

# Journal of Visualized Experiments

## Disrupting reconsolidation of fear memory in humans by a noradrenergic $\beta$ -blocker

--Manuscript Draft--

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<b>Abstract:</b>	<p>The basic design used in our human fear-conditioning studies on disrupting reconsolidation includes testing over different phases across three consecutive days. On day 1 - the fear acquisition phase, healthy participants are exposed to a series of picture presentations. One picture stimulus (CS1+) is repeatedly paired with an aversive electric stimulus (US), resulting in the acquisition of a fear association, whereas another picture stimulus (CS2-) is never followed by an US. On day 2 - the memory reactivation phase, the participants are re-exposed to the conditioned stimulus without the US (CS1-), which typically triggers a conditioned fear response. After the memory reactivation we administer an oral dose of 40 mg of propranolol HCl, a <math>\beta</math>-adrenergic receptor antagonist that indirectly targets the protein synthesis required for reconsolidation by inhibiting the noradrenaline-stimulated CREB phosphorylation. On day 3 - the test phase, the participants are again exposed to the unreinforced conditioned stimuli (CS1- and CS2-) in order to measure the fear-reducing effect of the manipulation. This retention test is followed by an extinction procedure and the presentation of situational triggers to test for the return of fear. Potentiation of the eye blink startle reflex is measured as an index for conditioned fear responding. Declarative knowledge of the fear association is measured through online US expectancy ratings during each CS presentation. In contrast to extinction learning, disrupting reconsolidation targets the original fear memory thereby preventing the return of fear. Although the clinical applications are still in their infancy, disrupting reconsolidation of fear memory seems to be a promising new technique with the prospect to persistently dampen the expression of fear memory in patients suffering from anxiety disorders and other psychiatric disorders.</p>
<b>Author Comments:</b>	<p>Editorial comments:</p> <p>1.We have changed steps 3.6.1 and 3.6.2 into steps 2.6.1 and 2.6.2.</p> <p>2.We have now un-highlighted the following steps: 2.7 _ 2.8 _ 2.9 _ 2.15 _ 3.4 _ 4.5 _ 4.2 _ 4.3.</p>

3. Journal names are now italicized throughout. Moreover we removed the extraneous comma from ref\_#3.

4. We clarified step 3.8.1. by adding "for the propranolol no-reactivation condition". Also we now explain in the introduction section on page 3 what the propranolol no-reactivation condition is for.

5. We replaced "...clinical applications are still in its infancy.." by "...clinical applications are still in their infancy..".

6. We have thoroughly proofread our manuscript to ensure that there are no spelling or grammar issues.

7. We do not use figures from previous publications.

Reviewer #1:

1. Following the suggestion of the reviewer we changed the term "erasure" for a less absolute term.

2. We have now replaced "...re-exposed to the unreinforced conditioned stimulus.." with "...re-exposed to the conditioned stimulus without the US..".

3. We now explain in the long abstract that propranolol HCl is administered orally.

4. Following the suggestion of the reviewer we now stated in the long abstract that "...propranolol indirectly targets the protein synthesis required for reconsolidation by inhibiting the noradrenaline-stimulated CREB phosphorylation".

5. Indeed we should also recognize that anisomycin may interfere with the release of neurotransmitters thereby causing the amnesic effect. We now clarified this on page 3.

6. We are not sure whether we understand what the reviewer means by an instructed prediction error. But if the reviewer means that we could tell the participants what exactly happens during reactivation [i.e., that the CS will no longer be followed by a US] reconsolidation will not be triggered [see Sevenster et al. 2012].

7. We now clarified in the "ethics statement" that a physician approves our protocol: this may help convincing the ethics commission to use electric shocks as well as propranolol HCl.

8. Shock intensities are individually set as different skin types may affect how the shock is experienced. Participants judge the pleasantness of the shock on 11-point rating scales - ranging from minus 5 [i.e., unpleasant] to 5 [i.e., pleasant] - at the end of acquisition: this is now clarified in step 2.15. As long as the groups do not differ on this evaluation of the US we feel that this method is valid.

9. Solely because a physician is not present during our conditioning studies the use of an anti-depressant drug - and not depression per se - is an exclusion criterion: we have now changed this accordingly in step 2.2. Hence this exclusion criterion will not be a determining factor in the application of this method.

10. Following the suggestion of the reviewer we now comment on the loud noises at the end of the introduction section on page 3.

11. We now explain in step 2.8 that the mouse is used to position the cursor and that the participants have to press the left button in order to confirm their expectancy.

12. Our "single-blind" option is for the propranolol no-reactivation condition. We now clarified this in step 3.8.1. Also we now explain at the end of the introduction section on page 3 what this condition is for.

13.A physician on call with knowledge of the protocol is indeed considered necessary. We now clarified this in step 3.8.

14.Our first two studies [i.e., Kindt et al. 2009 Soeter and Kindt 2010] indeed involved a group of participants that received the drug without prior retrieval to control for any non-specific effects [i.e., propranolol no-reactivation condition]. Already we included this in the protocol but in an unclear fashion. At the end of the introduction section on page 3 we now explain why there should be such a condition. Further we clarified in step 3.8.1 that the "single blind" option is meant for this control condition.

15.We now explain at the end of the introduction section on page 3 that test phases should be separated by at least 24 hr in order to (1) support the (re)consolidation of the memories and (2) allow the drug to wash out before testing.

16.Gender differences are not to be expected. Following the suggestion of the reviewer we now suggest this at the beginning of the discussion section on page 10.

17.We have replaced "form" with "from".

Reviewer #3:

1.Following the suggestion of the reviewer we now refer to Mineka and Zinbarg 2006.

2.Ample evidence indicates that startle potentiation reflects a specific and reliable index of fear whereas the SCR used by Schiller et al. is much less specific. While startle responding is potentiated in reaction to a CS that is associated with an US of negative valence [e.g., electrical stimulation] it can typically not be observed with USs of neutral valence [e.g., vibrotactile stimulation or reaction time-task] [e.g., Lipp et al. 1994 Hamm and Vaitl 1996]. Contrary the affective valence of the US does not modify SCR conditioning as it can occur irrespective of the valence of the US [e.g., Lipp et al. 1994 Hamm and Vaitl 1996]. As such the SCR is considered a non-specific measurement of anticipatory arousal and we do not follow the suggestion of the reviewer.

3.A series of studies demonstrated that extinguished fear responding returned after unpredictable shocks or a context change or the passage of time while fear did not return after disrupting reconsolidation [e.g., Kindt et al. 2009 Soeter and Kindt 2010 2011 Sevenster et al. 2012 2013]. Based on those observations we feel that it is justified to say "support the superiority of disrupting reconsolidation over extinction learning".

4.Written informed consent is obtained from all participants before the beginning of the experiment. We now clarified this in step 2.1.

5.See our answer to comment 2.

6.Also following the suggestion of the reviewer we now refer to Agren et al. 2012 and Oyarzún et al. 2012.

7.Only when fear was fully extinguished during the reconsolidation window Schiller et al. found an effect on subsequent fear responding. Yet in clinical practice fear responding is often not extinguished within one-session and thus forms a potential limitation for this method. As a result we consider our statement "...would be that in clinical practice the fear...one-session exposure-treatment.." correct.

8.Strength of fear memory is not necessarily a boundary condition for reconsolidation. For instance some older and stronger memories have been observed not to undergo reconsolidation [Suzuki et al. 2004] leading several researchers to conclude that it reflects a lingering consolidation process that may eventually terminate [Alberini 2011]. However others have noted that the parameter-space for reactivation may be changed under these reconsolidation-resistant conditions as longer [Suzuki et al. 2004] or delayed [Wang et al. 2009] reactivation may still engage reconsolidation. However it is

	not self-evident that disrupting reconsolidation of older or stronger and broader memory networks such as in patients with PTSD is as effective as it has been shown in the laboratory for cued fear conditioning - see page 11.
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	
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Amsterdam - July 7 2014

Dear Dr. Upponi,

Attached please find a revised version of our manuscript entitled “Disrupting reconsolidation of fear memory in humans by a noradrenergic  $\beta$ -blocker” [JoVE52151R1]. We are grateful to the comments of the reviewers and respond to them in the accompanying letter. We hope that our manuscript satisfactorily addresses the concerns of the reviewers and is now suitable to be published in JoVE. Changes in the manuscript are marked up in [blue](#).

Sincerely,

Merel Kindt

**TITLE:**

Disrupting reconsolidation of fear memory in humans by a noradrenergic  $\beta$ -blocker.

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**KEYWORDS:**

Fear memory - reconsolidation - noradrenergic  $\beta$ -blocker - human fear conditioning - startle potentiation - translational research.

**SHORT ABSTRACT:**

Disrupting reconsolidation is a promising approach to dampen the behavioral expression of fear memory in patients with anxiety disorders or posttraumatic stress disorder. In a series of human fear conditioning studies we showed that disrupting reconsolidation by the noradrenergic  $\beta$ -blocker propranolol is very effective in erasing conditioned fear responding.

**LONG ABSTRACT:**

The basic design used in our human fear-conditioning studies on disrupting reconsolidation includes testing over different phases across three consecutive days. On day 1 - the *fear acquisition* phase, healthy participants are exposed to a series of picture presentations. One picture stimulus (CS1+) is repeatedly paired with an aversive electric stimulus (US), resulting in the acquisition of a fear association, whereas another picture stimulus (CS2-) is never followed by an US. On day 2 - the *memory reactivation* phase, the participants are re-exposed to the conditioned stimulus without the US (CS1-), which typically triggers a conditioned fear response. After the memory reactivation we administer an oral dose of 40 mg of propranolol HCl, a  $\beta$ -adrenergic receptor antagonist that indirectly targets the protein synthesis required for

reconsolidation by inhibiting the noradrenaline-stimulated CREB phosphorylation. On day 3 - the test phase, the participants are again exposed to the unreinforced conditioned stimuli (CS1- and CS2-) in order to measure the fear-reducing effect of the manipulation. This retention test is followed by an extinction procedure and the presentation of situational triggers to test for the return of fear. Potentiation of the eye blink startle reflex is measured as an index for conditioned fear responding. Declarative knowledge of the fear association is measured through online US expectancy ratings during each CS presentation. In contrast to extinction learning, disrupting reconsolidation targets the original fear memory thereby preventing the return of fear. Although the clinical applications are still in their infancy, disrupting reconsolidation of fear memory seems to be a promising new technique with the prospect to persistently dampen the expression of fear memory in patients suffering from anxiety disorders and other psychiatric disorders.

## **INTRODUCTION:**

Our brains are programmed to learn. Humans are well equipped with the ability to learn the potential dangers in life and perhaps even more important to learn the predictors of danger. Pavlovian aversive conditioning is an excellent tool to study associative fear learning not only in humans but also across a wide range of organisms<sup>1,2</sup>. This procedure involves presenting an innocuous biologically neutral conditioned stimulus (CS, e.g., a tone or picture) with a noxious or harmful unconditioned stimulus (US), typically a mild electrical shock. If the CS becomes a reliable predictor of the US, the CS will elicit species-typical conditioned behavioral responses (e.g., freezing in rats and potentiated startle reflex in humans), which are conceptualized as expressions of fear (see <sup>3</sup> for critical comments on this terminology). Not only does aversive conditioning research add to a better understanding of the molecular and cellular processes of associative fear learning and memory<sup>4</sup> but it also provides a foundation for understanding the etiology and course of anxiety disorders<sup>5</sup>. Though it bears mentioning that anxiety disorders do not necessarily result from direct conditioning experiences such as traumatic events. They may also result from indirect or vicarious fear learning experiences<sup>6</sup>. But irrespective of the learning history, associative fear memory lies at the core of anxiety disorders. The Pavlovian conditioning paradigm has not only proven its utility in understanding the origin of anxiety disorders, it is also an excellent translational model to develop and advance treatment for anxiety disorders<sup>5</sup>.

In the laboratory the two most extensively studied procedures to reduce learned fear are (1) extinction and (2) disruption of reconsolidation. Even though extinction training - i.e., the repeated unreinforced re-exposure to the CS<sup>7,8</sup> - is an effective anxiolytic strategy, animal and human fear conditioning studies reliably show that the conditioned fear response can easily return by re-exposure to unsignaled USs (i.e., reinstatement), a context change (i.e., renewal), or the passage of time (i.e., spontaneous recovery)<sup>7,9,10</sup>. A consensus has been building that extinction learning does not erase the original fear memory but instead reflects the formation of a new inhibitory memory. As a consequence, the fear memory may resurface resulting in a return of fear even after originally successful fear extinction. The various sources of relapse are still a major challenge for clinical practice.

An alternative approach to diminish conditioned fear responding is the more recently (re)discovered procedure of disrupting memory reconsolidation by pharmacological agents. This procedure is very promising as it not only diminishes conditioned fear responding but it even seems to erase associative fear memory, which might eventually solve the problem of relapse. Memory reconsolidation refers to a two-phase process by which previously consolidated memories transfer to a transient destabilized state at retrieval and require a time-dependent restabilization to persist<sup>14-18</sup>. Gene transcription, protein and RNA synthesis are necessary for this restabilization and offer a window of opportunity for amnesic agents to affect the fear memory. Amnesia for learned fear has been demonstrated in animals by drugs (e.g., anisomycin) targeting the required protein synthesis<sup>14,19,20</sup> or the release of neurotransmitters<sup>21,22</sup>. Protein synthesis may also be disturbed by the noradrenergic beta-blocker propranolol, which is supposed to inhibit the noradrenaline-stimulated CREB phosphorylation<sup>23-25</sup>. The fear reducing effects of propranolol have been demonstrated in animals and humans<sup>26-32</sup>. In a series of discriminative fear-conditioning studies, we consistently demonstrated that propranolol (40 mg) administered prior to or after memory reactivation effectively reduced the conditioned startle fear response and prevented the return of fear in healthy participants.

### **Proof of Principle**

The basic design used in our human fear-conditioning studies on disrupting reconsolidation includes testing over different phases across three consecutive days separated by at least 24 hr in order to (1) support (re)consolidation of the memories and (2) allow the drug to wash out before testing. On day 1 - the *fear acquisition* phase, healthy participants are exposed to a series of picture presentations. One picture stimulus (CS1+) is repeatedly paired with an aversive electric stimulus (US), resulting in the acquisition of a fear association, whereas another picture stimulus (CS2-) is never followed by an US. On day 2 - the *memory reactivation* phase, re-exposure to the unreinforced conditioned stimulus (CS1-) typically triggers a conditioned fear response. In this phase, we systemically administer 40 mg propranolol HCl, a  $\beta$ -adrenergic receptor antagonist that indirectly targets the protein synthesis required for reconsolidation<sup>25</sup>. In view of the peak plasma concentrations of propranolol<sup>33</sup>, we administered an oral dose of propranolol 90 min prior to the reactivation of the fear memory in our first experiments. However, for a more optimal test of reconsolidation, the amnesic agent should be administered after the memory reactivation. Therefore, in our latter experiments we always administered propranolol after memory reactivation with very similar results. On day 3 - the *test* phase, the retention of the fear memory is tested 24 hr after the intervention (i.e., first test trial day 3), followed by an extinction procedure and situational triggers to test for the return of fear (i.e., reinstatement, renewal, spontaneous recovery, rapid reacquisition). For determining whether the effect of propranolol requires active retrieval of the fear memory, the drug should be administered to another fear-conditioned group without reactivation of the memory (i.e., propranolol no-reactivation condition)<sup>27,28</sup>. We used the potentiation of the startle reflex as a measure for conditioned fear responding and online US expectancy ratings as an index for contingency learning. The potentiation of the startle reflex is conceived as a specific and reliable index of fear<sup>34</sup>, subserved by the amygdala<sup>35</sup>. The most commonly used startle-eliciting stimulus is the "startle probe", a loud noise that is presented through headphones during a stimulus or in the interval between two stimulus presentations (i.e., intertrial intervals)<sup>36</sup>. Stronger startle



responses to the loud noise during the fear conditioned stimulus (CS1+) as compared to the control stimulus (CS2-) reflects the fearful state of the participant elicited by the feared stimulus (CS1+).

In a series of 10 consecutive experiments in independent samples<sup>27-32,37-39</sup> we consistently replicated our original finding where disrupting reconsolidation of fear memory by a beta-adrenergic blocker propranolol HCl effectively reduced the conditioned startle reflex and prevented the return of fear<sup>27</sup>. The observations that exposure to primary reinforcers (i.e., reinstatement), a change in context (i.e., renewal) or simple the passage of time (i.e., spontaneous recovery) did not lead to the re-emergence of conditioned fear responding as is generally observed after extinction training, support the superiority of disrupting reconsolidation over extinction learning. In addition to these retrieval techniques, reacquisition learning did not reveal any savings of the previously learned fear response. In sum, these findings suggest that disrupting reconsolidation of associative fear memory by propranolol effectively reduced the emotional expression of fear memory.

Furthermore, we tested several boundary and necessary conditions for disrupting reconsolidation: (1) The memory reactivation session seems procedurally similar to extinction training (i.e., unreinforced exposure), but it should involve less unreinforced trials than extinction training because noradrenergic beta-blockers (i.e., propranolol) may also interfere with the formation of extinction memory instead of targeting the original fear memory<sup>40,41</sup>; (2) Memory retrieval is not sufficient for memory reconsolidation<sup>18,31,42,43</sup>. Propranolol only reduced the conditioned fear responding when there was something to be learned during the retrieval session<sup>31,32</sup>. A discrepancy between what has already been learned and what can be learned on a given retrieval session (i.e., prediction error) seems to be necessary for inducing reconsolidation of associative fear memory<sup>32</sup>. The results indicate that the occurrence of a prediction error is a necessary condition for reconsolidation and provide a useful instrument for developing and optimizing reconsolidation-based treatments for patients suffering from anxiety disorders.

## **PROTOCOL:**

**Ethics statement:** Our physician-approved protocol meets the requirements of the Ethical Committee of the Department of Psychology at the University of Amsterdam for testing human participants.

### **1. Attachment of the Fear Potentiated Startle and Schock Electrodes**

1.1) Ensure the experimental setting consists out of a sound-attenuated participants' room and a separated experimenter room.

1.2) Use two computers with four screens for recording and monitoring physiological responses as well as for presenting the experimental script to the participants.

1.3) Attach the double-sided adhesive collars to the EMG electrodes.

1.4) Clean the participant's skin below the right or left eye and on the forehead using an alcohol swab. Instruct the participants to keep their eyes closed to avoid potential eye irritation.

1.5) Fill the center of the EMG electrodes with highly conductive electrolyte gel using a syringe. Do not try to fill the electrodes by applying small amounts of gel successively as this increases the risk of air bubbles within the gel, which may affect the recordings negatively.

1.6) Attach one of the EMG electrodes below the lower eyelid of the right or left eye in line with the pupil in forward gaze. Attach the second one approximately 1 - 2 cm lateral to the first following the curvature of the lower eyelid. Attach a ground reference on the forehead approximately 1 cm below the hairline. Optimal placement of the electrodes relative to the muscle may require individual adjustments.

1.7) Apply a drop of the conductive gel on the shock electrodes and attach them on the left upper wrist using nylon velcro strap. Make sure the strap is not on too tight.

1.8) Adjust the intensity of the 2-ms electric shock for each participant individually. Inform the participants that the shock should be "uncomfortable but not painful".

1.9) Manually trigger the shocks. Start at an intensity of 1 mA and continue triggering single shocks - gradually increasing the intensity in 2 or 3 mA increments - until the participant indicates that the shock is "uncomfortable but not painful".

1.10) Write down the final shock level and maintain this level throughout the remainder of the experiment.

## **2. Fear Acquisition - Day 1**

*Duration about 45 minutes.*

2.1) Instruct the participants about the delivery of shocks and the possible administration of propranolol HCl. Further inform them that propranolol HCl is used to treat hypertension. Obtain written informed consent.

2.2) Screen the participants regarding their health and any conditions that would contra-indicate taking a single dose of 40 mg of propranolol HCl: pregnancy - seizure disorder - respiratory disorder - cardiovascular diseases - cardiovascular diseases in first-line relatives - diabetes - liver or kidney disorder - current use of an anti-depressant drug - psychosis.

2.3) Obtain blood pressures and heart rate measurements using an electronic sphygmomanometer. Ask the participants to remove their arm from the sleeves and put the inflatable cuff securely on the upper arm. Instruct the participants to relax and sit with their arm slightly bent on the same level as their heart and resting comfortably on a table or other flat

surface. BP should be above 90-60 mmHg and heart rate above 60 BPM for inclusion in the experiment.

2.4) Administer the State-Trait Anxiety Inventory<sup>44</sup> and the Anxiety Sensitivity Index<sup>45</sup> when a participant is medically cleared. Also administer the Spider Phobic Questionnaire<sup>46</sup> when using pictures of spiders as the conditioned stimuli.

2.5) Position the participants in front of a computer screen and attach the EMG and shock electrodes: see **2. Attachment of the Fear Potentiated Startle and Shock Electrodes**.

2.6) Use two different learning procedures - see steps 2.6.1 and 2.6.2. Inform the participants about the two different pictures they will see on the computer screen. Instruct them to monitor the relationship between the pictures they are seeing and when a shock is received.

2.6.1) *Non-Asymptotic Learning* - Inform the participants that one of the pictures will be followed by the electric shock in most cases whereas the other will never be followed by the shock.

2.6.2) *Asymptotic Learning* - Inform the participants which of the two pictures will always be followed by the electric shock and which picture will never be followed by the shock (i.e., instructed fear learning).

2.7) Inform the participants about the loud noises they will hear through the headphones. Order them to pay no attention to the noises.

2.8) Instruct the participants to report the expectancy of an electric shock during the presentation of all pictures by shifting a cursor on continuous rating scales by using a mouse and to push the left mouse button within 5 sec following stimulus onset in order to confirm their expectancy. Ensure scales consist of 11-points labeled from “certainly no electric shock” through “uncertain” to “certainly an electric shock”. Present the scales continuously at the bottom of the computer screen in order to support the participants to focus their attention on the pictures. When the pictures disappear the cursor should automatically return to the “uncertain” position.

2.9) Direct the participants to keep their eyes on the screen and sit as still as possible with both arms placed in a relaxed position with wrists up on the desk in front of them. Put on the headphones. Dim the lights and shut the door. Start the experiment from the other room.

2.10) During fear acquisition one of the two pictures co-terminates with the shock: CS1, whereas the other one is never followed by the shock: CS2. Make use of fear relevant stimuli in order to strengthen the fear association during acquisition. For instance pictures of spiders: IAPS numbers 1200 - 1201.

2.11) Present both the CS1 and CS2 for 8 sec. Loud noises or startle probes - 40 ms and 104 dB - are presented 7 sec after CS onset and - for the CS1 - are followed by the 2-ms electric shock 500

ms later.

2.12) Start the actual fear learning by presenting ten baseline startle probes to diminish initial startle reactivity:

2.12.1) *Non-Asymptotic Learning* - Present a total of 8 CS1 trials on a 75% reinforcement scheme: 6 CS1 trials are reinforced by a shock - the first and the fifth trial are unreinforced. Further present 8 CS2 trials as well as 8 NA trials during the inter-trial-intervals.

2.12.2) *Asymptotic Learning* - Present a total of 3 CS1 trials on a 100% reinforcement scheme: all CS1 trials are reinforced by a shock. Further present 3 CS2 trials as well as 3 noise alone startle probes (NA) during the inter-trial-intervals.

2.13) Counterbalance the assignment of the pictures as CS1 and CS2 across participants. Randomize the order of trial type within blocks of CS1 - CS2 - NA such that no trial-type repeats more than two times. Vary the inter-trial-intervals - during which participants view a black screen - between 15 sec 20 sec and 25 sec with a mean of 20 sec.

2.14) After completion of the experiment detach the electrodes and let the participants clean off the gel.

2.15) Instruct the participants to judge the pleasantness of the shock on 11-point rating scales ranging from *minus* 5 (i.e., unpleasant) to 5 (i.e., pleasant).

2.16) Explicitly instruct the participants to remember what they had learned during fear acquisition in order to augment retention of the CS-US contingency the following days as well as to prevent them from erroneously expecting a different contingency scheme during subsequent testing. Furthermore - to facilitate the salivary samples the next day - instruct the participants to refrain from exercise caffeine and alcohol during the 12 hr and to avoid food and drinks other than water as well as smoking and tooth brushing during the 2 hr prior to the next session.

2.16) Clean the electrodes thoroughly with water after usage: get rid of all the gel without scratching any portion of the AgCl layer. After cleaning do not wipe off the water but use a different set of electrodes on the next participant and allow the other set to dry over time.

### **3. Memory Reactivation - Day 2**

*Duration about 105 minutes.*

3.1) Randomly assign the participants to either the propranolol HCl or placebo pill condition with the restriction that conditions are matched on STAI scores as close as possible given that trait anxiety determines the fear-reducing effects of disrupting reconsolidation<sup>47</sup>. Match the participants on SPQ scores as well when using pictures of spiders as the conditioned stimuli.

3.2) Obtain blood pressures and heart rate measurements as well as salivary samples: instruct the participants just to place the swab in their mouths for about 3 minutes. Keep the Salivettes at *minus* 25°C after removal.

3.3) For the *propranolol no-reactivation* condition go to step 4.8.1.

3.4) Position the participants in front of the computer screen and attach the EMG and shock electrodes: see **2. Attachment of the Fear Potentiated Startle and Shock Electrodes**. Report to the participants that the intensity of shock will remain the same.

3.5) Inform the participants that the same two pictures will be presented on the computer screen and ask them to remember what they had learned the previous day - one of the pictures will again be followed by the electric shock in either *most* (i.e., non-asymptotic learning) or *all* (i.e., asymptotic learning) cases whereas the other will never be followed by the shock. Instruct the participants to report the expectancy of an electric shock during the presentation of all pictures. Put on the headphones and dim the lights. Shut the door and start the experiment from the other room.

3.6) Begin the memory reactivation by presenting 10 baseline startle probes to diminish initial startle reactivity. Next present a single unreinforced CS1 trial followed by a single NA trial.

3.7) Detach the participants from the experimental set-up and let them clean off the gel. Seat them behind the other table.

3.8) Administer “double-blind” an oral dose of placebo pill or 40 mg of propranolol HCl. Pills should be blinded by a pharmacy or colleague independent of the study. Further a physician on call with knowledge of the protocol is considered necessary after drug administration.

3.8.1) Administer “single-blind” an oral dose of 40 mg of propranolol HCl for the *propranolol no-reactivation* condition.

3.9) Insert a resting period of about 90 minutes. Offer the participants magazines to read. Clean the electrodes in the meantime.

3.10) Again obtain BP and heart rate measurements as well as salivary samples.

#### **4. Test - Day 3**

*Duration about 45 minutes.*

4.1) Obtain BP and heart rate measurements.

4.2) Position the participants in front of the computer screen and attach the EMG and shock electrodes. Again report to the participants that the intensity of shock will remain the same.

4.3) Instruct the participants that the same two pictures will be presented on the computer screen. Again instruct the participants to report the expectancy of an electric shock during the presentation of all pictures. Put on the headphones and dim the lights. Shut the door and start the experiment from the other room.

4.4) Start the experiment by presenting 10 baseline startle probes. During extinction present about 12 unreinforced CS1 and CS2 trials as well as 12 NA trials. After extinction participants get three unsignaled USs: reinstatement. Again present the participants with about 4 unreinforced CS1 and CS2 trials as well as 4 NA trials following the unsignaled USs: reinstatement testing.

4.5) Detach the electrodes and let participants clean off the gel. Pay the participants a small amount for their participation in the experiment. Clean the electrodes thoroughly with water.

## REPRESENTATIVE RESULTS:

**Manipulation check propranolol:** Contrary to pill placebo, both the systolic and diastolic BP as well as the salivary alpha amylase should decrease 90 min after the propranolol intake during memory reactivation, which indicates that the drug manipulation exerted its intended physiological effect. BP and salivary alpha amylase should again return to baseline levels at the test on day 3.

**US expectancy ratings:** With the current protocol you may not expect any effects of the propranolol treatment on the US expectancy ratings. Irrespective of the pharmacological treatment, the differential US expectancy ratings should increase during *fear acquisition* and decrease during *extinction learning* 48 hr later. Moreover the presentation of the unsignaled USs is expected to result in a return of the differential expectancy ratings (Fig. 1A-C).

**Prerequisite for reconsolidation:** In order to trigger the process of reconsolidation, the memory trace should firstly be destabilized as a necessary condition for the restabilization phase. For fear memory destabilization, an expectation of threat at the moment of reactivation (i.e., intact CS1-US expectancy) is necessary<sup>31</sup> though not sufficient. Pursuing on the idea that the function of reconsolidation is to update the memory trace to an ever-changing environment, the memory reactivation should also involve a prediction error (PE: a mismatch between what is expected and what actually happens). Given that the US expectancy ratings remain unaffected by the propranolol manipulation, changes in threat expectations from acquisition to test (PE) may serve as an independent indicator of memory destabilization (see <sup>32</sup> for more details). But only when the memory reactivation follows *asymptotic* learning, these changes in threat expectation may be observed. In case of partially reinforced *non-asymptotic* learning, a shift in threat expectancies is not necessary for memory destabilization. Hence a retrieval trial may also trigger reconsolidation without a change in expectancies after non-asymptotic learning.

**Startle fear responding:** Successful *fear acquisition* on day 1 is demonstrated by an increase in differential startle fear responding (i.e., CS1 vs. CS2) from the first to the last trial of acquisition (Fig. 1D-F). Higher startle fear responding to the stimulus that was previously coupled with the shock (CS1) compared to noise alone during *memory reactivation* on day 2 further indicates that the fear memory is well consolidated.

During the *test* phase on day 3 you may expect intact startle fear responding in the placebo group: startle responding to the CS1 should be higher compared to the CS2 at the beginning of test. Extinction training subsequently reduces the differential startle responding (i.e., CS1 vs. CS2). Moreover the presentation of the unsignaled USs should result in a return of fear responding to the CS1 compared to the CS2 (Fig. 1D).

Contrary to pill placebo, the administration of propranolol HCl is expected to result in an elimination of the differential startle fear responding (i.e., CS1 vs. CS2) at the beginning of the *test* phase on day 3. Furthermore you may expect that the unsignaled USs will not recover the startle fear responding (Fig. 1E).

Note that propranolol should not have any fear-reducing effects when the pill is administered without memory reactivation on day 2 (Fig. 1F).

#### FIGURE LEGENDS:

**Figure 1:** US expectancy ratings to the CS1 and CS2 trials and startle fear responses to the CS1, CS2 and noise alone (NA) trials during acquisition, memory reactivation, extinction and test for the placebo (A,D), propranolol (B,E), and propranolol no- reactivation (C,F) groups. R-CS1<sup>-</sup> refers to an unreinforced reactivation trial.

#### DISCUSSION:

In a series of studies we consistently demonstrate that - irrespective of gender - 40 mg of the noradrenergic beta-blocker propranolol either administered prior to or after memory reactivation effectively neutralized the conditioned fear responding (i.e., defensive startle response). None of the four mechanisms of relapse - reinstatement, renewal, spontaneous recovery and rapid reacquisition - were observed after disrupting reconsolidation with propranolol<sup>27-32,37-39</sup>. It is noteworthy that the fear-neutralizing effects were only observed for the defensive startle response, but neither for the threat expectancy rating nor for the skin conductance. The data for the electrodermal conditioning are not reported here as the general patterns for the SCR did not significantly deviate from the expectancy ratings. In human fear conditioning research, multiple indices of conditioned responding (e.g., US-expectancy, SCR, startle response, pupil dilation, neural activity) are usually obtained for reasons of cross-validation<sup>48</sup>. However, there is now convincing evidence that these different response levels do not necessarily act in concert and may even dissociate from each other<sup>3,27-32,34,37,49</sup>. Note that the startle response is an automatic defensive reflex, which is potentiated in response to a CS that is associated with a US of negative valence and can generally not be observed with USs of

neutral or positive valence<sup>49,50</sup> (e.g., vibrotactile stimulation or reaction time task). Hence, potentiation of the defensive startle reflex is a reliable and specific index of aversive conditioning<sup>34</sup>. In contrast, SCR conditioning can occur irrespective of the valence of the US<sup>49,50</sup>. Given that affective valence of the US does not modify SCR, electrodermal conditioning is a non-specific measure of anticipatory arousal. We therefore believe that the SCR is less suited as behavioral measure in human aversive conditioning research.

If we speculate on translating these findings into clinical practice, several issues and potential limitations are to be considered. First, it may be questioned whether a pharmacological manipulation by the noradrenergic beta-blocker is really necessary to disrupt the process of reconsolidation or whether a behavioral procedure aimed to interfere with reconsolidation would yield a similar neutralizing effect. Even though a one-session treatment of a low dose of propranolol is clearly nontoxic, an entirely behavioral procedure is always preferable over a pharmacological intervention. There is indeed an alternative method where extinction training is presented within the window of reconsolidation<sup>51</sup>. Several studies failed however to replicate these original findings by Schiller et al.<sup>29,52-54</sup> but see <sup>55,56</sup>. In addition to these conflicting results, another potential limitation of the extinction within reactivation procedure would be that in clinical practice the fear response is generally not extinguished in a one-session exposure treatment. For instance, imaginary exposure for patients with PTSD traditionally takes ten sessions before the fear subsides<sup>57</sup>. Thus, even acknowledging the obvious disadvantages of a simple pharmacological treatment in comparison to an entirely behavioral intervention, we believe that the noradrenergic manipulation of memory reconsolidation seems to be more feasible than extinction within the reconsolidation window.

A second issue concerns the optimal conditions to trigger memory reconsolidation. There is growing evidence that the mechanisms mediating the behavioral expression of fear are clearly dissociated from the mechanisms mediating the process of reconsolidation<sup>31,32,58-62</sup>. For instance, recent animal studies uncovered differential and dissociable receptors in the basolateral amygdala mediating the expression, destabilization and restabilization of previously conditioned fear memories<sup>63,64</sup>. A behavioral expression of fear memory is not only dissociated from processes mediating memory reactivation (i.e., access to a memory trace), it seems also not being indispensable for reconsolidation to occur<sup>63</sup>. As such, fear expression during memory reactivation is not informative on whether the memory trace enters a labile phase. Given that memory destabilization is a prerequisite for the noradrenergic beta-blocker to interfere with the restabilization process, an important question is how we can infer successful memory destabilization in clinical practice. A host of findings indicate that a crucial factor in inducing reconsolidation and in demarcating reconsolidation from either memory retrieval or the consolidation of a novel memory<sup>40,65</sup> is the degree of *prediction error* induced during memory retrieval<sup>31,32</sup>. But given that no objective criterion is available to determine the optimal degree of prediction error in clinical practice, the current laboratory findings cannot easily be translated to treatment protocols.

Another challenge for translating the neuroscience literature into clinical practice concerns the ecological validity of the paradigm. The evidence for disrupting reconsolidation has mainly been



shown in animals and humans for relatively new (one day old) and simple fear memories (i.e., tone → shock; picture → shock). It is not self-evident that disrupting reconsolidation of older, stronger and broader memory networks such as in patients with PTSD is as effective as it has been shown in the laboratory for cued fear conditioning. Also with respect to the dependent variable it is still unclear whether the observations from the animal and human laboratory studies generalize to patients with anxiety disorders. The fear reducing effects are thus far mainly demonstrated for the behavioral expression of aversive conditioning (i.e., freezing behavior in rodents or defensive startle reflex in humans), with only one exception where we demonstrated that also the subjective feelings of distress were significantly neutralized by noradrenergic blockade of memory reconsolidation<sup>37</sup>. It may be questioned whether these fear-reducing effects in the laboratory are indicative of the typical experiences of fear and avoidance behavior characteristic of patients with anxiety disorders. Future research should investigate whether the current findings indeed generalize to avoidance behaviors, one of the central symptoms of anxiety disorders.

In sum, although the Pavlovian aversive conditioning procedure is an excellent tool to study the basic mechanisms of fear learning and memory, we cannot easily translate the laboratory findings into clinical practice. The insights that we have been acquiring on the optimal, boundary and necessary conditions for memory reconsolidation should only be considered as a starting point for the development of reconsolidation-based treatments. On the other hand, the extensive research on extinction training has resulted in extinction-based exposure interventions, which still belong to the most effective treatments for anxiety disorders and other related disorders. Given that the noradrenergic blockade of memory reconsolidation overshadows the anxiolytic effect of extinction learning, disrupting reconsolidation points to a promising new intervention to effectively reduce excessive and irrational fears.

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#### **DISCLOSURES:**

Authors have nothing to disclose.

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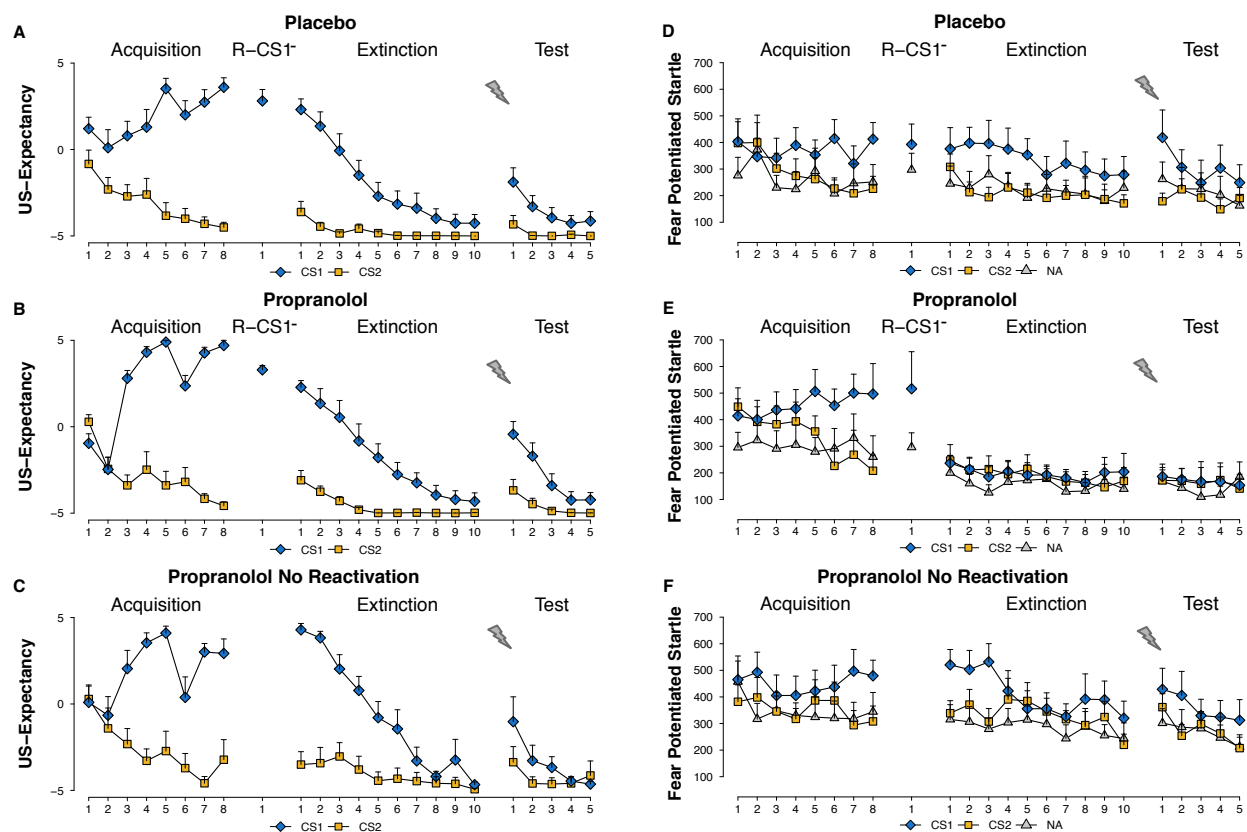
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Figure

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Name of Material - Equipment	Company	Catalog Number
2 computers with 4 screens	Dell	Optiplex 9010
Amplifier	Developed by B. Molenkamp - University of Amsterdam	
VSSRP98	Developed by B. Molenkamp - University of Amsterdam	
MATLAB	MathWorks	
Presentation	NeuroBehavioral Systems Inc. - USA	
Constant current stimulator	Digitimer - UK	DS7A
Shock electrodes	Made by B. Molenkamp - University of Amsterdam	
Headphones	Sennheiser Electronic GmbH & CO - Germany	HD 25-1 II
EMG electrodes	Made by B. Molenkamp - University of Amsterdam	
Double-sided adhesive collars	MedCaT - the Netherlands	848125
Conductive gel	Signa Gel - Parker Laboratories Inc. - USA	224.550.011
Alcohol swabs	Sanadep 0.5 % - Microtek Medical - the Netherlands	3053800
Sphygmomanometer	Omron Healthcare Europe B.V. - the Netherlands	M4-I HEM-752-E
Cotton salivettes	Sarstedt - Germany	511.534
Curved tip syringe	Monoject - Covidien - USA	412
Propranolol HCl - 40 mg and placebo pills	Huygens Apotheek - the Netherlands	

Comments - Description
Recording and monitoring physiological responses. Presenting the experimental script.
Designed around a Burr Brown INA101 amplifier and ISO103 isolation stage.
Physiological registration software. Record electromyography - EMG - activity using a bundled pair of electrodes wires connected to a front-end amplifier with an input resistance of 10 MΩ and a bandwidth of DC-1500 Hz. Raw EMG signals are integrated in the amplifier. Integrated EMG signals are sampled at 1000 Hz and used for data analysis.
Analyzing data. Peak amplitudes are determined by taking the baseline 50 ms before probe onset to peak differences within 30 - 150 ms following probe onset and are recorded in microvolt.
Stimulus presentation.
Generates electrical stimulation.
Ag electrodes of 20 mm * 25 mm with fixed inter-electrodes mid-distance of 45 mm.
Presentation of startle probe and background noises.
Three 7 mm sintered Ag-AgCl electrodes.
13-mm x 5-mm. For attaching the EMG electrodes to the skin.
Facilitates conduction from the skin to both the EMG and shock electrodes.
For cleaning the skin of the participant.
Measuring blood pressures and heart rate.
Obtaining salivary samples.
Applies gel to EMG and shock electrodes.
Pills should be identical in exterior.





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Author(s): Merel Kindt, Marieke Soeter, Dieuwke Sevenster

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**Editorial comments:**

1. We have changed steps 3.6.1 and 3.6.2 into steps 2.6.1 and 2.6.2.
2. We have now un-highlighted the following steps: 2.7 \_ 2.8 \_ 2.9 \_ 2.15 \_ 3.4 \_ 4.5 \_ 4.2 \_ 4.3.
3. Journal names are now italicized throughout. Moreover we removed the extraneous comma from ref\_#3.
4. We clarified step 3.8.1. by adding “for the propranolol no-reactivation condition”. Also we now explain in the introduction section on page 3 what the propranolol no-reactivation condition is for.
5. We replaced “..clinical applications are still in its infancy..” by “..clinical applications are still in their infancy..”.
6. We have thoroughly proofread are manuscript to ensure that there are no spelling or grammar issues.
7. We do not use figures from previous publications.

**Reviewer #1:**

1. Following the suggestion of the reviewer we changed the term “erasure” for a less absolute term.
2. We have now replaced “..re-exposed to the unreinforced conditioned stimulus..” with “..re-exposed to the conditioned stimulus without the US..”.
3. We now explain in the long abstract that propranolol HCl is administered orally.
4. Following the suggestion of the reviewer we now stated in the long abstract that “..propranolol indirectly targets the protein synthesis required for reconsolidation by inhibiting the noradrenaline-stimulated CREB phosphorylation”.

5. Indeed we should also recognize that anisomycin may interfere with the release of neurotransmitters thereby causing the amnesic effect. We now clarified this on page 3.
6. We are not sure whether we understand what the reviewer means by an instructed prediction error. But if the reviewer means that we could tell the participants what exactly happens during reactivation [i.e., that the CS will no longer be followed by a US] reconsolidation will not be triggered [see Sevenster et al. 2012].
7. We now clarified in the “ethics statement” that a physician approves our protocol: this may help convincing the ethics commission to use electric shocks as well as propranolol HCl.
8. Shock intensities are individually set as different skin types may affect how the shock is experienced. Participants judge the pleasantness of the shock on 11-point rating scales - ranging from *minus* 5 [i.e., unpleasant] to 5 [i.e., pleasant] - at the end of acquisition: this is now clarified in step 2.15. As long as the groups do not differ on this evaluation of the US we feel that this method is valid.
9. Solely because a physician is not present during our conditioning studies the use of an anti-depressant drug - and not depression per se - is an exclusion criterion: we have now changed this accordingly in step 2.2. Hence this exclusion criterion will not be a determining factor in the application of this method.
10. Following the suggestion of the reviewer we now comment on the loud noises at the end of the introduction section on page 3.
11. We now explain in step 2.8 that the mouse is used to position the cursor and that the participants have to press the left button in order to confirm their expectancy.
12. Our “single-blind” option is for the propranolol no-reactivation condition. We now clarified this in step 3.8.1. Also we now explain at the end of the introduction section on page 3 what this condition is for.

13. A physician on call with knowledge of the protocol is indeed considered necessary. We now clarified this in step 3.8.
14. Our first two studies [i.e., Kindt et al. 2009 Soeter and Kindt 2010] indeed involved a group of participants that received the drug without prior retrieval to control for any non-specific effects [i.e., propranolol no-reactivation condition]. Already we included this in the protocol but in an unclear fashion. At the end of the introduction section on page 3 we now explain why there should be such a condition. Further we clarified in step 3.8.1 that the “single blind” option is meant for this control condition.
15. We now explain at the end of the introduction section on page 3 that test phases should be separated by at least 24 hr in order to (1) support the (re)consolidation of the memories and (2) allow the drug to wash out before testing.
16. Gender differences are not to be expected. Following the suggestion of the reviewer we now suggest this at the beginning of the discussion section on page 10.
17. We have replaced “form” with “from”.

**Reviewer #3:**

1. Following the suggestion of the reviewer we now refer to Mineka and Zinbarg 2006.
2. Ample evidence indicates that startle potentiation reflects a specific and reliable index of fear whereas the SCR used by Schiller et al. is much less specific. While startle responding is potentiated in reaction to a CS that is associated with an US of negative valence [e.g., electrical stimulation] it can typically not be observed with USs of neutral valence [e.g., vibrotactile stimulation or reaction time-task] [e.g., Lipp et al. 1994 Hamm and Vaitl 1996]. Contrary the affective valence of the US does not modify SCR conditioning as it can occur irrespective of the valence of the US [e.g., Lipp et al. 1994 Hamm and Vaitl 1996]. As such the SCR is considered a non-specific

measurement of anticipatory arousal and we do not follow the suggestion of the reviewer.

3. A series of studies demonstrated that extinguished fear responding returned after unpredictable shocks or a context change or the passage of time while fear did not return after disrupting reconsolidation [e.g., Kindt et al. 2009 Soeter and Kindt 2010 2011 Sevenster et al. 2012 2013]. Based on those observations we feel that it is justified to say “support the superiority of disrupting reconsolidation over extinction learning”.
4. Written informed consent is obtained from all participants before the beginning of the experiment. We now clarified this in step 2.1.
5. See our answer to comment 2.
6. Also following the suggestion of the reviewer we now refer to Agren et al. 2012 and Oyarzún et al. 2012.
7. Only when fear was fully extinguished during the reconsolidation window Schiller et al. found an effect on subsequent fear responding. Yet in clinical practice fear responding is often not extinguished within one-session and thus forms a potential limitation for this method. As a result we consider our statement “..would be that in clinical practice the fear...one-session exposure-treatment..” correct.
8. Strength of fear memory is not necessarily a boundary condition for reconsolidation. For instance some older and stronger memories have been observed not to undergo reconsolidation [Suzuki et al. 2004] leading several researchers to conclude that it reflects a lingering consolidation process that may eventually terminate [Alberini 2011]. However others have noted that the parameter-space for reactivation may be changed under these reconsolidation-resistant conditions as longer [Suzuki et al. 2004] or delayed [Wang et al. 2009] reactivation may still engage reconsolidation. However it is not self-evident that disrupting reconsolidation of older or stronger and broader memory networks such as in patients with PTSD is as effective as it has been shown in the laboratory for cued fear conditioning - see page 11.