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# The Amygdala, Fear and Reconsolidation

## *Neural and Behavioral Effects of Retrieval-Extinction in Fear Conditioning and Spider Phobia*

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ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2017

ISSN 1652-9030
ISBN 978-91-554-9863-4
urn:nbn:se:uu:diva-317866

Dissertation presented at Uppsala University to be publicly examined in Gunnar Johansson salen, Blåsenhus, von Kraemers allé 1A, Uppsala, Friday, 12 May 2017 at 13:00 for the degree of Doctor of Philosophy. The examination will be conducted in English. Faculty examiner: Emily Holmes (Karolinska institutet, Institutionen för klinisk neurovetenskap; University of Oxford, Department of Psychiatry).

**Abstract**
Björkstrand, J. 2017. The Amygdala, Fear and Reconsolidation. Neural and Behavioral Effects of Retrieval-Extinction in Fear Conditioning and Spider Phobia. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Social Sciences* 140. 72 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-9863-4.

The amygdala is crucially involved in the acquisition and retention of fear memories. Experimental research on fear conditioning has shown that memory retrieval shortly followed by pharmacological manipulations or extinction, thereby interfering with memory reconsolidation, decreases later fear expression. Fear memory reconsolidation depends on synaptic plasticity in the amygdala, which has been demonstrated in rodents using both pharmacological manipulations and retrieval-extinction procedures. The retrieval-extinction procedure decreases fear expression also in humans, but the underlying neural mechanism have not been studied. Interfering with reconsolidation is held to alter the original fear memory representation, resulting in long-term reductions in fear responses, and might therefore be used in the treatment of anxiety disorders, but few studies have directly investigated this question.

The aim of this thesis was to examine the effects of the retrieval-extinction procedure on amygdala activity and behavioral fear expression in humans. The work presented here also investigated whether findings from studies on recent fear memories, established through fear conditioning, extends to naturally occurring long-term phobic fears.

**Study I**, combining fear conditioning and a retrieval-extinction procedure with functional magnetic resonance imaging (fMRI), demonstrated that memory retrieval shortly followed by extinction reduces later amygdala activity and fear expression in healthy subjects. In **Study II**, these subjects were re-tested 18 months later. The results showed that the effects on fear expression were still present and that initial amygdala activity predicted long-term fear expression. Using an adapted version of the retrieval-extinction procedure, **Study III** showed that memory retrieval shortly followed by exposure to spider pictures, attenuates subsequent amygdala activity and increases approach behavior in subjects with life-long fear of spiders. In **Study IV**, these subjects were re-tested 6 months later, and the results showed that effects on amygdala activity as well as approach behavior were maintained.

In summation, retrieval-extinction leads to long-lasting reductions in amygdala activity and fear expression. These findings are consistent with the hypothesis that retrieval-extinction alters an amygdala dependent fear memory. Retrieval-extinction can also attenuate long-term phobic fears, indicating that this manipulation could be used to enhance exposure-based treatments for anxiety disorders.

*Keywords:* Fear conditioning, phobia, memory reconsolidation, retrieval-extinction, exposure therapy, amygdala, fMRI

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ISSN 1652-9030
ISBN 978-91-554-9863-4
urn:nbn:se:uu:diva-317866 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-317866)

*To Frida, Arvid and Nora*

# List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Agren, T., Engman, J., Frick, A., Björkstrand, J., Larsson, E. M., Furmark, T., Fredrikson, M. (2012) Disruption of reconsolidation erases a fear memory trace in the human amygdala. *Science*, 337(6101),1550-1552

II Björkstrand, J., Agren, T., Frick, A., Engman, J., Larsson, E. M., Furmark, T., Fredrikson, M. (2015) Disruption of memory reconsolidation erases a fear memory trace in the human amygdala: an 18-month follow-up. *PLoS ONE*, 10(7), e0129393

III Björkstrand, J., Agren, T., Åhs, F., Frick, A., Larsson, E. M., Hjorth, O., Furmark, T., Fredrikson, M. (2016) Disrupting reconsolidation attenuates long-term fear memory in the human amygdala and facilitates approach behavior. *Current Biology*, 26(19), 2690-2695.

IV Björkstrand, J., Agren, T., Åhs, F., Frick, A., Larsson, E. M., Hjorth, O., Furmark, T., Fredrikson, M. (2016) Think twice, it's alright: Long lasting effects of disrupted reconsolidation on brain and behavior in human long-term fear. *Behavioural Brain Research,* 324, 125-129.

# Contents

# Abbreviations

| | |
|---|---|
| ACC | Anterior cingulate cortex |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| AMPAR | AMPA receptor |
| BA | Basal amygdala |
| BAT | Behavioral approach test |
| BLA | Lateral and basal amygdala |
| BNST | Bed nucleus of the stria terminalis |
| BOLD | Blood oxygen-level dependent |
| CE | Central amygdala |
| CeM | Central medial amygdala |
| CI-AMPAR | Calcium impermeable AMPAR |
| CP-AMPAR | Calcium permeable AMPAR |
| CR | Conditioned response |
| CS | Conditioned stimulus |
| CS+ | Conditioned stimulus, reinforced |
| CS- | Conditioned stimulus, not reinforced |
| EMG | Electromyography |
| EPSP | Excitatory post-synaptic potential |
| fMRI | Functional magnetic resonance imaging |
| FPS | Fear potentiated startle |
| GABA | Gamma-Aminobutyric acid |
| HR | Heart rate |
| IL | Infralimbic cortex |
| ITC | Intercalated cell masses |
| LA | Lateral amygdala |
| LTD | Long term depression |
| LTP | Long term potentiation |
| mPFC | Medial prefrontal cortex |
| MRI | Magnetic resonance imaging |
| NMDA | N-Methyl-D-aspartic acid |
| NMDAR | NMDA receptor |
| NS | Neutral stimulus |
| OCD | Obsessive-compulsive disorder |
| PAG | Periaqueductal gray |
| PD | Panic disorder |
| PL | Prelimbic cortex |

| | |
|---|---|
| PTSD | Post-traumatic stress disorder |
| SAD | Social anxiety disorder |
| SCL | Skin conductance level |
| SCR | Skin conductance response |
| UR | Unconditioned response |
| US | Unconditioned stimulus |
| vmPFC | Ventromedial prefrontal cortex |
| VR | Virtual reality |

# Introduction

Fear memories are easily formed but hard to get rid of. When a neutral stimulus is paired with an aversive outcome, the previously neutral stimulus will start to elicit defensive responses, and become a conditioned stimulus (CS). These conditioned defensive responses can be ameliorated by repeatedly exposing the subject to the CS without the aversive outcome, thereby inducing extinction learning. Extinction learning, however, does not remove the fear memory completely, as defensive responses tend to return even after initially successful fear extinction. Extinction, thus, induces an inhibitory safety memory that temporarily suppresses the expression of the original fear memory. The general aim of this thesis is to explore whether the effects of extinction can be made more permanent by exploiting memory reconsolidation mechanisms. When a memory is retrieved it enters a labile state and may, for a brief time period, be updated before it returns to a stable state, a process called reconsolidation (Alberini & LeDoux, 2013). By causing the fear memory to be retrieved shortly before extinction, the fear memory will be in a labile state and extinction can cause the original fear memory to be updated, thus producing a more permanent suppression of defensive responses. This mechanism could be translated to exposure based treatments for anxiety disorders, possibly improving immediate outcomes and reducing risk of relapse.

The following introduction summarizes the literature on reconsolidation disruption and its neural correlates and modulators in animals and humans. For the purpose of setting the stage for the empirical studies forming this dissertation, the human studies could be reviewed separately as the text blocks are stand-alone sections, but for the sake of completeness and for informing translation research the animal literature is included.

## Learning and memory

Learning from a biological perspective depends on connections between neurons, i.e. synapses, and consequently is mediated by synaptic plasticity. Learning occurs when an experience causes the synaptic strength between neurons to be altered, and in this sense, memory is the persistence of these changes. Although this process is not fully understood, a large body of research in the field of neuroscience has made some strides towards explaining what actually happens in the brain during learning. Most of this research has been performed

on non-human animals, but it may well extend to humans as many of the mechanisms involved appear to be fundamental to neuronal functioning and well conserved across species.

As postulated by Donald Hebb (Hebb, 1949), associative learning can be explained by temporal patterns in firing in connected neurons, as the strength of the connections between two neurons is increased by their simultaneous depolarization, often referred to as Hebbian plasticity. Although the biological substrates for Hebbian plasticity are not fully known, on a cellular level this type of learning has been linked to the process of long-term potentiation (LTP). This process involves pre-synaptic release of the neurotransmitter glutamate, the major excitatory neurotransmitter in the brain, and post-synaptic glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-Methyl-D-aspartic acid (NMDA). When the pre-synaptic cell depolarizes, it releases glutamate that binds to AMPA and NMDA receptors on the post-synaptic cell. This will cause the AMPA receptor (AMPAR) to open an ion channel admitting positively charged potassium ions (Na+), causing an excitatory post-synaptic potential (EPSP). The NMDA receptors (NMDARs), on the other hand, are blocked by magnesium ions and are therefore initially unable to admit ions to the post-synaptic cell. However, if the increased Na+ influx sufficiently increases the positive charge of the postsynaptic neuron this will cause NMDARs to discharge their magnesium ions and open up ion channels allowing the influx of positively charged calcium ions (Ca+). Increased Ca+ concentration will cause a further increase in post-synaptic depolarization, and also trigger plastic processes involving immediate insertion of additional AMPARs in the post-synaptic membrane. The increased density of AMPARs on the post-synaptic membrane will cause the cell to be more sensitive to subsequent presynaptic glutamate release, thereby potentiating the synaptic connection between these two cells (Malenka & Nicoll, 1999).

This process is often referred to as early LTP and lasts a couple of hours at most, and could therefore be a neural substrate for short term memory. In addition to the insertion of AMPARs on the synaptic membrane, increased Ca+ concentration also triggers other processes involving intracellular signaling that leads to gene translation, protein synthesis and ultimately structural alterations to the cell, such as increased number of dendritic spines and larger synapses, a process called late LTP. These changes last much longer than early LTP, perhaps indefinitely, providing a potential neural substrate for long-term memory. Whereas early LTP is a very fast process causing immediate changes to synapses, late LTP develops slower, taking minutes or hours to complete. In short, NMDARs function as coincidence detectors that trigger plastic processes like AMPAR trafficking and protein synthesis dependent structural alterations that ultimately affects the long-term connections between neurons, and are thus central to associative learning. As has become apparent during

the last couple of decades, memory is not a passive and static saving of information but a dynamic process, as the persistence of memories is actively regulated by neural and synaptical processes that is in constant flux (Haubrich & Nader, 2016; Malenka & Nicoll, 1999).

## Consolidation and reconsolidation

After a learning experience has occurred and a memory has been acquired, it is does not immediately enter a stable state. As noted above, learning triggers various cellular processes that stabilizes the memory over the course of a couple of hours, a process often referred to as memory consolidation or synaptic consolidation. This has been known for several decades as various procedures affecting memory consolidation, such as administration of amnesic drugs, for example protein synthesis inhibitors, electroconvulsive shocks or competing learning experiences, are detrimental to later memory performance if administered shortly after learning. Also, memory performance can be enhanced by various substances if given shortly after the learning experience. Of note, these types of manipulations only affect memory performance if they are applied within a time-window of a couple of hours after learning. If administered after a longer delay they have no effect. This indicates that acquired memories are initially in an unstable state and are sensitive to manipulation until consolidation is completed. Previously, the stable state achieved after completed consolidation, was believed to be permanent. After a memory trace had been stabilized it would no longer be amenable to attempts at manipulation, but recent findings have cast doubt on this conclusion (Alberini & LeDoux, 2013; Haubrich & Nader, 2016).

There are now numerous studies that confirm that memories are sensitive to manipulation even after initial consolidation has been completed. This line of research has used very similar procedures as those that have investigated consolidation. First memory acquisition is performed and the memory is allowed to consolidate over a period of 24 hours or more. Then the memory is reactivated, by inducing the subject to retrieve the memory, and some manipulation is performed with aim of affecting the reconsolidation of the memory. Using the same type of manipulations as in research on consolidation, it has been demonstrated that post-activation administration of protein-synthesis inhibitors or other amnesic drugs, electroconvulsive shocks, and competing learning experiences decreases performance at later retentions tests. Also, post-activation administration of various drugs that are thought to facilitate LTP-related processes, enhance later memory performance. Again, these types of manipulations only affect memory performance if they are administered shortly after the memory activation. Delayed manipulation produces no effects on memory performance. This indicates that when consolidated mem-

ories are activated they enter a destabilized state and are amenable to modification. In order for the memory to persists it must go through an LTP-related process in order to return to its formerly stable state. Memory reconsolidation has over the last 15 years been demonstrated in rodents, humans and several other species, using a variety of different amnesic procedures and learning paradigms. Much of this work has employed fear conditioning procedures, investigating the persistence of fear memories, which is the focus of this thesis (Alberini & LeDoux, 2013; Haubrich & Nader, 2016).

## Fear conditioning

Fear conditioning is a form of associative learning that allows animals to predict aversive outcomes based on cues in the environment that precedes those outcomes. Fear learning is adaptive and conducive to survival as it allows animals to escape and avoid environmental cues that are potentially harmful. This form of learning is evolutionary old and conserved across species as it has been demonstrated in a wide array of organisms from rodents to humans (LeDoux, 2000). Despite being an adaptive ability, in humans fear learning is also believed to contribute to the development of fear and anxiety related disorders (Mineka & Zinbarg, 2006). These type of disorders, such as specific phobia, social anxiety disorder (SAD), panic disorder (PD), post-traumatic stress disorder (PTSD), and obsessive-compulsive disorder (OCD), are characterized by exaggerated and irrational fears of what is essentially harmless cues and situations. When fears become excessive this may result in suffering and avoidance of situations important for quality of life. Therefore, an increased understanding of fear learning mechanisms may serve to increase the effectiveness of interventions aimed to reduce anxiety disorders and alleviate human suffering (Craske, Treanor, Conway, Zbozinek, & Vervliet, 2014; Vervliet, Craske, & Hermans, 2013).

Classical conditioning occurs when a neutral stimulus (NS) is paired with another biologically important stimulus, called an unconditioned stimulus (US), that has the capability to elicit some innate physiological or behavioral response (an unconditioned response; UR). Through pairing, the subject learns that the NS predicts the US and the NS also comes to elicit some physiological or behavioral response (a conditioned response, CR) in anticipation of the US, thus becoming a conditioned stimulus (CS) (Craske, Hermans, & Vansteenwegen, 2006). This was first demonstrated in dogs, in the beginning of the 20th century (Pavlov, 1927), using a sound as the NS and food as the US, causing a salivary response (UR). After several parings, the sound itself came to elicit a salivary response (CR), and the bell had thus become a CS.

When the US constitutes some threat to the subject, causing a defensive response, this is termed fear conditioning, or threat conditioning. This form of learning has been studied extensively both in rodents and humans. In rodents,

the most common experimental procedure uses a tone (NS/CS) preceding an electric chock (US), then measuring freezing or avoidance behavior (CR) to the tone, this procedure is often referred to as cued fear conditioning. In rodents, it is also common to pair a specific environment, instead of discrete cues, with aversive stimuli, a procedure referred to as contextual fear conditioning. In experiments on humans, typically, visual stimuli are presented to the subject (NS/CS) followed by an electric chock (US), while measuring some index for physiological arousal to the CS, such as skin conductance response (SCR), heart rate (HR), or fear potentiated startle reflex (FPS) (Craske et al., 2006). The increased physiological arousal level is mediated by the sympathetic branch of the autonomous nervous system, and serves as a way for the organism to prepare for fight or flight, thus increasing the chances of survival in the face of danger. To control for non-learning related changes in physiological arousal, experiments in humans often include a control stimulus that is not paired with the US. This is called differential fear conditioning, and in these cases, the stimulus paired with the US is referred to as the CS+ and the control stimulus is referred to as the CS- (Craske et al., 2006). Although, it has been suggested that responses to the CS- also reflect learning related processes, since the CS- predicts the non-occurrence of the US, thus becoming a safety cue (Grillon, 2008). Due to its simplicity and ability to induce fast learning, the fear conditioning procedure is well suited to study the biological underpinnings of learning and research over the last couple of decades have led to a detailed understanding of what happens in the brain when fear associations are formed (LeDoux, 2000).

## Neural substrates of fear conditioning

Fear conditioning is thought to depend on a distributed neural circuit, involving separate sensory input pathways transmitting information of the CS and US, that converge in the amygdala, an almond shaped bilateral structure situated at the anterior section of the medial temporal lobe. Output pathways from the amygdala then projects to other regions that control defensive responses. Experimental work, primarily using cued and contextual fear conditioning paradigms in rodents, has described this learning pathway in detail. Although learning related synaptic plasticity may take place in several sites along these input and output pathways, the amygdala has been shown to be of particular importance and is thought to be the location were the CS-US association is formed (Johansen, Cain, Ostroff, & LeDoux, 2011; LeDoux, 2000; Pape & Pare, 2010).

The amygdala can be divided into different areas, most commonly the lateral (LA), basal (BA), and central (CE) areas, constituting distinct subnucleui differing in cellular composition and connectivity. Often, the lateral and basal areas are collectively referred to as the basolateral area (BLA). These subdivisions have been shown to be relevant in the context of fear conditioning

since they have been implicated in different stages of the learning process (LeDoux, 2000; Pape & Pare, 2010).

The LA is thought to be the input station of the amygdala. Sensory input from the thalamus and cortex that convey information of the CS and US are routed to the LA where the US and CS signals are thought to converge. Thus, the LA is held to be the site were the CS-US association is formed through synaptic plasticity. LA then projects to the CE that is believed to be the output region of the amygdala. The CE has long range connection that in turn projects to the hypothalamus, the bed nucleus of the stria terminalis (BNST) and areas in the brain stem, for example the periaqueductal gray (PAG), that control defensive responses, such as freezing, physiological arousal, stress hormone secretion and behavioral avoidance (LeDoux, 2000; Pape & Pare, 2010).

In support of the central role of the LA in fear conditioning, studies in rodents have shown that lesions or reversible inactivation of the LA blocks fear conditioning. Also, electrophysiological studies show that LA neurons are responsive to both CS and US input and that these responses are modulated by fear conditioning. Before fear conditioning, the US elicits strong depolarization of LA neurons, leading to activation of the output pathway causing defensive responses, whereas the CS only elicits weak depolarization. When the CS is paired with the US, the strong depolarization caused by US input on LA neurons will potentiate CS-input synapses on these LA neurons due to their simultaneous activation. After fear conditioning the CS-elicited depolarizations are enhanced, and the CS alone can trigger activation of the output pathway, resulting in defensive responses (Johansen et al., 2011; LeDoux, 2000).

Thus, fear conditioning likely involves Hebbian plasticity in the LA, and in further support of this, various LTP-related processes have been implicated in the formation of fear memories. For example, it has been demonstrated that microinjection of NMDAR-antagonist into the LA blocks fear conditioning, indicating that NMDARs on LA neurons may function as coincidence indicators, triggering plastic processes during fear learning. Furthermore, indices of increased calcium concentration have been observed in spines of LA neurons following fear conditioning and also increased membrane AMPAR-insertion in thalamic-LA synapses, both of which appear to be necessary for fear conditioning to occur (Johansen et al., 2011).

These findings support the role of the LA in the initial acquisition of fear memories but synaptic plasticity in the LA has also been linked to the consolidation of fear memories. For example, protein synthesis in the LA has been shown to be necessary for the consolidation of fear memories since microinjection of protein synthesis inhibitors into the LA following fear conditioning blocks long-term fear expression but not short-term fear expression. Also, various indices of gene transcription and protein translation in the LA have been linked to the long-term retention of fear memories and structural alterations of LA neurons have been observed following fear conditioning, such as increases in synapse size and number (Johansen et al., 2011).

For obvious reasons, most of the methods used to study the neural aspects of fear conditioning in animals is prohibited in humans due to their harmfulness and invasive nature. Nonetheless, there is support for the central role of the amygdala also in humans. For example, fear conditioning studies on individuals with pre-existing brain damage have observed deficits in fear learning in individuals with amygdala lesions (Bechara et al., 1995) or unilateral temporal lobe lesions including the amygdala (LaBar, LeDoux, Spencer, & Phelps, 1995). The finding that amygdala is central to fear learning has also been extended to humans by studies using in-vivo brain imaging techniques, most commonly functional magnetic resonance imaging (fMRI). These studies have found increased amygdala activation to the CS+ compared to the CS- in the early phases of fear conditioning, and amygdala activity has also been shown to be correlated with CS-elicited increases in physiological arousal measured with SCR. Apart from the amygdala, increased activation in the anterior cingulate cortex (ACC), anterior insula and hippocampus have also been observed during fear conditioning (Greco & Liberzon, 2016). Also a recent meta-analysis showed increased activation in the thalamus and in sub-cortical structures related to fear expression, for example the hypothalamus and various brain-stem regions including PAG (Fullana et al., 2016). Whereas amygdala activity have been shown to decrease over the course of fear conditioning, activity in the ACC and insula remain high throughout, possibly indicating that amygdala is specifically involved in the acquisition of fear responses whereas the ACC and insula are more related to fear expression and activation of the ACC has also been found to correlate with SCRs (Greco & Liberzon, 2016; Shin & Liberzon, 2010).

## Fear extinction

Conditioned fear responses can be diminished through extinction. During extinction, the CS is presented repeatedly without being followed by the US and thus the subject learns that stimuli that previously predicted an aversive outcome no longer does so, causing the CR to decrease or disappear. However, extinction is not believed to undo the original CS-US association but instead is thought to be caused by new inhibitory learning. This mainly relies on observations showing that the decreases in fear responses that occur after extinction are not permanent. Fear responses will return with the passing of time, called spontaneous recovery, and also if the US is presented alone without pairing it with the CS, called reinstatement. In addition to this, extinction learning appears to be context specific which is not true for fear conditioning. If you establish fear responses to a cue by repeatedly pairing it with a US, the subject will continue to display defensive responses even if you expose them to that stimulus in a different environment. Extinction however, is sensitive to

shifts in context. If you extinguish a stimulus in a particular context, fear responses tend to return if the stimulus is presented in a different context, a phenomenon often referred to as renewal. Also, if you after successful extinction again pair the CS with US, relearning of the CR goes faster than if no previous learning has occurred, called reacquisition. These phenomena suggest that fear memories are not removed through extinction. Because the fear response so easily returns, extinction is not considered to cause "unlearning" or forgetting of the original fear memory, but rather is a separate learning process in and of itself leading to the formation of a context dependent inhibitory safety memory. From a neural perspective, extinction is thought to induce a separate memory trace that suppresses the expression of the fear memory, thus extinction does not cause the fear memory to disappear, but rendering it temporarily dormant (Bouton, 2002; Dunsmoor, Niv, Daw, & Phelps, 2015; Vervliet et al., 2013).

## Neural substrates of fear extinction

Although extinction is regarded as a separate learning process from fear conditioning it involves an overlapping network of brain areas, including the amygdala, hippocampus and the ventromedial prefrontal cortex (vmPFC). The amygdala appears to be essential for forming extinction memories as procedures that inhibit neuronal activity or interferes with synaptic plasticity in the BLA weakens extinction learning. Electrophysiological studies also show that extinction reduces CS elicited activity in the LA. Although both fear and extinction learning is dependent on the amygdala this does not indicate that extinction abolishes the fear memory trace. In fact, studies have shown that separate neuronal populations within the BLA are responsible for fear and extinction learning. For example, neurons in the dorsal LA show reduced responses during extinction, whereas neurons in the ventral LA show no activity reductions. Furthermore, these different types of neurons have been shown to have different long range connectivity, specifically to the vmPFC regions. Fear neurons in the BLA project to the prelimbic region (PL) of the vmPFC, that has been shown to mediate fear expression, whereas extinction neurons project to the the infralimbic region (IL) which appears to have a dampening effect on fear expression (Duvarci & Pare, 2014; Orsini & Maren, 2012; Tovote, Fadok, & Luthi, 2015).

The vmPFC has been heavily implicated in extinction learning, and specifically the IL is thought to be particularly important for suppressing fear responses after extinction. The IL is a structure in the rat brain, held to be roughly equivalent to the subgenual ACC in humans, that is reciprocally connected to the amygdala. Research in rodents have shown that extinction induces NMDAR dependent plasticity in IL neurons, and IL neurons show increased CS-elicited activity during extinction recall, i.e. when the CS is pre-

sented in the extinction context at a later time point. Also, electrical stimulation of the IL inhibits activity in the central medial amygdala (CeM), and could thereby decrease fear expression, since most of the amygdala connections that project to areas involved in fear expression originate in the CeM. This inhibitory effect may also involve an amygdala subregion called the intercalated cell masses (ITC), which is innervated by the IL. ITC lies between the BLA and CE and consists of GABAergic cell groups that can inhibit signaling between these two areas. Stimulating the IL causes neuronal activity in the ITC and activation of ITC decreases activation in target cells in the CE, which could serve to dampen fear responses. The decrease in fear expression that is observed after extinction learning thus involves both inhibitory circuits within the amygdala and regulatory signaling from the vmPFC (Duvarci & Pare, 2014; Orsini & Maren, 2012; Tovote et al., 2015).

Also the hippocampus is involved in extinction learning. As noted above, extinction is context specific, and since the hippocampus has been shown to be important for processing of context related information it is believed to involved in the contextual control of the expression of extinction memories. The hippocampus is connected to both the vmPFC and the amygdala and could thus modulate fear expression. In support of this, pharmacological deactivation of the hippocampus prior to a extinction retention test, blocks renewal, as does post-extinction hippocampal lesions (Orsini & Maren, 2012; Tovote et al., 2015).

Similar to fear conditioning, the neural substrates of extinction learning in humans have mainly been studied using in-vivo brain imaging. In line with findings in rodents, increased CS-elicited amygdala activation have been observed in the beginning of extinction that then deceases with time, supporting that extinction attenuates amygdala activity. Also, increased vmPFC activation has been found during extinction implicating this region in the encoding of extinction memories. Similar findings have also been reported during extinction recall. CS-elicited amygdala activations have been found during extinction recall as well as vmPFC activations. Notably, vmPFC has been linked to the retention of extinction memory as vmPFC activity during extinction recall has been found to be negatively correlated to fear expression measured with SCR. Although not as well studied, hippocampus has been implicated in fear renewal in humans. One study found increased activations in the hippocampus when the CS was presented in the same context as extinction took place but not when presented in the fear conditioning context. This study also found that activity in the hippocampus was positively correlated to activity in the vmPFC, indicating that the hippocampus might provide contextual control of fear expression through connections to prefrontal regions that suppress fear responses (Greco & Liberzon, 2016; Shin & Liberzon, 2010).

## Fear conditioning, extinction and anxiety disorders

Associative learning experiences, akin to experimental fear conditioning, is believed to contribute to the development of several fear and anxiety related disorders, such as specific phobia, panic disorder (PD), social anxiety disorder (SAD), post-traumatic stress disorder (PTSD) and obsessive-compulsive disorder (OCD)(Bouton, Mineka, & Barlow, 2001; Craske et al., 2014; Jacoby & Abramowitz, 2016; Mineka & Zinbarg, 2006; Rapee & Spence, 2004; Vervliet et al., 2013). In short, these types of disorders are thought to have their origin in learning experiences in which some frightening or aversive experience is associated with otherwise harmless cues or contexts, either through direct experience, verbal instruction or through observing others (Mineka & Zinbarg, 2006). This fear association is then generalized to include similar stimuli and leads to avoidance behavior, which perpetuates the problems since no extinction is allowed to occur (Craske et al., 2006, Dymond, Dunsmoor, Vervliet, Roche, & Hermans, 2015; Mineka & Zinbarg, 2006; Mowrer, 1960).

In relation to this, fear extinction has also served as an experimental model for behavioral therapy for anxiety disorders, specifically exposure therapy. In exposure therapy, the patient gradually approaches feared cues and situations, allowing extinction to occur, and thereby learns that they are not predictive of aversive experiences which decreases fear, anxiety and avoidance. Exposure therapy was developed during the middle of the 20$^{th}$ century based on learning theory derived from fear conditioning and extinction research (Mineka & Zinbarg, 2006; Vervliet et al., 2013; Wolpe, 1958) and is still one of the best validated and most effective treatments for many anxiety disorders (Ougrin, 2011). Thus, fear conditioning and extinction may be used as experimental models for anxiety disorders, and theory derived from basic fear learning experiments could increase our understanding of how these problems are acquired, maintained and alleviated (Craske et al., 2014; Mineka & Zinbarg, 2006; Scheveneels, Boddez, Vervliet, & Hermans, 2016; Vervliet et al., 2013).

In support of this, it has been shown that individuals with anxiety disorders differs from healthy individuals on several variables related to fear conditioning and extinction. A recent meta-analysis (Duits et al., 2015) showed that during fear conditioning, patients with anxiety disorders, including PTSD, specific phobia, OCD, PD, and generalized anxiety disorder (GAD), have larger fear responses to the CS-, but not the CS+ compared to healthy controls. This may reflect that patients show less inhibition of fear responses to stimuli signaling safety, or exaggerated generalization of the fear responses to safety cues, since in these types of studies the CS- is often perceptually similar to the CS+. The same meta-analysis also showed that during extinction, patient with anxiety disorders have increased fear responses to the CS+, but not the CS-, and larger differences between the CS+ and CS-, compared to healthy controls. This indicates that extinction learning is slower in people with clinical anxiety. Also, patients with PD and GAD show greater fear generalization

compared to healthy controls (Dymond et al., 2015). These findings suggest that deficiencies in fear and extinction learning may constitute vulnerability factors, increasing the risk of developing an anxiety disorder. However, since all these studies are cross-sectional designs it cannot be ruled out that this an acquired, rather than a pre-existing trait, although there are a few longitudinal studies that show that extinction learning deficiencies pre-dates the development of anxiety symptoms (Duits et al., 2015).

Findings from brain imaging studies also support a link between fear learning and anxiety disorders, showing that fear conditioning in healthy subjects and fear provocation in anxiety disorders results in similar brain activation patterns, suggesting that both rely on common underlying neural circuitry. One meta-analysis showed that enhanced amygdala and anterior insula activity during exposure to negative emotional stimuli is often found in patients with PTSD, SAD and specific phobia compared to healthy controls (Etkin & Wager, 2007). Also, a recent study investigated neural activation during fear provocation in several anxiety disorders in the same experiment. Compared to healthy controls, patients with PD, SAD, PTSD and dental phobia showed increased activation in the amygdala when watching disorder relevant negative emotional pictures, with no differences between disorders. Furthermore, amygdala activation was positively correlated to subjective anxiety ratings across all disorders. Apart from the amygdala, patients also showed higher activation in other areas believed to be involved in fear processing such as the anterior insula, ACC, medial prefrontal cortex (mPFC), thalamus and the brainstem (Feldker et al., 2016).

A recent meta-analysis looking only at specific phobia showed that patients had increased activations in the bilateral amygdala and insula when looking at phobic stimuli compared to neutral stimuli. When compared to healthy controls, patients showed increased activations in the left amygdala and insula and thalamus when looking at phobic stimuli (Ipser, Singh, & Stein, 2013). These findings are in line with the previous meta-analyses and implicates fear learning related regions specifically in phobias. Also, in specific phobia, exposure therapy has been found to impact neural responses in fear related circuitry. One recent study showed that a 2-hour exposure session resulted in reduced activity in the amygdala, ACC, and anterior insula, as well as decreases in self-rated spider fear and increased approach behavior in subjects with spider phobia. The study also found that these results were maintained at a 6-months re-test (Hauner, Mineka, Voss, & Paller, 2012). This indicates that fear provocation in specific phobia is related to increased amygdala activity which can be attenuated through exposure treatment.

It should be noted that it is not unproblematic to equate the type of learning that is studied in fear conditioning experiments with the type of learning that is believed to be involved in anxiety disorders. Fear conditioning is a simplistic experimental model, and being such, does not necessarily capture all relevant aspects of human fear and anxiety. In a recent paper, Joseph Ledoux, a

pioneer in research on the neuronal basis of fear conditioning, points out that the subcortical neural circuit that has been shown to mediate fear learning and expression might only be indirectly related to conscious subjective experiences of fear and anxiety in humans. Although this system has been shown to control the behavioral and physiological expression of fear, conscious experiences of fear and other emotions likely relies on other cortical structures that are unique to humans. In support of this, although humans with amygdala damage show deficits in fear conditioning they are still able to experience fear and panic, and also, fear conditioning is still possible even when stimuli are not consciously perceived. Thus, manipulations aimed at diminishing the behavioral and physiological aspects of fear expression does not necessarily impact conscious experiences of fear and anxiety, which is perhaps the most prominent problem in anxiety disorders (LeDoux & Pine, 2016). Indeed, LeDoux argues that the term fear conditioning is something of a misnomer and should more appropriately be called threat conditioning (LeDoux, 2014).

Also, fear conditioning research has largely been focused on learning through direct experience of the CS-US contingency. As noted above, the development of anxiety disorders may well depend on vicarious learning, such as learning thorough observation or verbal instruction (Mineka & Zinbarg, 2006), the underlying processes of which have not been studied to the same extent. Furthermore, whereas fear conditioning research typically studies acquired defensive responses to discreet cues and contexts, anxiety disorders are characterized by fear and avoidance of a large and sometimes fuzzy category of cues and environments, which might entail different memory processes and underlying neural circuitry. In general, the processes involved in the development and expression of clinical fear and anxiety might well be more complex than the processes involved in fear conditioning and therefore careful translational research is needed in order to determine to what extent basic findings in fear conditioning can be generalized to human fear and anxiety.

Lately, it has been suggested that incorporating findings from the field of fear extinction in the application of exposure therapy could improve outcomes in these types of treatment, an idea that has received some support in recent translational research (Craske et al., 2014; Vervliet et al., 2013). A lot of this research has focused on return of fear, a concept referring to the well-established finding that even after successful extinction, fear responses tend to return as an effect of spontaneous recovery, reinstatement, renewal and reacquisition. In line with findings from experimental fear extinction, return of fear has also been observed following exposure treatment in individuals with fears or anxieties. For example, increased self-reported fear, behavioral avoidance, and physiological arousal has been found in subjects with spider phobia if presented with spiders in a novel context following exposure treatment, equivalent to a renewal effect (Bandarian-Balooch, Neumann, & Boschen, 2015; Mystkowski, Craske, & Echiverri, 2002). Also, relapse after successful treat-

ment is not uncommon in anxiety disorders (Vervliet et al., 2013). Consequently, transferring manipulations that have been found to decrease return of fear following extinction to exposure-based interventions may improve treatment effects. The work presented in this thesis follows this line of research examining whether mechanisms pertaining to reconsolidation disruption can be used to reduce return of fear.

## Disrupting the reconsolidation of fear memories

In a seminal paper by Nader, et al. (Nader, Schafe, & Le Doux, 2000) it was demonstrated that reconsolidation occurs for fear memories in rats, and that it is dependent on protein synthesis in the LA. The study used a cued fear conditioning procedure, pairing a tone with aversive electric shocks. 24 hours after fear conditioning, the memory was activated by an un-reinforced CS presentation and reconsolidation was disrupted by injecting the protein synthesis inhibitor anisomycin directly in to the LA. The following day, the fear memory was tested by presenting the CS again and measuring freezing behavior. In the rats that had received anisomycin injection shortly after the memory activation, freezing behavior was reduced. In contrast, performing the manipulation 6 hours after memory activation or administering anisomycin without a previous memory activation had no effect on freezing behavior. This demonstrates that memory activation causes a consolidated fear memory trace localized in the LA to enter a destabilized state that requires protein synthesis to return to a stable state. This study sparked a renewed interest in the reconsolidation phenomenon and the findings have been replicated several times (Baldi & Bucherelli, 2015). However, subsequent studies have also shown that the effect does not always appear and have identified several boundary conditions pertaining to aspects of the fear memory itself and how it is activated.

### Age and strength of fear memories

Firstly, aspects of the initial fear memory determine whether amnesic procedures following memory retrieval decreases later fear expression. Specifically, age and strength of the memory have been shown to modulate the effects of post-activation administration of amnesic drugs. Although several studies have shown that it is possible to disrupt reconsolidation of fear memories older than 24 hours, memories appear to become more resistant to this manipulation as the time between acquisition and activation increases (Alberini, 2011; Bustos, Maldonado, & Molina, 2009; Milekic & Alberini, 2002; Nader et al., 2000; Suzuki et al., 2004; Wang, de Oliveira Alvares, & Nader, 2009). Also, the strength of the memory influences reconsolidation disruption as stronger memories appears to be more resistant to this manipulation (Suzuki et al., 2004; Wang et al., 2009).

Secondly, the length of the activation session influences the effect of post-activation disruption and also interacts with the age and strength of the initial fear memory. For example, using a contextual fear conditioning paradigm, Suzuki et al. (2004) showed that a relatively weak fear memory, established by delivering 1 foot shock in the training context during acquisition, can be disrupted by systemic anisomycin administration in conjunction with a 3-minute exposure to the training context, thereby destabilizing the memory. However, this manipulation had no effect on stronger memories, established by delivering three foot shocks during acquisition, but when increasing the length of the memory activation to 10 minutes the effects appeared again. Length of memory activation also influences the effect of reconsolidation disruption in older memories. Suzuki et al. (2004) also found that anisomycin administration in conjunction with a 3-minute memory activation decreases subsequent freezing behavior in 1-week and 3-week-old memories, but not 8-week-old memories. Increasing the length of the reminder to 10 minutes, however, produces the effect also in 8-week-old memories. These results suggest that older and stronger fear memories require longer memory activation in order to be destabilized.

Wang et al. (2009) have also investigated boundary conditions related to the strength of the initial fear memory using cued fear conditioning. During memory acquisition, tones (CS) were paired with electrical shocks (US). One group received one CS-US pairing, establishing a weak fear memory, and one group received 10 CS-US pairings, inducing a strong fear memory. 48 hours after acquisition, the memory was reactivated by one non-reinforced CS presentation immediately followed by anisomycin injection directly into the BLA. The integrity of the fear memory was then tested by measuring freezing behavior during CS presentation 24 hours later. In line with Suzuki et al. (2004), decreased freezing was observed only in the group that had received one CS-US pairing during acquisition and not in the group that received 10 CS-US pairings. This study also evaluated if the age of the memory influences whether strong fear memories undergo reconsolidation, by administering anisomycin following one CS presentation, either 7 days, 30 days or 60 days after strong fear acquisition. The results showed no effect on subsequent freezing for 7 day old memories, but surprisingly did find significant effects both for 30-day and 60-day old memories. These results show that even if strong fear memories do not undergo reconsolidation when activated shortly after acquisition they will do so after a longer delay, and thus it appears that the boundary condition of memory strength is transient. These studies are important, in that they illuminate possible boundary conditions that pertains to the reactivation of old and strong fear memories. If this mechanism is to be translated to clinical settings it is paramount that it is possible to influence old and strong memories, as clinical fears are often old, and arguably, strong.

## Length of memory activation

It has been observed that either very short or very long memory activations does not lead to fear memory reconsolidation. Pertaining to short memory activations, studies using contextual fear conditioning paradigms have found that administration of amnesic agents following a 1 min exposure to the conditioning context does not decrease subsequent fear expression even for young fear memories, whereas a 3 min exposure does (Bustos et al., 2009; Suzuki et al., 2004). On the other hand, studies that use cued fear conditioning have shown that administration of amnesic drugs following a single non-reinforced CS presentation readily produces decreased fear expression (Nader et al., 2000). One line of reasoning that could explain these findings is the supposition that the memory retrieval must cause some prediction error in order for destabilization to take place.

Prediction error, simply put, refers to an instance when the predicted outcome does not match the actual outcome. Thus, in order for destabilization to occur the memory retrieval must include some potential for new learning (Sevenster, Beckers, & Kindt, 2013). Using a non-reinforced CS presentation as a memory activation would fulfil this requirement as the subject under study would expect to receive a shock upon CS presentation because of having previously received one or several CS-US pairings. However, a recent study has also shown that not only expectations referring to the occurrence/absence of the US can induce prediction error, but also expectations relating to the timing of US delivery. Dias-Mataix et al. (2013), investigated this by subjecting rats to an auditory cued fear conditioning protocol consisting of 10 CS-US pairings, the CS being a 60 second tone with the US delivered after 30 seconds. The subsequent day they induced memory retrieval by a reinforced CS presentation with the US delivered after either 30 seconds in one group or 10 seconds in another group, followed by anisomycin injection directly into the LA, thereby disrupting reconsolidation. At a re-test 24 hours later, freezing behavior was reduced only in the group that had received the US 10 seconds after the CS onset during memory retrieval, demonstrating that if the predicted timing of US delivery is not violated during memory activation, no destabilization occurs. The same results were obtained also when using a weaker fear conditioning protocol consisting of one or two CS-US pairings. This supports that inducing prediction error during memory activation is necessary in order to destabilize fear memories and also demonstrates that during fear conditioning it is not only the contingency/reinforcement rate that is learnt but also the US timing.

This conclusion could explain previous findings from Suzuki et al. (2004) and Bustos et al. (2009). These studies used contextual fear conditioning protocols in which the rats received un-signaled foot-shock either 2.5 or 3 minutes after being placed in the training context. In light of the Dias-Mataix study it seems logical that during subsequent memory activation, a 1-min presentation

of the training context would be insufficient to induce prediction error, as the expected delivery of the US occurs later. Possibly, this could also explain why increasing the length of memory activation counteracts the boundary conditions of age and strength of memory in studies using contextual fear conditioning protocol since longer exposure to the conditioning context during memory activation might increase prediction error.

On the other hand, if the memory reactivation is too long, it will not induce memory reconsolidation. Studies on contextual fear conditioning in rodents have shown that if protein synthesis inhibitors are given after a 30-min reactivation session, this will cause increased freezing behavior during a later retest compared to control subjects receiving saline solution (Mamiya et al., 2009; Suzuki et al., 2004). Similar findings have been observed in studies using cued fear conditioning and administration of NMDAR antagonists prior to memory activation to interfere with reconsolidation (Lee, Milton, & Everitt, 2006; Merlo, Milton, Goozee, Theobald, & Everitt, 2014). One of these studies found that systemic administration of NMDAR antagonist reduced later fear expression compared to a control group that received saline solution, if followed by a single non-reinforced CS presentation, but if followed by four CS presentations there were no differences between groups. Also, if the memory activation consisted of seven or ten CS presentations the results where inverted, with increased freezing behavior in the group that received NMDAR antagonist compared to the saline group (Merlo et al., 2014). The reason for this is believed to be that a long memory activation will induce the formation of an extinction memory instead of triggering reconsolidation of the fear memory. Thus, these findings indicate that extinction and reconsolidation are dissociative processes. Consequently, amnesic manipulations given in conjunction with long memory activations will interfere with the consolidation of the extinction memory instead of disrupting reconsolidation of the fear memory, resulting in increased fear expression compared to control subjects.

## Neural substrates of fear reconsolidation

As noted above, protein synthesis is necessary for reconsolidation to occur, implicating processes related to late LTP in the re-saving of fear memories following retrieval (Baldi & Bucherelli, 2015; Nader et al., 2000; Wang et al., 2009). NMDA receptors are also important for reconsolidation as systemic administration NMDAR antagonist after memory activation reduces later freezing behavior in a dose dependent manner, as demonstrated by Suzuki et al. (2004), using a contextual fear conditioning procedure in rats. Also, NMDAR antagonist have been shown to reduce later fear expression if given before memory activation, whereas administration of NMDAR agonists results in increased fear expression (Lee et al., 2006; Merlo et al., 2014). Com-

pounds affecting other types of receptors have also been shown to disrupt reconsolidation. Beta-blockers acting as an antagonist on beta-adrenergic receptors, reduce later fear expression if administered after memory activation within the reconsolidation window, linking the noradrenergic system to fear memory reconsolidation (Debiec & Ledoux, 2004). Likewise, studies investigating post-activation administration of benzodiazepines have produced similar effects, implicating that the GABAergic system modulates reconsolidation (Bustos, Giachero, Maldonado, & Molina, 2010; Bustos et al., 2009). Importantly, in contrast to synthesis protein inhibitors and NMDAR antagonists, beta-blockers and benzodiazepines are safe for use in humans, providing possible translational opportunities for experimental and clinical studies in human subjects.

Studies with this type of design indicate what processes are implicated in the re-saving of the memory, but does not show what neural processes that drives the initial destabilization. This is especially interesting since memory does not always destabilize as a result of exposure to learning related cues. Information on what cellular processes drive memory destabilization might help in understanding the reason for this. This question can be investigated by looking at which cellular processes are engaged immediately after memory activation, and also by examining if pharmacological manipulations prior to memory activation can block or facilitate the effect of post-activation administration of amnesic compounds.

Similar to reconsolidation disruption, LTP-related processes are also implicated in the destabilization process. For example, NMDAR antagonists infused into the amygdala prior to memory activation, blocks the effect of post-activation anisomycin injection on later fear expression (Mamou, Gamache, & Nader, 2006). Interestingly, Wang et al. (2009) have shown that the boundary condition induced by strong training may be mediated by NMDAR signaling. Specifically, they found that strong training reduces expression of NMDARs containing the NR2B subunit in the LA if tested 2 days after training, when destabilization is not possible, but that this is not so when tested 60 days after training, when destabilization is possible. This indicates that the action of NMDA receptors may be crucial for memory destabilization to occur. In further support of this, a study by Bustos et al. (2010) showed that administration NMDAR agonists can facilitate memory destabilization. They found that exposing rats to a stressful experience 24 hours prior to fear conditioning resulted in a memory that was insensitive to reconsolidation disruption using post-activation administration of benzodiazepines. However, if NMDAR agonist D-cycloserine was administred prior to memory activation they did find an effect of post-activation benzodiazepines on later freezing behavior, indicating that NMDAR activity can facilitate destabilization in fear memories otherwise insensitive to memory activation.

Also, AMPA receptors have been linked to memory destabilization. One study has found that AMPA receptor trafficking in the amygdala tracks

memory destabilization after retrieval. The results showed that memory activation induces decreased density of calcium impermeable AMPA receptors (CI-AMPARs) and increased density of calcium permeable AMPA receptors (CP-AMPARS) following the same temporal pattern as the reconsolidation window (Hong et al., 2013). Since calcium acts as a plasticity trigger, high calcium influx caused by increased density of CP-AMPARs could potentiate synaptic plasticity specifically at activated synapses, providing a mechanism for memory updating following activation. Hong et al. (2013) also demonstrated the causal role for these processes in memory destabilization, as blocking CI-AMPAR endocytocis prior to memory activation counteracts the effect of post-activation anisomycin administration, and inhibits insertion of additional CP-AMPARs. In addition to this, they also showed that NMDAR antagonist blocked CI-AMPAR – CP-AMPAR exchange, demonstrating that the effect of NMDAR antagonists on memory destabilization, observed by Mamou, et al. (2006) and Bustos et al. (2010), may be mediated by the effect of NMDAR activity on AMPA receptor exchange.

## Pharmacological reconsolidation disruption in humans

Since memory traces established by fear conditioning are thought to be an important aspect of the development of anxiety disorders, this line of research might have important applications in the treatment of anxiety. Extinction training does lead to reduced fear expression but these effects are transient, since it does not alter the original fear memory, but leads to the formation of an inhibitory safety memory (Bouton, 2002; Vervliet et al., 2013). Mechanisms affecting fear memory reconsolidation may therefore be used to increase the effectiveness of exposure-based treatments for anxiety disorders as this would allow alterations of the fear memory itself. However, many of the manipulations and compounds used in experimental work on rodents, such as microinjection of protein synthesis inhibitors are too invasive and not safe for use in humans. Therefore, alternative methods of disrupting reconsolidation have been explored.

As noted above, in rodents, compounds blocking beta-adrenergic receptors, has been shown to disrupt reconsolidation of fear memories, producing similar effects as protein-synthesis inhibitors (Debiec & Ledoux, 2004). These findings have been successfully translated to humans, as administering the beta-adrenergic blocker propranolol in conjunction with the activation of a consolidated fear memory reduces later fear expression, as measured with FPS (Kindt, Soeter, & Vervliet, 2009; Lonergan, Olivera-Figueroa, Pitman, & Brunet, 2013; Soeter & Kindt, 2011). In line with the rodent literature, the effects of this manipulation disappeared if the memory activation was omitted

or if propranolol administration was delayed by 6 hours after memory activation, providing evidence that the drug has a specific effect on memory reconsolidation.

This effect is not specific to the reactivated stimulus but also generalizes to perceptually similar stimuli. In one study (Soeter & Kindt, 2011), human subjects went through a fear conditioning paradigm in which pictures, depicting spiders and guns were paired with electrical shocks. 24 hours after fear conditioning subjects received a memory activation consisting of a non-reinforced presentation of one of the pictures previously paired with the US, whereas the other stimulus was not activated. In conjunction with the memory activation an oral dose of propranolol was administered to disrupt reconsolidation. At a re-test the next day, subjects were re-exposed to both pictures presented during fear conditioning and also to similar pictures belonging to the same categories, i.e. other pictures of spiders and guns, thereby evaluating generalization. The results showed reduced fear potentiated startle to the activated stimulus as compared to the non-activated stimulus, and also that this effect generalized to other stimuli belonging to the same category. Interestingly, a similar study also demonstrated that memory destabilization is not dependent on exposure to the actual CS during memory activation. This study paired spider pictures with electrical shocks during fear conditioning and found that presenting participants with the written word "spider" during memory activation followed by propralonol administration reduced CS-elicited FPS 24 hours later (Soeter & Kindt, 2015). These findings are promising for possible clinical translation of interventions targeting reconsolidation disruption, since it indicates that the effect is not specific to cues presented during memory retrieval, and that fear memories can be destabilized by cues belonging to the same conceptual category.

## Updating fear memories using retrieval-extinction

In that extinction learning provides a conflicting learning experience as opposed to fear conditioning it was hypothesized that performing extinction within the reconsolidation window, when the fear memory is destabilized, would permanently update the fear memory and decrease later fear expression. This manipulation is often referred to as the retrieval-extinction procedure. This effect was first demonstrated in rats by Monfils and colleagues (Monfils, Cowansage, Klann, & LeDoux, 2009). First they established a fear memory using cued auditory fear conditioning, and 24 hours later the memory was activated by one non-reinforced CS presentation. Then subjects underwent extinction either 10 minutes, 1 hour, 6 hours or 24 hours after memory activation. CS-elicited freezing behavior was then measured 24 hours and 30 days after extinction training. Although none of the experimental groups showed increases in freezing behavior compared to the end of extinction training at the

24-hour re-test, after 30 days the 6-hour and 24-hour groups, but not the 10-min and 1-hour groups, showed substantial increases, demonstrating spontaneous recovery. These results show that memory activation 10 minutes or 1 hour prior to extinction training results in long term decreases in fear expression. Additional experiments showed similar results when fear expression was evaluated using renewal, reinstatement and reacquisition (Monfils et al., 2009). This manipulation, then, appears to be robust even after switching context and hampers relearning of the CS-US association. The study also identified a possible synaptic mechanism underlying this effect as they observed increased expression, in the LA of AMPARs containing the subunit GluR1, that are permeable to calcium, 3 min and 1 hour after memory activation, implicating AMPA receptor trafficking in memory destabilization. These results where replicated and extended by Clem and Hugnair (2010) as they showed similar behavioral effects following post-activation extinction and that this effect is accompanied by evidence of long term depression (LTD) in the LA, meditated by CP-AMPAR removal.

Although the effect of the retrieval extinction procedure has been replicated several times, a recent meta-analysis did not find a significant aggregated effect of this manipulation in rodents (Kredlow et al., 2016). However, there were several methodological differences that modulated the outcomes. Most importantly, whether animals were housed together or not, and time between manipulation and re-test emerged as significant predictors of the effect. In studies were animals were housed together there was a small negative non-significant effect of the manipulation whereas in studies were animals were housed alone there was a large positive significant effect, possibly indicating that social learning can counteract the effect. Also, in studies where the time between extinction and re-test exceeded 6 days there was a large positive significant effect whereas studies with a shorter delay (24-72 hours) found no effect of the manipulation, showing that in rodents the long-term effects are more pronounced than short term effects when post-activation extinction is performed. This is largely in line with the original Monfils-study in that there was no indication of group differences 24 hrs after extinction but clear differences after 30 days. This may be due to that for these experimental procedures, extinction induced inhibition of fear expression is still present for several days after the manipulation, masking the effect.

## Translating retrieval-extinction to humans

Because retrieval-extinction is a safe procedure that could have important clinical implications, these studies have been translated to humans. This was first demonstrated in human subjects in a study by Schiller et al. (2010). The first day subjects underwent cued fear conditioning, pairing neutral images with aversive electrical shocks. The second day the memory was activated by one

non-reinforced CS presentation and followed by extinction after 10 minutes in one group, or 6 hours in another group, and also one group received extinction without previous memory activation. After 24 hours, the persistence of the fear memory was evaluated by measuring SCRs during non-reinforced CS presentations. The 10-min group, that had received extinction while the fear memory was destabilized, showed little evidence of fear expression, whereas the 6-hour group, that had received extinction outside of the reconsolidation window, and the no-activation group, showed enhanced SCRs as compared to the 10-minute group, and also significant increases from the end of extinction, demonstrating spontaneous recovery. The effect was shown to be long-lasting, as a one-year follow-up in a subset of the original sample showed similar results using a reinstatement procedure, which supports the hypothesis that this procedure alters the original fear memory.

Using similar experimental designs several other studies have replicated these initial findings (Agren, Furmark, Eriksson, & Fredrikson, 2012; Agren et al., 2012; Asthana et al., 2015; Björkstrand et al., 2015; Johnson & Casey, 2015; Liu et al., 2014; Oyarzún et al., 2012; Schiller, Kanen, LeDoux, Monfils, & Phelps, 2013; Steinfurth et al., 2014) but there have also been a number of studies that have found conflicting results (Fricchione et al., 2016; Golkar, Bellander, Olsson, & Ohman, 2012; Kindt & Soeter, 2013; Klucken et al., 2016; Meir Drexler et al., 2014; Soeter & Kindt, 2011). A recent meta-analysis (Kredlow, Unger, & Otto, 2016) found the effect to be significant and of small to medium size when aggregating the results of several of these studies. The meta-analysis also investigated several aspects of study design that might explain why some studies find the effect and some don't. Moderator analysis showed that length of US and the number of CS+ presentations during the acquisition phase significantly predicted observed effect sizes. Length of US was positively related to finding the effect, with longer US duration being related to higher effect-size. This runs counter to studies in rodents studying the effect of post-activation protein synthesis inhibition, as a longer US would arguably induce stronger fear learning and therefore the established memory would be harder to destabilize. Kredlow et al. (2016) suggest that maybe if the US is to short it does not engage unconscious amygdala dependent associative learning, but rather engages conscious learning processes that do not depend on the amygdala. Since the effect of reconsolidation disruption of fear memories has been shown to be amygdala dependent, a short US might then render the manipulation ineffective. Number of CS+ presentations during fear learning was also positively related to effect-size, again indicating that stronger learning increases the effect. However, number of CS-US pairings during acquisition did not predict the effect, which speaks against this conclusion. In contrast to findings in the rodent literature on protein synthesis inhibition, length of reminder did not moderate the effect. However, length of reminder has not been examined experimentally in humans, so other methodological differences in the included studies may cofound this analysis.

The meta-analysis does not include all published studies however. The effect has been replicated in several additional studies. Steinfurth et al. (2014), evaluated whether post-activation extinction had an effect on 7-day old fear memories. The study included one arm evaluating 1-day old fear memories and one arm evaluating 7-day old fear memories (i.e. acquisition was performed either 1 day or 7 days prior to activation and extinction), using a between-subject design for both arms. They found significant effects of post-activation extinction for both 1-day and 7-day old fear memories, indicating that memories older than 24 hours are susceptible to this manipulation. Johnson & Casey (2015), evaluated whether the post-retrieval extinction effect also is present in adolescents, investing both an adolescent and an adult sample, using a between-subject design. They found a significant effect of post-retrieval extinction in both age groups with no difference between age-groups. Liu et al. (2014) investigated whether US presentation can be used to activate the fear memory, and found that both CS and US presentation prior to extinction leads to decreased fear expression at re-test, but that the effect of US presentation generalizes to all stimuli paired with that US, whereas the effect of CS activation is specific to the activated cue. Ashtana et al. 2016, investigated whether genetics modulates the retrieval-extinction effect. They did observe an effect of post-activation extinction, but only in a subgroup with a specific variant of a polymorphism related to brain derived neurotropic factor (BDNF). This is in line with a previous study by Agren et al. (2012) that also found that genetics modulate the effect, but then looking at serotonergic and dopaminergic polymorphisms. Also, Agren et al. (2016) recently investigated whether imaginal extinction can be used to update a destabilized fear memory. In this experimental set-up one group received regular post-activation extinction and one group received imaginal extinction, where instead of showing the actual cues presented during fear conditioning, a recorded voice instructed them to repeatedly imagine viewing the stimuli. They observed decreased fear responses at re-test in both groups, as compared to individuals who had received regular and imaginal extinction 6 hours after memory activation.

Of note, two recently published studies, did not find the retrieval-extinction effect. Klucken et al. (2016) investigated the effect of post-activation extinction using a within-group design, and measuring SCR and brain responses using fMRI in all phases of the experiment. They found no effects of post-activation extinction in either measure. This study used a relatively short US of 100ms, but notably they found CS-induced increased amygdala activity during acquisition, suggesting that even a fairly short US does engage the amygdala during the learning stage. Also, Fricchione et al. (2016) found no effects of retrieval-extinction using a within-group design, and a particularly strong fear learning paradigm, where they recruited non-phobic subjects with elevated fear of spiders and used short film-clips of moving tarantulas as CSs, and a 500ms electrical shock as US. However, since the reinforcement rate during conditioning was 50% and memory retrieval consisted of a single non-

reinforced CS-presentation, the authors suggests that this might not have produced a sufficiently large prediction error to destabilize a strong fear memory.

In summary, although numerous studies confirm the existence of the retrieval-extinction effect in humans, the overall heterogeneity of the findings suggests that there probably exist, as of yet unknown, boundary conditions. Although several boundary conditions have been suggested, there is no clear consensus as to which ones may be most relevant. Understanding the underlying neural mechanisms may help answer this question, but few studies have investigated this in humans. Because many of the methods used in rodents to elucidate neural mechanisms are to invasive to use in humans, this is no easy task. One of the aims for this thesis is to explore the mechanisms of this effect by investigating how amygdala activity measured by fMRI is affected by reconsolidation manipulation of fear memories.

## Disrupting reconsolidation in anxiety disorders

Because reconsolidation disruption potentially has the ability to diminish amygdala located fear memories, there have been hopes of applying this mechanism in clinical practice in the treatment of various anxiety disorders. Although this procedure shows experimental promise, it's use in clinical conditions may be hampered by several boundary conditions. Specifically, research in rodents have shown that it is harder to obtain these effects in old and strong memories (Alberini, 2011; Bustos et al., 2009; Milekic & Alberini, 2002; Nader et al., 2000; Suzuki et al., 2004; Wang et al., 2009). Anxiety disorders are often long-lasting conditions that have been present for a significant amount of time before treatment is started, and therefore their age might preclude the use of this mechanism in clinical anxiety. Also, they are arguably strong memories, established through multiple learning experiences over an extended period of time, and possibly often retrieved and reconsolidated which would further strengthen these memories. Nonetheless, there are studies that suggests that reconsolidation disruption may indeed be applicable in clinical anxiety. Especially PTSD has been given a lot of attention, since this debilitating disorder has its origin in an identified traumatic event, and likely is established through associative processes akin to fear conditioning. PTSD occurs after the individual has been exposed to some serious trauma and is characterized by persistent re-experiencing of the trauma, through intrusive thoughts or flash-backs; avoidance of trauma related cues; and heightened arousal, for example irritability or hypervigilance (American Psychiatric Association, 2000). These symptoms then lead to considerable personal distress and decreased functioning. Several studies have investigated the use of post-activation propranolol administration in PTSD patients. Also, a few studies have investigated reconsolidation disruption in spider phobia, characterized by a pervasive and irrational fear of spiders.

## PTSD

The first study that investigated reconsolidation disruption in anxiety disorders evaluated the effect of a single dose of propranolol following trauma memory activation in a sample with chronic PTSD (Brunet et al., 2008). First, to activate the memory, subjects gave a written account of their traumatic experience in a 20-minute session, and then, to disrupt memory reconsolidation, were given a dose of propranolol. A control group also recounted their trauma but were given placebo. One week later, subjects listened to recorded accounts of their traumatic experience, and SCRs, HR and electromyogram (EMG) of the left corrugator (frowning) muscle were measured, serving as physiological indexes of fear expression. The results revealed significant group differences with greater physiological responses in the placebo group, in both SCR and HR but not EMG. These results suggest that even old and strong fear memories may be diminished by post-activation propranolol administration. Notably, this study lacked a no-activation control group, so the specific effect of reactivation followed by propranolol administration cannot be established, in that propranolol may have a general dampening effect on physiological responding in this procedure. A subsequent study (Wood et al., 2015) used a similar design but with a no-activation control group that also received propranolol, and found no group differences on physiological responses while listening to recorded trauma scripts. This suggests that the effects of the Brunet study may have been driven by a general dampening effect of propranolol, but since the subsequent study (Wood, 2014) did not include a placebo control this can only be inferred. Differences in the sample composition and the fact that Wood et al. (2008) administered propranolol prior to activation could also explain the discrepant results.

Brunet and colleagues have also conducted a series of uncontrolled studies more closely resembling clinical treatment. In these studies, patients with PTSD were given a dose of propranolol and 90 minutes later gave a written or oral account of their trauma lasting 15-20 minutes. This procedure was then repeated once a week for a duration of 6 weeks, and outcomes were evaluated with self-report and clinician administered questionnaires measuring PTSD symptoms. Results showed large improvements both post-treatment and at follow-up in all studies (Brunet et al., 2011). This suggests that this may be a promising treatment for PTSD, but given that the studies lacked control groups and were non-blinded, this only provides tentative evidence for the efficacy of the procedure. It is uncertain whether these effects depend on reconsolidation disruption, in that spontaneous remission, regression to the mean, expectancy effects, effects of imaginal trauma exposure alone, effect of propranolol administration alone, and possibly a facilitating effect of propranolol on imaginal trauma exposure could also explain these results.

A very small uncontrolled neuroimaging study has also examined the effect of this type of treatment on neural activations. Seven PTSD patients received

6 weekly sessions of trauma reactivation concurrent with propranolol administration, as described above, and were scanned with fMRI before and after treatment (Mahabir, Tucholka, Shin, Etienne, & Brunet, 2015). During scanning they watched pictures of faces displaying fearful, happy or neutral expressions, a procedure that has previously been shown to elicit increased amygdala activity in PTSD-patients (Shin et al., 2005). The results showed decreases in activity in the thalamus and amygdala pre- to post-treatment, indicating that this intervention attenuates fear-circuit activity. Although, given the small sample size, the lack of control groups, and that the fMRI procedure did not include fear provocation, this conclusion should be considered preliminary. The same possible confounds cited above also apply here.

## Spider phobia

A more convincing demonstration of the effect of post activation reconsolidation disruption in anxiety disorders can be found in a study by Soeter and Kindt (Soeter & Kindt, 2015). They investigated whether a single dose of propranolol following a brief exposure to a live spider affects later approach behavior and self-reported spider fear, in a sample with spider phobia. They employed a randomized double blind pre-post design, including both a control group receiving placebo after activation as well as a no-activation propranolol control group. First, in order to establish pre-treatment levels of behavioral approach, all participants were subjected to behavioral approach test (BAT), in which they were asked to gradually approach a live baby-tarantula in a stepwise manner. Five days later, subjects were briefly exposed to another spider, standing in front of a terrarium containing a large tarantula for 2 minutes, thereby destabilizing the fear memory, and then received a single oral dose of either propranolol or placebo. Also, one group received propranolol without previous memory activation. The outcome was measured with a BAT utilizing the spider presented during the memory activation session, conducted 4 days after the manipulation. The results showed that the post-activation propranolol group displayed more approach behavior and lower subjective fear ratings than the two control groups, who did not differ from each other, clearly demonstrating that post-activation memory disruption can affect strong and long-present fear memories. Importantly, in contrast to previous investigations, this study included adequate controls to show that neither memory activation, nor propranolol, by themselves are sufficient to produce this effect, supporting the conclusion that the effect is mediated through disruption of reconsolidation.

This study also evaluated generalization and long-term persistence of the effects. To do this, another BAT utilizing the baby-tarantula presented during the pre-treatment measurement, was performed 11 days, 3 months, and 1 year after the manipulation respectively. The results showed a substantial increase

in approach behavior from pre-treatment to post-treatment in the post-activation propranolol group, and these increases were maintained both at the 3-month and 1-year follow-up measurement. In contrast, neither the post-activation placebo group nor the no-activation propranolol group showed any change in approach behavior from pre- to post-treatment, or the follow-up. All groups scored similarly on the BAT at pre-treatment, but the post-activation propranolol group showed significantly more approach behavior both at post-treatment and at follow-up compared to the two controls. This demonstrates that the effects are long-lasting and that they are not specific to the activated cue, but generalizes to other anxiety provoking stimuli within the same category, which supports that this manipulation is a promising candidate for clinical application. Also, self-reported spider fear was measured using a validated questionnaire pre-treatment, as well as 11 days, 3 months and 1 year after the manipulation. There were no group differences for self-reported spider fear either pre- or post-treatment. However, the post-activation propranolol group showed decreases on this measure from post-treatment to the 3-month follow-up, scoring lower than the controls, and this effect persisted to the 1-year follow-up. This demonstrate that this manipulation does not have an immediate effect on the subjects' conscious self-perception of their fears in a more global sense, although this effect appears after a delay. This could indicate that this manipulation has no direct effect on conscious self-perception of fear, but rather has a specific effect on defensive responses such as behavioral avoidance and physiological arousal, and that the subjects alter their conscious perception of their fears only after having observed themselves act in a non-fearful way for some time. This would be in line with previous research indicating that reconsolidation disruption is an amygdala dependent process, and that the amygdala may not directly mediate conscious fear expression but rather dictates defensive responses such as cue elicited physiologic arousal and avoidance behavior (LeDoux & Pine, 2016).

To date, there is only one published study (except for Study III of this thesis) that have examined the retrieval-extinction procedure in anxiety disorders. Shiban and colleagues (Shiban, Brutting, Pauli, & Muhlberger, 2015) tested the effect of post-activation virtual reality (VR) exposure to spiders in a sample with spider phobia. They evaluated the outcome with physiological responses in the form of skin conductance level (SCL) during VR spider exposure, subjective online fear ratings during VR spider exposure, approach behavior measured with a BAT using a live spider, and self-reported spider fear using a validated questionnaire. First, to obtain pre-treatment comparisons, subjects performed a BAT and completed the self-report spider fear questionnaire. Then, in one of the groups, the fear memory was activated by a 5-second spider presentation in the VR-environment, while the control group were shown a VR- rendered plant. Then, after 10 minutes, both groups underwent a graded exposure protocol in the VR-environment, being shown 10 different scenes with increasing amounts of spiders, for 30 minutes. After 24 hours,

spontaneous recovery was evaluated by exposing the participants to one spider for 3 minutes in the VR-environment, while simultaneously recording SCL and obtaining online fear-ratings. This was followed by a second BAT and then subjects completed the spider fear questionnaire. In order to facilitate generalization of the VR-exposure to the real world, both groups received a non-standardized exposure session, using a real spider, 7 days after the spontaneous recovery test. This involved gradually approaching a live spider and lasted about 40 minutes on average. In conjunction with the live-exposure, subjects again completed the spider fear questionnaire. This questionnaire was also administered to a subset of the sample at a 6-month follow-up measurement.

Overall, the results did not indicate that post-activation exposure is more effective than exposure since there were no group differences for any measure at any time-point, though several of the outcome measures indicated that both groups decreased in their fear of spiders. Results from the spontaneous recovery test on day 2 in the VR environment showed no group differences in SCL. However, analyses of the data from the VR-exposure session on day 1 showed no decreases in SCL for any group, so the exposure procedure used appears not to have an effect on physiological responding as measured with SCL. Online fear-ratings did decrease during the VR exposure session, and these decreases were still present also at the spontaneous recovery test but no group differences emerged. Analyses of the data from the BAT using a real spider, showed increased approach behavior from pre- to post-treatment in both groups with no significant group differences. This suggests that the VR-exposure procedure had a positive effect on approach behavior to real spiders similar across groups, but since the design did not include a no-exposure control group this could also be an effect of repeated testing. Analyses of the spider fear questionnaire showed a gradual decrease for each time point with a significant pre-treatment to follow-up differences, with no differences between groups. Although this might indicate that the VR-procedure had an effect on self-perceived spider fear, the result could also be due to the live exposure session conducted a week after the VR-exposure session. Since, the live exposure did not include a prior memory activation for any group, it may also have masked possible group differences at the follow-up, by providing both groups with additional fear extinction. Also, since the design did not include a no-exposure control group, this decrease in self-perceived fear might also be explained by spontaneous remission, regression to the mean, repeated testing or expectancy effects. In summary, although the results suggest that activating the memory before the repeated exposure does not have an effect on subsequent fear expression in long-lasting fears, it is hard to draw firm conclusions since it is unclear whether the VR-exposure procedure actually had an impact on any of the outcome measures, with the possible exception of the online fear-ratings. As noted above, the fear ratings did decrease during the VR-exposure session and this effect was still present 24 hours later, but there was no

indication that activating the fear memory prior to exposure had an additional effect on fear-ratings as compared to no activation-exposure.

This result stands in contrast to the study by Soeter and Kindt (2015) that did find an effect of post-activation reconsolidation disruption on online fear ratings. This discrepancy could be explained by several methodological differences, the most obvious one being the different methods used for interfering with reconsolidation. Since Soeter and Kindt used a pharmacological manipulation instead of exposure training, these results might indicate that post-activation propranolol is a more effective method than post-activation extinction when trying to ameliorate anxiety disorders. However, there are also several other differences that might explain the discrepant results. One important difference is the length of memory activation. The 2-minute memory activation used in the Soeter and Kindt-study was 24 times longer than the 5-second memory activation Shiban et al. used. Considering previous work in rodents showing that older and stronger memories may require longer memory activation in order to be destabilized (Bustos et al., 2009; Suzuki et al., 2004), this could be a possible explanation for why Soeter and Kindt obtained the effect but Shiban et al. did not. Another potentially important methodological difference concerns the time between the manipulation and the re-test. In the Kindt and Soeter-study there was a 4-day delay between manipulation and re-test, whereas in the Shiban et al. study there was a delay of 24 hours. Research in rodents using the retrieval-extinction procedure suggests that effects are not observed 24 hours after manipulation (Kredlow et al., 2016; Monfils et al., 2009) as the extinction induced safety-memory could still be present at this time-point. Therefore, 24 hours might be too short a delay in that it does not allow enough time for spontaneous recovery in the control group, having also received exposure.

In summary, the evidence for an effect of reconsolidation manipulation in anxiety disorders is so far limited. There is some evidence that post-activation propranolol has an impact on later fear expression in spider phobia, but no support that the retrieval-extinction procedure is effective in this respect. Furthermore, there is little research on the underlying neural mechanisms when it comes to anxiety disorders. This thesis aims to investigate this by evaluating the effect of post-activation exposure on amygdala activity and approach behavior in spider phobia. In that LTP-related processes and neural activity in the amygdala have been shown to mediate the effect of reconsolidation manipulation in studies using fear-conditioning procedures, it would be very interesting to investigate if this also occurs in anxiety disorders.

# Aims

The aim of this thesis is to investigate underlying neural mechanisms involved in reconsolidation disruption of fear memories in humans, specifically examining the effect of the retrieval-extinction procedure on subsequent amygdala activity, a brain structure that has previously been shown to be crucial for the acquisition, consolidation, and reconsolidation of fear memories. Also, this thesis aims to extend previous findings on experimentally induced recent fear memories, established through fear conditioning, to long-term phobic fears.

I Using fear conditioning and a retrieval-extinction procedure, the first study investigated whether memory retrieval followed by extinction after 10 minutes, i.e. within the reconsolidation time window, decreases subsequent CS-elicited amygdala activity and fear expression measured with SCR, and also whether amygdala activity predicts later fear expression.

II The second study investigated whether the effect of the retrieval-extinction procedure on fear expression is long-lasting by performing an 18-month follow-up test on the participants from Study I, and also examined the relationship between long-term effects on fear expression and initial amygdala activity.

III Using an adapted version of the retrieval-extinction procedure, the third study examined whether memory retrieval 10 minutes prior to repeated exposure to feared cues diminishes subsequent amygdala activity and facilitates behavioral approach in a sample with long-term fear of spiders.

IV The fourth study investigated whether the effects of reconsolidation disruption on phobic fears are long lasting, by performing a re-test on the participants from Study III, six months after the original manipulation.

# Methods

## Skin conductance responses

Skin conductance responses (SCRs) is the one of the most commonly used outcome measure of physiological arousal in human fear conditioning research. SCRs measures phasic shifts in in the amount of sweat secreted by eccrine sweat glands. This is accomplished by placing a pair of electrodes close to each other on either the palms of the hands or the or soles of the feet, were eccrine sweat glands are primarily located, and passing a weak electrical current between them. For Study I and II, electrodes where placed on the hypothenar eminence of the left hand. When sweat secretion increases this reduces the resistance of the skin leading to increased conductance. Secretion of the eccrine sweat glands is controlled by the sympathetic autonomous nervous system, and thus by evaluating stimulus induced shifts in conductance, SCRs can be used as an event related index for sympathetic arousal (Boucsein, 2011; Sequeira, Hot, Silvert, & Delplanque, 2009). As noted above, SCR in combination with functional brain imaging has previously been used to establish a link between central nervous system activity and fear responses, showing that SCRs correlate with activity in fear related brain circuits (Greco & Liberzon, 2016; Shin & Liberzon, 2010).

## fMRI

Functional magnetic resonance imaging (fMRI) is one of the most common methods used today to study in-vivo neural activity in humans. It is a non-invasive method with good spatial and temporal resolution that can measure regional changes in blood flow, which is considered a valid index of neuronal activity. Measurement with fMRI is possible in deep subcortical structures of the brain and is not restricted to cortical regions like other methods for recording neuronal activity, for example electroencephalography (EEG).

When performing MRI the subject under study will be placed in a strong static magnetic field that will cause protons in the underlying tissue to be aligned along the same axis. By applying a radiofrequency pulse this will cause some protons to align opposite to the magnetic field and also will cause the protons to rotate in synchrony along their axis. When the pulse is switched off the protons will gradually return to their previous state and when they do

so they will emit a signal that can be measured by the scanner. Due to the fact that different types of tissue emit different signals when they return to their original state, this signal can be used to reconstruct a structural image of the brain, although this image contains no information of underlying neuronal activity.

Functional MRI depends on the fact that deoxy- and oxyhemoglobin have different magnetic properties and thus emits different signals. Neuronal activity will cause an increased consumption of oxygen and consequently a local increased inflow of oxygenated blood. The increased blood flow will cause a shift in the signal emitted from that region that can be detected by the scanner, and consequently can be used as an index for underlying neuronal activity. This signal is referred to as the blood oxygen level dependent (BOLD). By comparing the BOLD signal during some task or stimulation to a baseline, it is then possible to evaluate what brain regions are involved in the process under study (Heeger & Ress, 2002; Schild, 1990).

# Summary of studies

## Study I

### Background and aim

The amygdala has been shown to be crucially involved in the acquisition, retention and expression of fear memories (Johansen et al., 2011). Likewise, the reconsolidation of fear memories depends on plastic processes in the amygdala, which has been demonstrated in rodents using post activation administration of protein synthesis inhibitors to disrupt reconsolidation (Nader et al., 2000) and also using retrieval-extinction procedures (Clem & Huganir, 2010; Monfils et al., 2009). The retrieval-extinction procedure has been demonstrated to decrease fear expression also in humans (Schiller et al., 2010). Study I aimed to investigate whether reconsolidation disruption in humans is amygdala dependent by combining fear conditioning and a retrieval-extinction procedure with brain imaging using fMRI. Specifically, the study aimed to examine if extinction performed during the reconsolidation window results in decreased CS-elicited amygdala activity and if amygdala activity is related to fear expression measured with skin conductance responses (SCRs).

### Methods

30 healthy participants (13 women) underwent a 4-day experimental protocol, consisting of fear conditioning (day 1), a retrieval-extinction procedure (day 2), re-extinction during fMRI (day 3), and a retention test using a reinstatement procedure (day 5), see Figure 1A. On day 1, participants underwent fear conditioning. Sitting in front of a computer the subjects watched a display of a neutral environment containing a lamp that lit up in the colors blue or red. One of the colors (CS+) was repeatedly paired with an aversive electrical shock to the right forearm, while the other color (CS-) was never paired with a shock. On day 2, subjects returned to the lab and the fear memory was activated with a two-minute CS+ presentation. Then, after 10 minutes, half the sample received extinction consisting of 8 non-reinforced CS+ and CS- presentations, thereby interfering with reconsolidation. The other half, serving as a control group, received extinction after 6 hours when the fear memory had been allowed to reconsolidate. On day 3, subjects underwent a renewal

session consisting of 8 non-reinforced CS+ presentations and 8 CS- presentations, while fMRI data was collected. On day 5, subjects were given a reinstatement protocol, in which they received two un-signaled electrical shocks followed by a single non-reinforced CS+ presentation. Skin conductance was measured during all phases of the experiment except on day 3 in the MR-scanner, due to technical constraints.

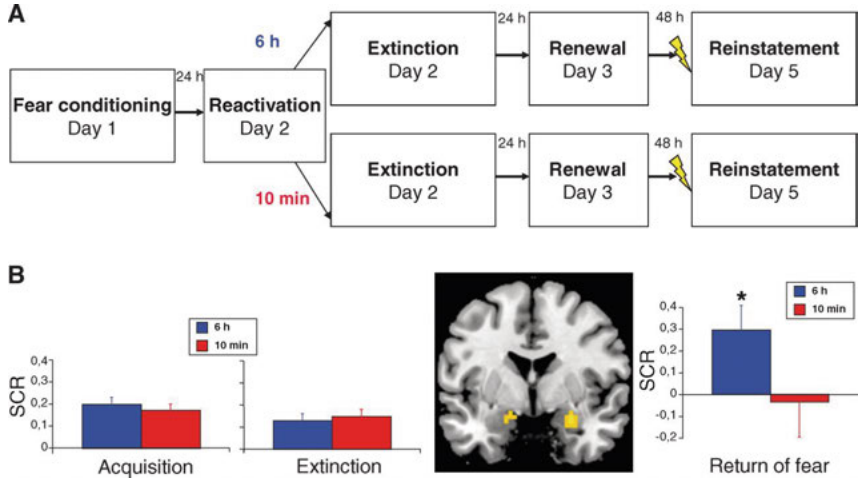


*Figure 1.* Extinction during reconsolidation blocks reinstatement of fear and abolishes a memory trace in the amygdala. (A) After fear conditioning on day 1, when 16 shocks were paired with a visual cue, a memory reminder was given on day 2, and extinction was performed after 10 min or 6 hours, by exposure to eight conditioned cues with no shocks. On day 3, amygdala activity was assessed with functional magnetic resonance imaging (fMRI) during renewal-induced fear. On day 5, return of fear was evoked by presenting unpaired shocks before CSs were again presented. (B) Groups were equivalent in acquisition [$t(20) = 0.66$, $P = 0.51$] and extinction [$t(20) = 1.03$, $P = 0.31$]. Return of fear was confirmed in the 6 hours group [blue bar; $t(10) = 2.72$, $P = 0.02$] but not in the 10 min group [red bar; $t(8) = 0.23$, $P = 0.82$]. fMRI demonstrated a remaining fear memory representation in the amygdala after reactivation and normal reconsolidation but not after reactivation followed by disrupted reconsolidation. The voxels reflecting the bilateral memory trace, encompassing the basolateral amygdala, indicate superior memory representation in the 6 hours as compared with the 10 min group (brain coordinates: $x, y, z = 27, 5, -17$; $Z$-score = 2.46; $P = 0.007$; 378 $mm^3$; $x, y, z = -15, -1, -14$; $Z = 2.22$; $P = 0.013$; 162 $mm^3$). The autonomic nervous system measure of fear is the SCR. The CNS measure of amygdala activity is BOLD activity. Brain coordinates are according to the Montreal Neurological Institute (MNI). Error bars are standard error of means. From Agren et al. (2012). Reprinted with permission from AAAS.

## Results

Following fear conditioning, 22 of the participants attained reliable fear responses as measured by SCRs and were included in the subsequent analyses. The two groups achieved similar levels of fear conditioning and extinction, as there were no group differences in SCRs on day 1 or day 2. On day 3, however, the 6h group (n=11) showed increased amygdala activity during CS+ presentation as compared to the 10min group (n=11). On day 5, the 6h group demonstrated return of fear, signified by increased SCRs to the CS+ following reinstatement as compared to responses during the end of extinction, whereas the 10min group showed no increases, see Figure 1B. Moreover, in the 6h group, amygdala activity on day 3 was positively correlated to return of fear following reinstatement on day 5, whereas no such relationship was observed in the 10min group. The 10min group also had decreased functional amygdala coupling to other areas of the fear network, as covariation of amygdala activity and activity in the ACC, insula and the hippocampus was greater in the 6h group compared to the 10min group.

## Discussion

The results showed that extinction performed shortly after memory activation decreases CS-elicited amygdala activity as well as subsequent fear expression, supporting that disrupting memory reconsolidation through extinction training can attenuate an amygdala dependent fear memory. In further support of this conclusion, amygdala activity in the 6h group, but not the 10min group, was correlated to fear expression following reinstatement, demonstrating that amygdala activity is related to later fear expression, and also the 6h group showed greater functional coupling to other parts of the fear network compared to the 10min group. In line with previous research in rodents, this suggests that fear memory reconsolidation in humans is, at least partially, amygdala dependent.

# Study II

## Background and aim

Disrupting the reconsolidation of fear memories is thought to alter the original fear memory representation so that defensive responses to fear conditioned cues are permanently attenuated. In support of this, disrupting reconsolidation has been shown to have long-lasting effects on fear expression. Disrupting reconsolidation through retrieval-extinction procedures have demonstrated decreased fear responses 30 days after the manipulation in rodents (Monfils et al., 2009), and after one year in humans (Schiller et al., 2010). Study II aimed to replicate these findings in humans by performing a follow-up measurement on the subjects in Study I, 18 months after the original manipulation, and also relating long-term effects on fear expression to initial amygdala activity.

## Method

Of the 22 original participants, 20 were available for the follow-up measurement and one additional subject had to be excluded due to a technical error. The follow-up test consisted of a reacquisition procedure using the same conditioned stimuli as in Study I, see Figure 2A. 18 months after the original manipulation subjects returned to the lab and were sat in front of a computer screen displaying a neutral environment containing a lamp that lit up in either blue or red. One of the colors (CS+) had previously, on day 1, been paired with electrical shocks, whereas the other color (CS-) had not. The reacquisition protocol consisted of 4 reinforced CS+ presentations and 4 CS- presentations. Skin conductance was measured to evaluate fear responses to the conditioned stimuli.

## Results

After reacquisition, the 6h group, having previously received extinction outside the reconsolidation window, showed successful re-learning of the fear response in that they had significantly larger SCRs to the CS+ as compared to the CS-. The 10min group however, having previously received extinction inside the reconsolidation window, did not discriminate between the CS+ and CS- during reacquisition. Using the same return of fear index as used in Study I, evaluating changes in CS+ elicited SCRs from the end of extinction on day 2 to reacquisition 18 months later, showed significantly larger responses in the 6h group as compared to the 10min group. Furthermore, in the 6h group, CS-elicited amygdala activity on day 3 was positively correlated to return of fear during reacquisition 18 months later, whereas no such relationship was observed in the 10min group, see Figure 2.

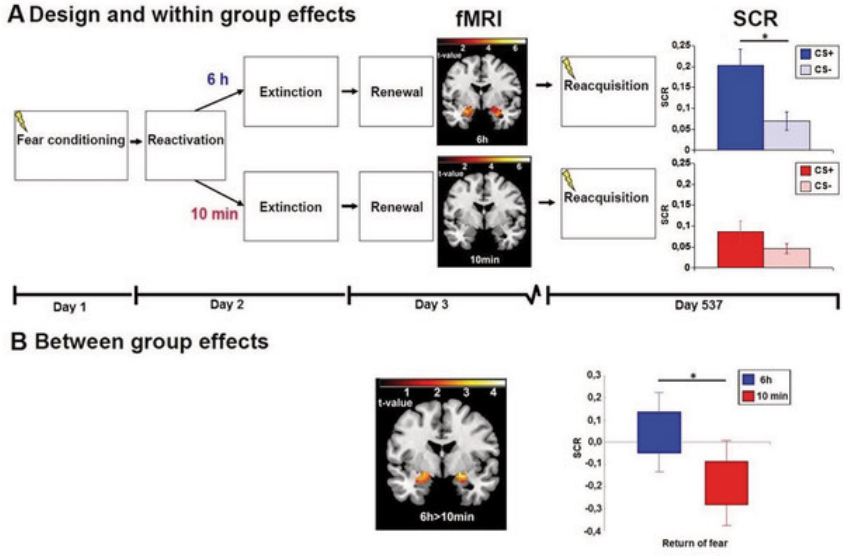


*Figure 2.* Amygdala activity predicts return of fear over 18 months. A) Fear conditioning on day 1 was established by pairing a visual cue with electric shocks and then the memory reminder was given on day 2 either 10 min or 6 hours prior to extinction was performed, through exposure to the conditioned cue without shocks. On day 3, memory related amygdala activity was evaluated using functional magnetic resonance imaging (fMRI) during renewal-induced fear, and return of fear was evoked on day 537. Skin conductance responses (SCR, the electrophysiological fear index) in the 6h group with undisrupted reconsolidation, but not the 10min group with disrupted reconsolidation, discriminated between the shock-reinforced (CS+) and unreinforced (CS-) cue during reacquisition. See the two right hand bars in row A. Bars represent means and error-bars are SEM. Return of fear was predicted by initial neural activity in the basolateral amygdala in the 6h but not the 10min group. (Coronal brain slices in the two top rows). B) As illustrated in the right panel in row B, return of fear was stronger after undisrupted (6h) than disrupted (10min) reconsolidation at 18 months follow-up, as reflected in enhanced reactivity to the cue predicting shocks. Boxes illustrate mean± SEM, whiskers represent SEMx1.96. The coupling between the electrophysiological fear measure and brain activity was significantly stronger in the 6h than in the 10min group as reflected by enhanced connectivity between SCR and BOLD activity in the basolateral amygdala; mapped in the coronal brain slice in row B. * indicates p<.05 one-tailed. The right side of the brain is depicted to the right. From Björkstrand et al. (2015).

## Discussion

The results replicated previous studies in that subjects that had received disrupted reconsolidation, even after 18 months, showed less return of fear than subjects having received undisrupted reconsolidation. Indeed, the 10min group did not show enhanced responding to the CS+ even after 4 reinforced stimulus presentations. Thus, the effect of the retrieval-extinction procedure

was long-lasting, which is consistent with the hypothesis that this manipulation alters the original fear memory trace. Also, in the 6h group, CS-elicited amygdala activity on day 3 predicted return of fear 18 months later, providing support that this mechanism is amygdala-dependent. Since the retrieval-extinction procedure appears to have long lasting effects on fear expression and counteract relearning of the CS-US association, this mechanism could have important clinical implications in the treatment of anxiety disorders.

# Study III

## Background and aim

Disrupting reconsolidation attenuates short-term fear memories established through fear conditioning and decreases subsequent fear expression in rodents and humans (Agren et al., 2012; Monfils et al., 2009; Nader et al., 2000; Schiller et al., 2013). This mechanism could be applied in the treatment of anxiety disorders. However, research in rodents has shown that older and stronger fear memories are less sensitive to reconsolidation disruption (Alberini, 2011; Bustos et al., 2009; Milekic & Alberini, 2002; Suzuki et al., 2004; Wang et al., 2009). Thus, it is uncertain whether reconsolidation disruption procedures can attenuate naturally occurring long-term fear memories that distinguish human anxiety. Study III aimed to investigate this question using an adapted version of the retrieval-extinction procedure in a sample with life-long fear of spiders, and also evaluated whether underlying neural mechanisms are similar to those found for experimentally established fear memories using fMRI. Specifically, this study investigated whether memory activation shortly followed by repeated exposure to feared cues attenuates later amygdala activity during fear provocation, and also whether this manipulation increases approach behavior to feared cues.

## Methods

45 subjects (33 women), highly fearful of spiders were recruited through public advertisements. All scored above the 95$^{th}$ percentile on a validated spider fear questionnaire. Over the course of two days, subjects underwent memory activation followed by exposure to spider cues (day 1), and after 24 hours, re-exposure to spiders followed by a behavioral approach test (day 2). On day 1, to activate the fear memory, subjects sat in front of computer screen and were briefly shown four different pictures of spiders. Then, subjects received repeated exposure to spider pictures in a MR-scanner after either 10 minutes, thereby disrupting reconsolidation, or after 6 hours, when the fear memory had been allowed to stabilize. During the exposure session, two of the spider pictures shown during memory activation were presented 7 times, and also two spider pictures not previously shown were presented 8 times, interleaved with 7 presentations of neutral pictures. On day 2, subjects were again placed in the MR-scanner and were shown all 6 pictures presented during the memory activation and exposure session day 1, along with two novel spider pictures for two presentations each, see Figure 3. This design allowed us to evaluate whether the effect of reconsolidation disruption is specific to activated and exposed stimuli or whether it generalizes to stimuli that were only activated,

only exposed, and novel stimuli. After the re-exposure session subjects underwent the behavioral test, in which they repeatedly had to choose whether to watch a picture of a spider or a neutral picture. Choosing the spider was rewarded with either 0, 0.5, 1, 2, or 5 SEK, whereas choosing the neutral picture was never rewarded. This allowed us to evaluate whether monetary reward differentially affects approach motivation subsequent to either disrupted or undisrupted reconsolidation. The behavioral test consisted of four choices at each reward level, the outcome measure being the average approach proportion at each level. To evaluate cue-elicited amygdala activity, fMRI was measured during the exposure session on day 1 and the re-exposure session day 2.

## Results

During the exposure session on day 1, both groups showed enhanced amygdala activation when viewing spider pictures as compared to neutral pictures, with no differences between groups. Also, both groups showed lower amygdala activation to spider pictures at the end of exposure as compared to the beginning of exposure with no differences between groups. During re-exposure on day 2, the 6h group, as compared to the 10min group, showed enhanced amygdala activation to the activated and exposed spider pictures. Similar group differences were also observed for spider pictures presented only during activation, only during exposure, as well as novel spider pictures, see Figure 3. The behavioral data showed a significant Group by Reward interaction, in that the 10min group chose to view more spiders than the 6h groups at the higher reward levels 2 and 5 SEK, see Figure 4A. Also, the 6h group showed a negative correlation between amygdala activity during re-exposure and subsequent approach behavior, whereas no such relationship was observed in the 10min group, see Figure 4B.

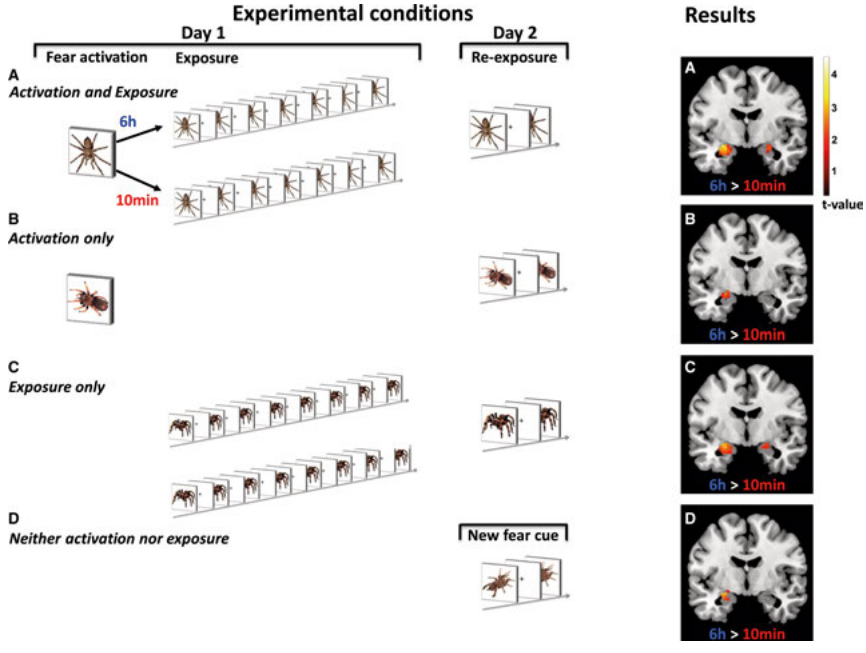


*Figure 3.* Exposure during reconsolidation attenuates amygdala-related return of fear. Experimental conditions: after fear activation followed by seven exposure trials on day 1, the memory was tested on day 2 (A). Exposure to spiders on day 1 was performed either within the reconsolidation window after 10 minutes (10 min group) or outside the window 6 hours (6 hr group) after fear activation. Another spider cue was activated but not presented during exposure (B). An additional cue was presented eight times during exposure day 1 but was not preceded by activation (C). On day 2, a new fourth spider slide was introduced (D). Within each group, subjects were exposed to all experimental conditions, and pictures were counter-balanced across subjects. Results: fMRI demonstrated increased amygdala activation from day 1 to day 2 in the 6 hr group (n = 23) as compared to the 10 min group (n = 22) for the activated and repeatedly exposed stimulus (xyz = −27, −7, −14; Z = 3.45; $p < 0.0001$; $p_{corrected} = 0.006$; 1,053 $mm^3$; xyz = 27, −1, −14; Z = 2.34; p = 0.010; $p_{corrected} = 0.107$; 432 $mm^3$) (A). For the activated but not exposed spider cue, amygdala reactivity tended to be higher in the 6 hr group than the 10 min group (xyz = −24, −7, −14; Z = 2.42; p = 0.008; $p_{corrected} = 0.091$; 243 $mm^3$) (B). The exposed but not activated slide also elicited enhanced amygdala responsivity in the 6 hr group as compared to the 10 min group (xyz = −24, −7, −14; Z = 3.17; p = 0.001; $p_{corrected} = 0.014$; 1,053 $mm^3$; xyz = 24, −10, −11, Z = 2.79; p = 0.003; $p_{corrected} = 0.039$; 378 $mm^3$) (C). The new slide introduced on day 2 elicited higher amygdala activation in the 6 hr group as compared to the 10 min group (xyz = −24, −7, −14; Z = 2.82; p = 0.002; $p_{corrected} = 0.035$; 567 $mm^3$) (D). The measure of amygdala activity is blood-oxygen-level-dependent (BOLD) activity. Brighter colors represent larger effects with higher significance. Brain coordinates are according to Montreal Neurological Institute (MNI). From Björkstrand et al. (2016). Reprinted with permission from Elsevier.

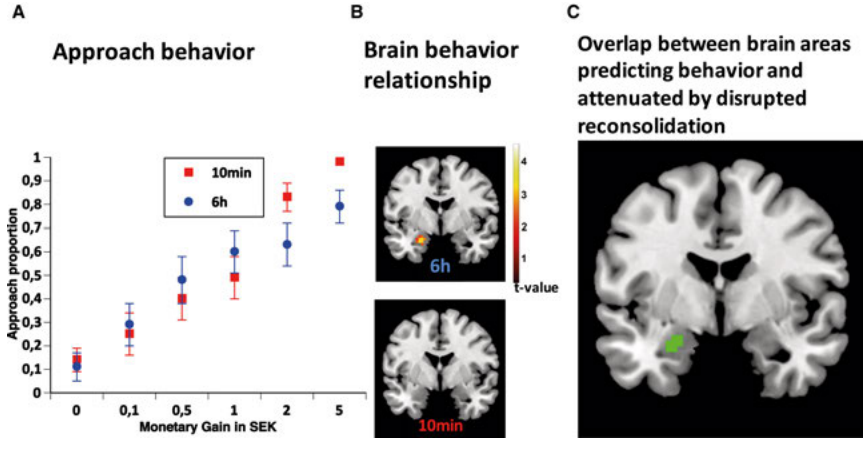


*Figure 4.* Disrupted reconsolidation facilitates approach and amygdala activity predicts behavior. Subjects chose between avoiding or viewing a spider slide while being paid nothing or 0.1, 0.5, 1, 2, or 5 SEK (Swedish Krona; 1 SEK ≈ 0.11 USD). (A) Approach behavior was facilitated by disrupted reconsolidation because the 10 min group (n = 21), as compared to the 6 hr group (n = 23), approached more spiders during high-gain conditions ($F(5, 210) = 3.23$; $p = 0.008$), being significant for the 2 and 5 SEK conditions ($t(42) > 2.86$; $p < 0.005$), and non-significant for the other conditions ($t(42) < 1.70$; n.s). Error bars indicate the SEM. (B) In the 6 hr group (top), which received undisrupted reconsolidation, amygdala activity during re-exposure was inversely related to approach behavior (xyz = −24, −1, −23; $Z = 3.05$; $p = 0.001$; $p_{corrected} = 0.018$; 540 $mm^3$), whereas in the 10 min group, which received disrupted reconsolidation, no such relationship was observed. (C) The amygdala areas coupled to disrupted reconsolidation (Figure 3A) overlapped with areas predicting approach behavior in a 378 $mm^3$ volume in the 6 hr group. From Björkstrand et al. (2016). Reprinted with permission from Elsevier.

## Discussion

The results showed that memory activation shortly followed by repeated exposure to feared cues attenuates subsequent amygdala activity and increases approach behavior. Thus, it appears that it is possible to disrupt reconsolidation even for decades old fear memories, and in line with previous studies on fear conditioning, the amygdala is implicated in this process. The effects were not specific to the activated and exposed cues, but generalized to the other stimuli, suggesting that reconsolidation disruption affects a "core" fear memory representation, thereby reducing fear responses to the conceptual category of spiders. Also, in the 6h group, amygdala activity during the re-exposure session predicted subsequent approach behavior, which indicates that behavioral approach may be regulated by the amygdala. Overall the results demonstrate that reconsolidation disruption could be applied in exposure treatment for anxiety disorders, possibly improving treatment outcomes and reducing relapse.

# Study IV

## Background and aim

Experimental research on fear conditioning has shown that memory retrieval shortly followed by extinction training, thereby interfering with memory reconsolidation, decreases subsequent fear expression (Agren et al., 2012; Kredlow et al., 2016; Monfils et al., 2009; Schiller et al., 2010), and that these effects persists for months or even years after the intervention (Björkstrand et al., 2015; Liu et al., 2014; Schiller et al., 2010). In Study III (Björkstrand et al., 2016) we showed that the retrieval-extinction procedure can also reduce the neural and behavioral expression of long term phobic fears 24 hours after the manipulation. Since the retrieval-extinction procedure is hypothesized to alter the original fear memory representation, rather than inducing an inhibitory safety memory, these effects are expected to be long-lasting. Study IV aimed to evaluate this question by performing a re-test on the participants from Study III, 6 months after the original manipulation.

## Methods

Of the original 45 subjects, 39 were available for the follow-up test. The test procedure was identical to the one used on day 2 in Study III, consisting of re-exposure to spider pictures and a behavioral approach test. Approximately 180 days after the original manipulation, subjects were called back to the lab, placed in the MR-scanner and were shown all 8 spider pictures shown on day 2, for two presentations each. Then they underwent the same behavioral approach test as used previously, in which they had to choose to watch a picture of a spider or a neutral picture. Choosing the spider was rewarded with 0, 0.5, 1, 2 or 5 SEK, and the subject had to make four choices at each reward level. During the re-exposure session, fMRI data was collected in order to evaluate amygdala activity. When evaluating the brain imaging data, we compared the amygdala activity on day 2 to day 180, using an analytical strategy equivalent to a 2x2 mixed ANOVA with Time (day 2 and day 180) and Group (10min group and 6h group) as factors. Because results from the previous study (Björkstrand et al., 2016) showed that amygdala activity was attenuated to all spider pictures following disrupted reconsolidation, the effects were evaluated by averaging brain activity over all spider pictures.

## Results

Analyses of the brain imaging data revealed that both groups had decreased in amygdala activity from day 2 to day 180, with a tendency towards a Group by

Time interaction, with the 10min group, that received disrupted reconsolidation, showing larger reductions in the right amygdala as compared the 6h group. We also found a main effect of group with the 6h group showing larger amygdala activity than the 10min group averaged across both re-exposure sessions. Taken together, this indicates that the group differences found previously are stable over 6 months. Also, the previously observed group difference in approach behavior persisted, as the 10min group still chose to view more spiders than the 6h group when paid the higher amounts 2 and 5 SEK. In contrast to previous findings, correlating amygdala activity to subsequent approach behavior in the 6h group, did not yield any significant results.

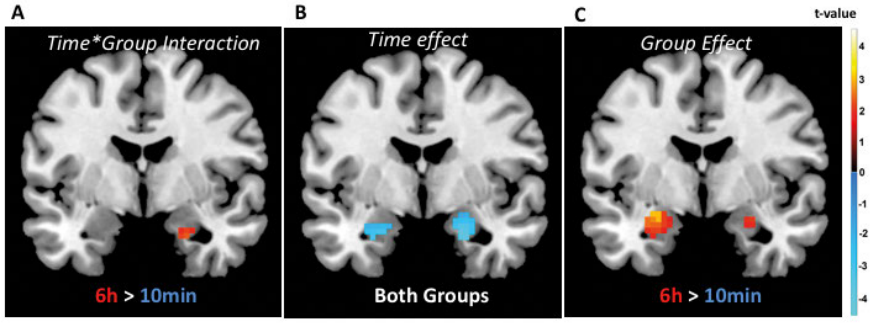


Figure 5. Disrupting reconsolidation produces long-lasting effects on amygdala activity. A. Between-group effects for the amygdala changes from day 2 to day 180 illustrating the Time by Group interaction. The results show similar decreases in the left amygdala and a trend towards greater reductions as a function time in the 10 min group as compared to the 6h. B. Within-group changes in amygdala activity from day 2 to day 180 across both groups representing the main effect of Time. The colors illustrate lower reactivity day 180 than day 2 across both groups. C. Between-group differences depicting the change in amygdala activity from the end of the exposure session day 1 to amygdala activity averaged over the re-exposure sessions on day 2 and day 180, representing the main effect of Group, demonstrating higher amygdala reactivity in the 6h than the 10min group. Taken together, this supports that the group differences are stable over 6-months, and possibly that amygdala activity is reduced more in the 10min as compared to the 6 h group. Most importantly, the effect of disrupted reconsolidation is clearly stable over time. The measure of amygdala activity is blood oxygenation level dependent (BOLD) signal changes, and statistical parametric mapping statistics display clusters reflecting significant group effects at $p<.05$ uncorrected exceeding 5 voxels for illustrative purposes. From Björkstrand et al. (2017). Reprinted with permission from Elsevier.

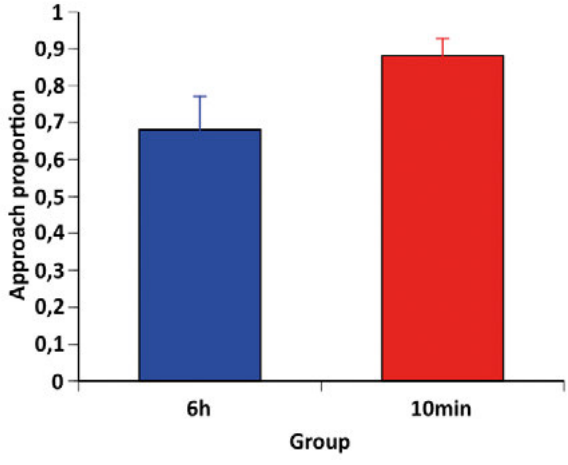


*Figure 6.* The effect of reconsolidation disruption on behavioral approach is stable over 6 months. The 10min group viewed significantly more spiders than the 6h group 6 months after memory activation and exposure, when paid higher amounts. The behavioral measure is the approach proportion averaged over the 2 and 5 SEK conditions. Means and standard error of the mean are shown. From Björkstrand et al. (2017). Reprinted with permission from Elsevier.

## Discussion

The results show that the previously observed short-term effects on amygdala activity and approach behavior following reconsolidation disruption (Björkstrand et al., 2016) are long lasting. This supports the hypothesis that memory retrieval followed by repeated exposure causes an amygdala localized fear memory representation to be permanently updated. Thus, the effect of reconsolidation disruption on long-term phobic memories is very similar to results obtained in studies using fear conditioning. In both cases, this manipulation leads to decreases in cue elicited amygdala activity and diminished behavioral fear expression, and these effects are stable over time. This provides further support that reconsolidation disruption procedures may be useful in the treatment of anxiety disorders, possibly improving treatment outcome and reducing relapse after an initially successful intervention.

# General discussion

## Main findings

Study I and II show that the reconsolidation of fear memories established through fear conditioning can be disrupted using a retrieval-extinction procedure. This manipulation decreases subsequent CS-elicited amygdala activity and fear expression, and the effects on fear expression are long-lasting. Also, amygdala activity is related to fear expression both in the short and long term in individuals having received extinction outside of the reconsolidation window, but not in individuals where reconsolidation was disrupted. Collectively this indicates that in humans, administering extinction training within the reconsolidation window permanently alters an amygdala localized fear memory, in line with previous research on rodents. Study III and IV extends these findings to long-term naturally occurring fear memories, demonstrating that memory activation prior repeated exposure to feared cues attenuate subsequent amygdala activity during fear provocation and facilitates approach behavior in individuals with a life-long fear of spiders. Effects on both amygdala activity and approach behavior were still present after 6 months, indicating that disrupting the reconsolidation of phobic fears may cause long-term alterations of an amygdala-dependent fear memory, in line with previous studies on fear conditioning.

## Discussion

In line with previous research in humans (Schiller et al., 2010) and rodents (Monfils et al., 2009), Study I found that a retrieval-extinction procedure can attenuate later fear expression as measured by SCR, a finding that has since been replicated multiple times by our lab (Agren et al., 2012; Agren, Björkstrand, & Fredrikson, 2017) and by others (Asthana et al., 2015; Johnson & Casey, 2015; Kredlow et al., 2016; Liu et al., 2014; Oyarzún et al., 2012; Schiller et al., 2013; Steinfurth et al., 2014) although, several studies have produced conflicting results (Fricchione et al., 2016; Golkar et al., 2012; Kindt & Soeter, 2013; Klucken et al., 2016; Kredlow et al., 2016; Meir Drexler et al., 2014; Soeter & Kindt, 2011). This suggests that although the retrieval-extinction effect is a reproducible phenomenon, there likely exist unknown boundary conditions. Although the overall design of these studies are very

similar, these discrepancies might be explained by subtle differences in methodology, such as the length of the US, type of US, exclusion criteria related to strength of initial learning, length of memory retrieval, different types of stimuli used as CS, use of online fear ratings, simultaneous use of fear potentiated startle, use of within- or between- group design, reinforcement rate during fear acquisition or other unknown factors. Since few studies have examined this systematically there is no clear evidence as to what differences are most important.

Study I also found that performing extinction within the reconsolidation window diminishes later CS-elicited amygdala activity and that amygdala activity predicts later fear expression in the group that received extinction outside of the reconsolidation window. This suggests that the retrieval-extinction effect on fear expression is amygdala-dependent in line with previous research in rodents showing that inhibiting protein synthesis in the LA disrupts fear memory reconsolidation (Baldi & Bucherelli, 2015; Nader et al., 2000), and that retrieval-extinction procedures engages plastic processes in the amygdala (Clem & Huganir, 2010; Monfils et al., 2009). The results in Study I have since been replicated in a study by Schiller et al. (2013), that showed decreased CS-elicited amygdala activity following extinction for stimuli that had been retrieved prior to extinction as compared to non-retrieved stimuli. Interestingly, this study also showed increased activity in vmPFC for non-activated as compared activated stimuli during the early part of extinction, suggesting that extinction during the reconsolidation window does not lead to formation of an inhibitory safety memory. In contrast to these findings, a later study (Klucken et al., 2016) also evaluating the effect of a retrieval-extinction intervention using fMRI, found no differences in amygdala activity between stimuli that were activated or not activated prior to extinction, either during the extinction phase or the re-test phase. However, since this study did not find any effect on fear expression measured with SCR either, this does not directly refute the finding that the amygdala mediates the retrieval-extinction effect on fear expression, but rather indicates that the manipulation they used did not accomplish the intended goal of destabilizing the fear memory and then disrupting its reconsolidation, possibly due to subtle differences in methods used in Study I and other studies (Klucken et al., 2016).

Study II showed that the effect of the retrieval-extinction procedure on subsequent fear expression are still present 18 months after the manipulation. This is in line with previous research in humans that have demonstrated the persistence of this effect 1 year (Schiller et al., 2010) and 6 months (Liu et al., 2014), after the manipulation. Interestingly, whereas previous studies used a reinstatement procedure, Study II used reacquisition for the long-term follow-up test, showing that even after 4 reinforced CS+ presentations, subjects having received disrupted reconsolidation still do not show increased SCRs. This indicates that the retrieval-extinction procedure counteracts later relearning of

the CS-US association, suggesting that if reconsolidation disruption techniques were successfully translated to exposure-based treatments of anxiety disorders, this could reduce relapse rates by inhibiting relearning of anxious and avoidant responses following successful treatment. The study also found that SCRs during the 18-month retest correlated with amygdala activity the day after the original manipulation. This indicates that long-term effects on fear expression are linked to acute attenuation of amygdala activity.

Research in rodents have shown that it is possible to disrupt the reconsolidation even of old and strong fear memories (Bustos et al., 2009; Suzuki et al., 2004; Wang et al., 2009). The result of Study III and IV confirm and extend these findings to humans, showing that a retrieval-extinction procedure increases approach behavior in individuals with long-lasting phobic fears. Similar to fear conditioning studies in rodents and humans (Agren et al., 2012; Baldi & Bucherelli, 2015; Clem & Huganir, 2010; Monfils et al., 2009; Nader et al., 2000; Schiller et al., 2013), this effect appears to be amygdala dependent. The behavioral results are in line with a previous study (Soeter & Kindt, 2015), showing that post-retrieval administration of beta-blockers can disrupt the reconsolidation of phobic fears, resulting in increased approach behavior and decreases in subjective self-rated fear levels in sample with spider phobia.

However, the findings are not consistent with results obtained by Shiban et al. (2015), which did not indicate that memory retrieval prior to VR-exposure to spiders had any effect on subsequent physiological arousal, approach behavior and subjective self-rated fear levels in spider fearful subjects. However, there are several methodological differences between this study and Study III that may explain this discrepancy. The study by Shiban et al., used a shorter memory retrieval, one 5s-presentation of a VR-rendered spider, as compared to four 6s-presentations of different spider pictures used in Study III. It is possible that this shorter memory retrieval may not have destabilized the memory, as research in rodents have shown that old memories may require longer memory retrieval in order to destabilize (Bustos et al., 2009; Suzuki et al., 2004). Also, the Shiban-study used a longer exposure session than Study III, consisting of a 30-minute exposure to 10 different VR-rendered scenes with an increasing number of spiders, as compared to a 10-minute exposure to spider pictures. This longer and more intense exposure may have resulted in a more persistent extinction induced safety memory in the control group, such that fear decreases were still present the subsequent day when the re-test was performed, masking group differences. Perhaps a longer and more intense exposure session necessitates a longer time-lag between intervention and re-test in order to find the effects, as has been demonstrated in the rodent literature (Kredlow et al., 2016; Monfils et al., 2009). The different outcome measures used by these studies might also explain the discrepant results. Whereas Study III measured amygdala activity and rewarded approach to cues of the same modality as used during exposure (i.e. spider pictures), Shiban and colleagues

measured physiological arousal with SCL, subjective fear-ratings and non-rewarded approach to cues of a different modality than used during exposure (i.e. live spiders). Possibly, amygdala activity and the behavioral assay used in Study III are more sensitive to subtle changes in spider aversion, as compared to the measures used by Shiban, et al.

In order for manipulations interfering with memory reconsolidation to be effective, the target memory must first be destabilized. In order for destabilization to occur, studies in both rodents and humans indicate that memory retrieval must induce prediction error (Diaz-Mataix, Ruiz Martinez, Schafe, LeDoux, & Doyere, 2013; Fernandez, Boccia, & Pedreira, 2016; Sevenster et al., 2013; Sevenster, Beckers, & Kindt, 2014). In fear conditioning research, it is fairly obvious how to induce prediction error since this relates to the expected occurrence and timing of the US after the presentation of the CS. However, when it comes to anxiety disorders the learning history is unknown and it is not entirely clear what the expected outcome is, or even what should be construed as the US. It might be related to aspects of the actual stimulus, such as the expected appearance or behavior of the spider in the case of spider phobia, or possibly aspects of the subject's own reactions, for example how fearful or anxious one feels when confronted with a spider. Although Study III of this thesis or the spider fear study by Soeter and Kindt (2015) do not directly investigate this question, the findings show that a mere brief exposure to feared stimuli can destabilize long present phobic fear memories, which in turn suggest that this type of manipulation also induces prediction error. This opens up avenues for further research, since it indicates that brief fear provocation might be used as a means for memory retrieval and destabilization when investigating reconsolidation disruption mechanisms in other anxiety disorders. These findings also raise some interesting questions pertaining to what aspects of memory retrieval are most relevant for memory destabilization in clinical populations. For example, can prediction error be manipulated so as to maximize the effect of manipulations aiming to interfere with reconsolidation? What is the optimal length of memory retrieval, so as to induce memory destabilization but not trigger extinction? Is the intensity of fear or anxiety elicited during memory retrieval related to the effect on subsequent fear responses? Does memory retrieval have to entail actual exposure to feared stimuli or can imaginal procedures serve the same purpose? Answering these types of questions could well be crucial for successful implementation of reconsolidation disruption techniques to the treatment of clinical anxiety.

## Limitations

Although the results from all studies indicate that the amygdala is involved in reconsolidation disruption in humans, the findings does not prove a causal role of the amygdala in these processes. Neuroimaging data collected with fMRI

is in essence correlational, and in order to show a causal role of amygdala related processes in the retrieval and subsequent interference of fear memory reconsolidation direct manipulation of such processes are necessary. Such manipulations, like lesions and micro-infusions of various compounds in discrete brain regions are not feasible in humans and therefore conclusion as to the causal role of the amygdala in reconsolidation disruption of fear memories must rely on extrapolation from studies in non-human animals.

For Study I, and consequently for Study II, the sample size is quite small. Although, the finding that the retrieval-extinction procedure attenuates CS-elicited amygdala activity has since been replicated in one small study (Schiller et al., 2013), further investigations on the effects of this manipulation on neural responses are warranted. Also in Study I, due to technical constraints, it was not possible to measure skin conductance during the re-test in the MR scanner on day 3, which would have strengthened the conclusions. Optimally, Study II should also have included simultaneous fMRI during the 18-month re-test, in order to establish long-term effects on CS-elicited amygdala activity.

Concerning Study III and IV, several aspects of the experimental design might preclude generalization of the findings to clinical treatments for specific phobia. The amount and type of exposure used is likely insufficient to produce clinically meaningful reductions in spider fear. Using still frames of spiders increased experimental control, allowing simultaneous brain imaging and detailed evaluation of generalization, but also decreased the ecological validity of the results. Although the effects generalized to novel spider pictures it is uncertain if these effects would generalize to live spiders. Also, the sample was recruited through public advertisements and not through clinical referral, and might not be representative of a clinical population. The studies demonstrate that memory retrieval followed by exposure can reduce even long-present phobic fears, but studies using methods more closely resembling clinical practice need to be performed before the clinical effectiveness of this manipulation can be verified.

In Study III, we performed neuroimaging not only during the re-test but also during the exposure phase on day 1, hoping to replicate a previous study on fear conditioning showing increased vmPFC activation to stimuli that had not been retrieved prior to extinction (Schiller et al., 2013). This would provide support that exposure during the reconsolidation time-window does not engage brain regions linked the formation of safety memories. Unfortunately, in this study, the signal from the vmPFC was very poor, likely due to inhomogenties in the static magnetic caused by the air-filled sinuses, and we were thus unable to evaluate this question.

## Future directions

Although it has been suggested for a long time that reconsolidation disruption procedures may find useful application in the treatment of anxiety disorders (Nader, 2000), very little research has been published that directly investigates this question. Findings over the last 15 years show that the reconsolidation of fear memories, even old and strong ones, can be disrupted following retrieval using various behavioral or pharmacological manipulations (Baldi & Bucherelli, 2015; Bustos et al., 2009; Diaz-Mataix et al., 2013; Kredlow et al., 2016; Monfils et al., 2009; Schiller et al., 2010; Soeter & Kindt, 2015; Suzuki et al., 2004; Wang et al., 2009) but research into clinical applications of this mechanism is still in its infancy. Establishing the clinical efficacy of procedures targeting memory reconsolidation will likely require a lot of research.

This thesis examined whether the retrieval-extinction procedure can be translated to phobic fears with a positive result. However, as mentioned above, these studies are pre-clinical in nature. Thus, it would be interesting to examine whether exposure treatment for specific phobia, conducted in a way more closely resembling clinical practice can be enhanced by a brief exposure shortly prior to the intervention, thereby inducing memory retrieval. This would entail using exposure treatment of much longer duration, and with real spiders, in a clinically recruited sample. Also, procedures using pharmacological disruption of reconsolidation could be further explored. One study (Soeter & Kindt, 2015) has demonstrated that propranolol administration following a brief live spider exposure, reduces self-rated spider fear and increases approach behavior. Thus, pharmacological reconsolidation disruption procedures are a promising treatment alternative for specific phobia, but in order to determine relative efficacy to existing therapeutic procedures, such interventions should be systematically compared to validated treatments, preferably exposure therapy, being the most well studied treatment for specific phobia to date (Wolitzky-Taylor, Horowitz, Powers, & Telch, 2008). If effects of post-activation propranolol are comparable to those of exposure treatment, the former might be the preferred choice, since exposure treatment can be demanding of the patient, takes longer, and requires extensive therapist training. Investigations examining both behavioral and pharmacological modes of reconsolidation disruption should include long-term follow-up measurements, since treatment differences might not be evident until long after the intervention.

A few studies have examined interventions targeting reconsolidation disruption in PTSD, and there are indications that propranolol administration in conjunction with memory activation may reduce symptoms in clinical patients, but this is based on uncontrolled or poorly controlled studies (Brunet, 2008; Brunet, 2011), which renders the conclusions tentative. Also, these studies have used memory activation that are much longer than has been previously used in fear conditioning trials, which might induce extinction related processes instead of reconsolidation. With regards to PTSD further studies are

needed that include adequate controls, such as memory activation with placebo administration, and also pharmacological intervention without previous memory activation in order to isolate the specific effect of reconsolidation disruption. Ideally, future studies might also use memory activations of shorter duration, which are less likely to induce extinction-like processes. Also in PTSD, further studies should include controls that receive already validated treatment interventions, in order to establish relative efficacy. So far, in PTSD only pharmacological disruption of reconsolidation has been studied. Since exposure based psychological treatment programs have been shown to be effective in treating PTSD (Cusack et al., 2016; Powers, Halpern, Ferenschak, Gillihan, & Foa, 2010) it would also be interesting to see whether reconsolidation disruption procedures could increase the effectiveness of these types of treatments. For example, one could evaluate whether the efficacy of prolonged script driven trauma imagery, which is a validated an effective treatment strategy for PTSD (Powers et al., 2010), could be enhanced by inducing memory retrieval shortly prior to the intervention.

Reconsolidation disruption procedures might also be applied in the treatment of other anxiety related disorders that are cue related, such as PD, SAD and OCD. These conditions are also believed, at least partly, to have their origin in associative learning experiences akin to fear conditioning (Bouton et al., 2001; Jacoby & Abramowitz, 2016; Rapee & Spence, 2004), and also treatment strategies for these disorders include exposure to cues or situations that elicit anxious reactions (Barlow, 2007). However, since these disorders does not always have their origin in an identified event, like PTSD, or are circumscribed to one type or stimuli or situation, like specific phobia, how to apply reconsolidation disruption strategies in these conditions is not obvious. One critical question that needs to answered, is what sort of experience might cause core fear memory representations to be retrieved, thereby destabilizing them. Most likely this would entail exposing the patient to some anxiety provoking cue or situation, but since anxiety in these disorders can be elicited by a wide variety of stimuli that are not always easy to produce in a therapeutic setting, answering this question will require some research.

Related to this question, is how to adapt the retrieval-extinction procedure to these disorders. Since treatments for these disorders currently in use often include exposure based strategies, this might be possible. However, as mentioned above, often the anxiety eliciting cues or situations are not always easy to recreate in the setting of the clinic, which makes translation harder. In therapy, this problem is solved by doing exposure treatment outside of the clinic, or more commonly, giving the patient self-exposure assignments to be performed on their own between sessions, but this might not be practical when doing controlled mechanistic studies. The easiest way may be to initially focus on exposure strategies that are currently used in-session, for example exposure to anxiety eliciting interoceptive cues in PD, exposure to public speaking in SAD, or exposure to contamination related cues in the absence of subsequent

anxiety reducing compulsions, such as washing, in OCD. Retrieval-extinction procedures could be readily translated to these types of exposure strategies, which would allow initial examination of the clinical usefulness of reconsolidation disruption in these disorders. A brief exposure to these types of situations could also serve as memory activations when investigating pharmacological modes of reconsolidation disruption. Whether investigating behavioral or pharmacological means of reconsolidation disruption, such studies should investigate to what degree the effects of the intervention generalize to other anxiety provoking cues and situations. This would provide information on the clinical potential for reconsolidation disruption and also shed light on whether exposure to these types of situation activate a core fear memory representation rather than a cue/situation specific memory.

There are also novel interventions that merits further investigation. Concerning the use of pharmacological manipulations to interfere with reconsolidation, mainly propranolol has been investigated in humans. Other compounds such as benzodiazepines (Bustos et al. 2009, Bustos et al., 2010) and NMDAR antagonists (Suzuki et al. 2004) have been shown to disrupt reconsolidation in rodents. This could also be investigated in humans, either experimentally using fear conditioning in healthy subjects or as treatment interventions in subjects with anxiety disorders. Also, in rodents NMDAR agonists have been shown to facilitate memory destabilization if given prior to memory retrieval, thus enhancing the effect of post-activation amnesic procedures (Bustos et al., 2010). This could also be investigated in humans and is of particular interest in clinical samples since this type of intervention might facilitate memory destabilization of old and strong fear memories that are likely more resistant to reconsolidation disruption.

Concerning behavioral interventions, mainly extinction has been studied as a means to update fear memories, but there are interesting findings suggesting that other types of interventions might also be useful. One study found that playing the computer game tetris subsequent to memory retrieval can reduce intrusive memories of trauma related footage (James et al., 2015). This indicates that manipulations taxing working memory resources might be used to interfere with the reconsolidation of maladaptive emotional memories, which may prove useful particularly in the treatment of trauma related disorders. Also, our lab recently demonstrated that imaginal extinction can be used to interfere with the reconsolidation of a fear memory (Agren et al., 2017). Similarly, another study found that retrieval followed by vicarious extinction, i.e. observing another person receiving extinction, can also be used to reduce physiological responses to fear conditioned cues (Golkar, Tjaden, & Kindt, 2017). Thus, adapting imaginal and vicarious retrieval-extinction procedures for use in exposure based treatments of anxiety disorders could be an interesting topic for future research.

In conclusion, the work presented in this thesis demonstrates that interfering with reconsolidation has long-lasting effects on both neural and behavioral

indices of human fear. Moreover, these effects are not circumscribed to recent fear memories, established through fear conditioning, but extends to life-long phobic fears. Thus, interventions targeting the reconsolidation of fear memories is may prove to be a useful tool in the treatment of anxiety disorders.

# Acknowledgements

First of all, I would like to thank my supervisors, Mats Fredrikson, Fredrik Åhs and Tomas Furmark. Truly, the best supervisors any doctoral student could ever wish for. Thank you for your invaluable advice, scientific curiosity, immense expertise, unrelenting support in all matters, for keeping me on my toes with harsh "devils-advocate" styled interrogations (Mats), and for letting me spend all your grant-money. During these years, you have provided me with a research environment that exceeds all expectations, and I only hope (probably in vain) that I see the likes of it again. Without your guidance, this work would not have been possible, and I sincerely hope that some little part of your scientific quality has rubbed off on me.

I would also like to extend a special thanks to my partners in crime, Drs. Thomas Ågren and Andreas Frick. Thank you, Thomas, for introducing me to the exciting world of reconsolidation research and the joy of shocking healthy volunteers. And Andreas, thank you for all the code, for your never ceasing helpfulness, analysis expertise, excellent scanning skills, and the many, many impromptu discussions on anything scientific.

I am also very grateful to all past and present members of the research group. It has been a joy and an inspiration to work with you all. Jörgen Rosén, for your helpfulness and commitment to well-commented code; Malin Gingnell, for your open door and no-nonsense advice; Vanda Faria, for the hugs and face-slaps (I'm certain I deserved them equally); Jonas Engman, for showing me my way around an MR data-set; Olof Hjorth; for being my companion and sole constant in this ever-changing life (when are you moving to Lund?); Johanna Motilla-Hoppe, for hard-core PET duty and for introducing me to student life when I first came to Uppsala; Iman Alaie, for joining me in my first uncertain steps as a PhD student and for soothing my anxieties with progressive rock and Finnish tango; Nathalie Peira, for the scary spider pictures and for curing my aversion to rats with unbridled enthusiasm; Ulrika Wallenquist, for interesting discussions, a contagious laugh and for teaching me about C57BL/6; and last (but certainly not least) Örjan Frans, whose encouraging words have always brightened my days at the department.

I also thank my fellow travelers and blob-hunters, Eva Stening and Jonas Persson, for great company, all your help and advice (and for letting me experiment on you occasionally).

I would also like to thank my fellow PhD students and all the wonderful people who work at the Department of Psychology in Uppsala, a place where

you never have to look long for an interesting discussion or a helping hand. I am very grateful to you all. Special thanks goes out to JoAnne Dahl and Per Lindberg for providing valuable feedback on my thesis.

I would also like to thank Elna-Marie Larsson, Britt-Mari Bolinder, Arvid Morell, Karin Åberg, Markus Fahlström, Johanna Mårtensson and all the staff at the 3T MR-lab. It has been a pleasure to work with you all, and your continuous support has been indispensable.

I'm very grateful to Ida Högdahl (research-assistant *extraordinaire*), Kristoffer Björkstrand, Irma Lejlic and Sara Hultberg, and all students over the years who have helped me collect the data. Without your hard work, I would never have gotten here.

I would also like to thank all the research participants who have endured my experiments and sacrificed their time and comfort in the name of science. I am also grateful to the Swedish Research Council, Heumanska stiftelsen, Boethius stiftelse, the Swedish Brain Foundation and the Swedish Council for Working Life and Social Research for providing the funds that made this research possible.

And, I would like to thank all my friends at Hilti Fightcenter and Crossfit Uppsala. Doing burpees together until exhaustion and strangling one another has really served to relieve my stress throughout this period. I would also like to thank you for being a reliable source of pilot research subjects. Having an endless pool of sensation seeking and pain-indifferent individuals with lots of spare time has been a great resource, especially when trying out new ways of scaring people in an MR-scanner.

I would also like to thank everyone at the Department of Psychology in Lund, who have made me feel so welcome during my recent exile, and also provided the desk at which this thesis was written.

I would like to thank my family, especially my parents, Eva and Bo, for their unconditional support and for showing me the way; my brother Kristoffer who took an active part in the work here presented (see above); and my sisters Linnea and Viola for making my life so enjoyable. I am also grateful to my in-laws, Marie and Olof, for support, encouragement and childcare.

And finally, my wonderful wife Frida, who has somewhat patiently endured my many absences during late evenings and weekends at the scanner, during conference-trips, and on the many occasions where my mind drifted towards research, leaving me staring in to thin air with a vacant expression. And of course, my lovely children Arvid and Nora, whose smiles, cheerfulness, and rambunctious behavior have made me one happy researcher. My love and gratitude for you knows no bounds.

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