposition to develop anxiety disorders (see chapter 1.5 Anxiety related disorders).

Another caveat arises from the inherent limitation of the employed basic-science approach that studies basic emotional mechanisms in a controlled laboratory setting. The doctoral thesis used a model for anxiety disorder and its therapy to obtain changes in basic emotional mechanisms associated with dopaminergic neurotransmission. Therefore, these results cannot be directly interpreted in terms clinical applications, simply because the effect on exposure-based therapy in anxiety related disorders were not examined. Furthermore, only relatively small groups were included. Another caveat lies in the etiological relevance of the US. The fear response to expectation of an electrical shock may differ from phobic fear reactions to spiders,

for example. Nevertheless, the model of fear conditioning and extinction has already given useful insights into anxiety related disorders. Furthermore, I consider research of basic mechanisms has to be a necessary first step in the development of new treatment strategies.

Further research on the augmentative effects of dopaminergic agonistic drugs on the consolidation of exposure-based therapy in patients with anxiety relates disorders may be promising.

6. Summaries

6.1 Summary

Anxiety disorders have the highest prevalence of mental disorders in the USA and Western Europe (Alonso et al. 2004, Kessler et al. 2005). Relapse after therapy occurs mostly through persistent fear memories that outlast psycho- or pharmacotherapeutical treatment.

In the laboratory, fear responses are acquired through presentation of a fear conditioned stimulus (CS) that is paired with an aversive stimulus (US). In analogy to exposure based psychotherapy, these fear responses are diminished using repeated exposure to the CS in the absence of the US. Extinction does not erase the conditioned fear memory (CS-UCS association) but generates a competing extinction memory (CS-noUCS association) that inhibits the fear memory. However, a dominance of fear over extinction memory retrieval and, thus, return of fear (relapse) is mainly observed if the extinguished CS is encountered outside of the extinction (therapy) context.

In an effort to find new ways to strengthen the extinction memory, this doctoral thesis focused on the phase after extinction learning, when the labile extinction memory is consolidated. In this phase, the influence of enhanced dopaminergic transmission was investigated. Return of fear at a later CS presentation was assessed using subjective fear and distress ratings, psychophysiological (skin conductance response (SCR)) and functional magnetic resonance imaging (fMRI) measurements.

The administration of the dopamine prodrug L-DOPA (INN: levodopa) directly after extinction learning reduced neural measures of return of fear (fear memory recall-related activation in the posterior hippocampus, the dorsomedial prefrontal cortex and the amygdala), enhanced activity in brain regions that have been implicated in fear inhibition (anterior hippocampus, ventromedial prefrontal cortex) and, in one of these studies, attenuated subjectively and psychophysiologically measured return of fear.

Together, these results provide evidence that dopaminergic neurotransmission can strengthen extinction memories and prevent the return of fear. These results encourage further studies investigating a possible pharmacological augmentation of psychotherapy of anxiety.

6.2 Zusammenfassung

Angsterkrankungen haben die höchste Prävalenz unter den psychischen Erkrankungen in den USA und Westeuropa (Alonso et al. 2004, Kessler et al. 2005). Ein großes Problem in der Behandlung ergibt sich aus den hohen Rückfallraten, das heisst, aus wiederkehrenden Angstsymptomen, die eine Psycho- oder Pharmakotherapie überdauern.

Die klassische Furchtkonditionierung gilt als Modell der Entstehung von Angsterkrankungen. Hierzu werden Furchtreaktionen durch Paarung eines Stimulus (konditionierter Stimulus, CS) mit einem intrinsisch aversiven Stimulus (unkonditionierter Stimulus, US) hervorgerufen. Die wiederholte Präsentation des CS ohne den US (Extinktion) lässt die Furchtreaktion auf den CS abnehmen und kann als Modell für Lernprozesse in der Psychotherapie von Angsterkrankungen angenommen werden. Konditionierung und Extinktion bilden zwei parallele Gedächtnisse, die mit dem CS verknüpft sind: Eines ruft eine Furchtreaktion hervor (CS-US-Assoziation), das andere signalisiert Sicherheit und inhibiert die Furcht (CS-keinUS-Assoziation). Das Furchtgedächtnis kann das Extinktionsgedächtnis dominieren und „Return of fear“ (Rückfall) auslösen, wenn der extinguierte CS ausserhalb des Extinktionskontexts (des Therapiekontexts) dargeboten wird.

Im Rahmen der Doktorarbeit wurde speziell die Phase nach dem Extinktionslernen, in der das Erlernte in ein stabiles Gedächtnis konsolidiert wird, untersucht, um so neue Ansätze für eine Verstärkung von Extinktionsgedächtnissen zu finden. Hierzu wurde insbesondere der Einfluss verstärkter dopaminerger Transmission betrachtet. Return of fear wurde während darauf folgender Wiederdarbietung des CS anhand subjektiver Furchtbewertungen, psychophysiologischer (Hautleitfähigkeitsantwort, SCR) und bildgebender (funktionelle Magnetresonanztomographie des Gehirns, fMRT) Indizes gemessen.

Die Verabreichung der endogenen Vorstufe von Dopamin, L-DOPA (INN:levodopa), direkt nach dem Extinktionslernen reduzierte neurale Maße des Return of fear (Furcht-Wiederabruf-assoziierte Aktivierung des posterioren Hippocampus, des dorsomedialen präfrontalen Kortex und der Amygdala), verstärkte die Aktivität von Hirnregionen, die mit Furchthemmung in Zusammenhang stehen (anteriorer Hippocampus, ventromedialer präfrontaler Kortex), und verringerte in einer der Studien auch den subjektiv und psychophysiologisch gemessenen Return of fear.

Zusammenfassend geben diese Ergebnisse einen ersten Hinweis, dass dopaminerge Neurotransmission Sicherheitsgedächtnisse verstärken und die Wiederkehr von Furcht verringern kann. Diese Ergebnisse können möglicherweise die Entwicklungen neuer Strategien der pharmakologischen Unterstützung der Psychotherapie von Angsterkrankungen anstoßen.

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Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

_____

Hamburg, den 01.Juni 2012 Unterschrift

Hiermit erkläre ich, dass die Dissertation nicht in einem früheren Promotionsverfahren angenommen oder als ungenügend erklärt wurde.

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