Postretrieval relearning strengthens hippocampal memories via destabilization and reconsolidation

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1 **Post-retrieval re-learning strengthens hippocampal memories via destabilization**

2 **and reconsolidation**

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Abstract

Memory reconsolidation is hypothesised to be a mechanism by which memories can be updated with new information. Such updating has previously been shown to weaken memory expression or change the nature of the memory. Here we demonstrate that retrieval-induced memory destabilization also allows that memory to be strengthened by additional learning. We show that for rodent contextual fear memories, this retrieval-conditioning effect is observed only when conditioning occurs within a specific temporal window opened by retrieval. Moreover, it necessitates hippocampal protein degradation at the proteasome and engages hippocampal Zif268 protein expression, both of which are established mechanisms of memory destabilization-reconsolidation. We also demonstrate a conceptually analogous pattern of results in human visual paired-associate learning. Retrieval-relearning strengthens memory performance, again only when relearning occurs within the temporal window of memory reconsolidation. These findings link retrieval-mediated learning in humans to the reconsolidation literature, and have potential implications both for the understanding of endogenous memory gains and strategies to boost weakly-learned memories.

Significance Statement

Memory reconsolidation allows existing memories to be updated with new information. Previous research has demonstrated that reconsolidation can be manipulated pharmacologically and behaviorally to impair problematic memories. In this paper, we show that reconsolidation can also be exploited to strengthen memory. This is shown both in rats, in a fear memory setting, and in a human declarative memory setting. For both, the behavioral conditions necessary to observe the memory strengthening match those that are required to trigger memory reconsolidation. There are several behavioral approaches that have previously been shown convincingly to strengthen memory. The present

45   demonstration that reconsolidation can underpin long-lasting memory improvements may both provide

46   an underlying mechanism for such approaches and provide new strategies to boost memories.

47

Introduction

Once acquired, memories are subject to modification. One mechanism by which this can be achieved involves the phenomenon of memory reconsolidation (Lee, 2009; Nader and Hardt, 2009; Lee et al., 2017). In reconsolidation, a memory is first destabilized (Ben Mamou et al., 2006). Following destabilization, the memory is restabilized, or reconsolidated, during which process the memory can be strengthened pharmacologically (Tronson et al., 2006) and new, updating information may be integrated (Lee, 2008, 2010; Inda et al., 2011; De Oliveira Alvares et al., 2013; Olshavsky et al., 2013).

The capacity of reconsolidation to update memories has been exploited behaviorally to weaken fear memory expression by combining memory retrieval with subsequent extinction training in a retrieval-extinction procedure. This was demonstrated initially in a tone fear setting dependent upon amygdala plasticity (Monfils et al., 2009), and subsequently shown to apply also to contextual fear memories (Flavell et al., 2011; Rao-Ruiz et al., 2011). These latter studies demonstrated that the retrieval-extinction phenomenon depended upon hippocampal L-type voltage-gated calcium channels (Flavell et al., 2011), which are known to be required for memory destabilization (Suzuki et al., 2008).

We hypothesised, based upon the apparent function of reconsolidation to update memories and the success of exploiting this to weaken memory expression, that reconsolidation might be similarly harnessed also to strengthen hippocampal memory expression. While simple additional learning in isolation certainly does strengthen memories (e.g. Lee, 2008), retrieval that induces destabilization can also be an effective method of increasing fear memory expression (Inda et al., 2011; De Oliveira Alvares et al., 2013). However, while both of these contextual fear memory-strengthening effects have been shown previously to involve hippocampal destabilization-reconsolidation (Lee, 2008; De Oliveira

71   Alvares et al., 2013), previous contextual fear memory studies have not attempted to combine

72   destabilization-inducing retrieval with additional relearning. Based upon the hypothesized conceptual

73   similarity between retrieval-extinction and the proposed retrieval-relearning, we would predict that any

74   memory-strengthening effect should be subject to the same temporal "reconsolidation window" of

75   effect, which includes 10-60-min intervals, but not a 6-hr interval, between retrieval and extinction

76   (Monfils et al., 2009).

77

78   Interestingly, studies of human associative memory have traditionally focused on the beneficial,

79   memory-enhancing effects of retrieval, rather than the destabilizing or updating effects. It is a well-

80   established observation in the cognitive psychology literature that memory testing (i.e., retrieval) is at

81   least as effective in supporting subsequent performance as is additional learning (Roediger and

82   Karpicke, 2006), and much more effective than additional learning when performance is assessed at long

83   delays, especially when combined with immediate feedback. In fact, it has recently been argued that

84   retrieval can act as a fast consolidating event for newly acquired memories (Antony et al., 2017). While

85   some empirical studies have confirmed that memory retrieval which likely induces destabilization can

86   itself strengthen memory (Forcato et al., 2011), it has not previously been shown that retrieval, via

87   destabilization and reconsolidation, opens a temporally-limited window of opportunity for a memory to

88   be strengthened by additional experience. We here test explicitly such a hypothesis using contextual fear

89   conditioning in rats, in which the cellular mechanisms of destabilization and reconsolidation are well

90   delineated, and associative learning in humans.

91

92   For the present series of experiments, we predicted that the combination of a single destabilization-

93   inducing memory retrieval with a single additional relearning session shortly thereafter would confer

94 greatest memory enhancement when arranged in a manner to engage reconsolidation (i.e. relearning

95 occurring after, rather than before, retrieval and within the reconsolidation window). Moreover, we

96 predicted that this retrieval-relearning double experience would exceed any memory gains afforded by

97 retrieval practice alone and would both rely upon memory destabilization and recruit cellular

98 mechanisms of reconsolidation. Recent evidence using inhibitory avoidance memories supports the

99 behavioral prediction (Du et al., 2017), but does not show a conclusive dependence upon destabilization

100 and reconsolidation. Therefore, using near-threshold parameters of conditioning (in order to avoid

101 ceiling effects), we exposed rats to subsequent retrieval and relearning within an uninterrupted session

102 or with varying inter-trial intervals. We also employed a reverse order condition (i.e. relearning followed

103 by retrieval) as a comparative approach to strengthen memories. Following confirmation that the

104 combination of retrieval and relearning strengthened hippocampal contextual fear memories in a

105 reconsolidation-dependent way, we applied the same strategy to weakly-learned human episodic paired-

106 associate memories, which are similarly dependent upon the hippocampus (Eichenbaum, 2000; Konkel

107 et al., 2008).

108

109

Materials and Methods

Experimental Design and Statistical Analysis

Rodent sample size was determined by power analyses assuming the effect size would be equivalent that that observed in memory disruption studies. Sample size for the human studies was arbitrarily set a level 50% greater than that used in previous human memory reconsolidation studies (Hupbach et al., 2007). Given the aim of showing memory strengthening, rats that showed >50% freezing after learning were excluded; pilot studies showed that the mean freezing after learning was 27.7%, and ¼ of rats increased % freezing levels by >50 from learning to test. The principles for exclusion criteria in the human study were that initial learning performance should not preclude detection of a population mean strengthening effect; specific details are included in the statistical analysis section. No outliers were excluded from the analyses (all data fell within 2 sd of the mean). Reported endpoints and statistical analytical approach were determined prospectively.

The original objectives of the research were to demonstrate whether relearning within the reconsolidation window strengthens contextual fear memory (Fig 1A), and whether this depends upon mechanisms of destabilization and reconsolidation. Following the outcomes of these experiments, the further objective of the research was to show analogous results in human paired associate memory. Research subjects and experimental design are described below. Subjects were randomly allocated to experimental group within each cohort of subjects, using a random sequence generator. Experimenters were not strictly blinded to allocation during the conduct of the experiments, but all data processing and analysis was conducted blind to the intervention.

Statistical analyses were conducted in JASP (JASP Team, 2016). Contextual freezing was analysed using mixed 2-way ANOVA across both test sessions, with separate one-way ANOVA analysis of freezing during retrieval/reconditioning (either the full retrieval session or the pre-shock period of the re-

conditioning session). Due to the groupings of cohorts, and a substantial time interval between cohorts, the data are analysed primarily within cohort, starting with core comparisons, followed by the wider analysis including additional groups. Raw uncorrected p values are presented, but all analyses survive Bonferroni correction for repeated analyses within each cohort. Within the wider analysis, Tukey-corrected post-hoc pairwise comparisons were used to explore group differences. We also conducted an exploratory comparison across cohorts, focussing on the effect of delay between retrieval and conditioning. η^2_p was used as an estimate of effect size, and $BF_{10}/BF_{Inclusion}$ is also reported as the outcome of Bayesian analyses for the estimation of posterior probability. Western blot and flow cytometry analyses were conducted using one-way ANOVAs, with Bonferroni-corrected post-hoc pairwise comparisons. For the human episodic memory task, a memory improvement score was calculated by the simple numerical difference between the number of correct object associates reported at the final test and the number reported immediately after learning on the first day of training. Data for participants scoring >32/40 in the immediate test on the first day of training were excluded to avoid individual ceiling effects, with the criterion determined by the average improvement score of 7.4 in the core experimental group without exclusions. These improvement scores were compared across groups using a series of one-way ANOVAs, each with Tukey-corrected post-hoc pairwise comparisons.

Subjects

121 experimentally-naïve adult male Lister Hooded rats (Charles River, UK) weighed either 200-225 g (for non-surgical experiments) or 275-300 g (for cannulated rats) at the start of the experiment. Rats were housed in quads (save for a 24 h recovery period following surgical procedures) under a 12 h light cycle (lights on at 0700) in a specialist animal facility. Individually-ventilated cages contained aspen chip bedding and a plexiglass tunnel for environmental enrichment. Rats had free access to food and

156 water other than during behavioral sessions. Experiments took place between 0900 and 1600 in a

157 behavioral laboratory. At the end of the experiment, animals were humanely killed using a rising

158 concentration of CO_2 to render the animal unconscious, followed by dislocation of the neck and

159 extraction of the brain if required. All procedures were approved by the local animal welfare and ethical

160 review board and carried out in accordance with the United Kingdom 1986 Animals (Scientific

161 Procedures) Act, Amendment Regulations 2012 (PPL P8B15DC34).

162 171 undergraduate students from the University of Birmingham participated in the study. All

163 participants were recruited through the Psychology Research Participation Scheme and received course

164 credit for their participation. Participants gave their informed consent, and all procedures were approved

165 by the University of Birmingham Science, Technology, Engineering and Mathematics (STEM) Ethics

166 Review Committee.

167

168 *Surgical procedures*

169 29 rats were implanted with chronic indwelling stainless steel cannulae (Coopers Needleworks, UK)

170 according to our established procedures (see Exton-McGuinness and Lee, 2015, for full details). The

171 cannulae targeted the dorsal hippocampus (Lee and Hynds, 2013). At the end of the experiment,

172 extracted brains were drop-perfused in 4% paraformaldehyde for 7 days and then processed for

173 histological assessment of cannula placements by Nissl staining.

174

175 *Rodent Behavioral procedures*

176 All behavioral procedures were carried out in conditioning chambers (MedAssociates, VT) as previously

177 described (Lee and Hynds, 2013), with freezing behavior automatically recorded by Videotracking

178   software (Viewpoint Life Sciences, France). Rats were randomly allocated to experimental group within

179   each experiment.

180   All rats (whether cannulated or not) received the same behavioral training. Conditioning consisted of a

181   single 3-min session, without any prior exposure to the context, in which rats were exposed to a single

182   0.35-mA footshock for 2 s after 2 min. This near-threshold footshock intensity generated appreciable

183   conditioning, in the form of later contextual freezing, in only a subset of rats, and so allowed for the

184   observation of memory strengthening. On the next day, the experimental retrieval-relearning groups

185   received a non-reinforced retrieval session (2 min re-exposure to the conditioning context), followed at

186   varying times later by a re-conditioning session (Fig 1A). Memory strengthening, assessed at tests on

187   days 4 & 11, was compared against a group that had no interval between the retrieval and relearning

188   (retrieval-0min-relearning; operationally, this consisted of a single conditioning session with footshock

189   delivered after 4 min that acted also as a relearning-only control), given that an interval is necessary to

190   engage the behavioral modification of a destabilized memory (Monfils et al., 2009). Additional control

191   groups included a double retrieval (retrieval-retrieval) group that received two retrieval sessions

192   separated by the same 15 min interval, both to control for the double experience and act as a retrieval-

193   only comparison, and the reversal of the order of presentation of the retrieval and reconditioning

194   sessions (relearning-retrieval). A final control consisted of two spaced reconditioning sessions

195   (relearning-relearning) that was expected to increase freezing maximally. During all intervals, rats were

196   returned to their homecage in the holding room. Contextual freezing was subsequently assessed in 2-min

197   test sessions 2 and 9 days later.

198   Cannulated rats were habituated to a dummy infusion procedure (with the injectors loaded with

199   phosphate-buffered saline, but no infusion taking place) on the day of conditioning. They were then

200   infused (1 μl/side) with clasto-lactacystin-β-lactone (β-lac; 32 ng/μl) or its vehicle (2% DMSO in 1 M

201 HCl diluted in PBS and adjusted to pH 7.0–7.4 with NaOH) (Lee, 2010) immediately prior to either the

202 retrieval session or the relearning session within the retrieval-1hr-relearning condition on day 2.

203

204 *Biochemical procedures*

205 36 rats were conditioned on day 1. On day 2, there were 5 conditions: (i) no behavioural session [non-

206 reactivated]; (ii) retrieval only; (iii) retrieval-1hr-relearning; (iv) relearning only; (v) relearning-1hr-

207 retrieval. The rats were killed 2 hr after the initial behavioural session on day 2 and their brains rapidly

208 extracted for assessment of Zif268 protein levels. The dorsal hippocampus was dissected and frozen on

209 dry ice. For flow cytometry, the tissue was subjected to a standard nuclear extraction protocol and the

210 nuclear fraction was re-suspended in 10% normal donkey serum. 5 of these samples were unable to be

211 processed by flow cytometry. Flow cytometry was conducted largely based upon established procedures

212 (Li et al., 2014). Samples were then incubated with rabbit anti-Zif268 (Santa Cruz Biotechnology, sc-

213 110, 1:500) and mouse anti-NeuN (Millipore, MAB377, 1:1000) primary antibodies, followed by

214 secondary antibodies (donkey anti-mouse IgG PE, Santa Cruz Biotechnology, sc-3744, 1:100; donkey

215 anti-rabbit IgG A488, Abcam, AB150073, 1:1000) and DAPI (Cell Signalling, 0.5 μg), and then run

216 through a flow cytometer. All gates were set at a fixed position across samples in order to include the

217 most fluorescent group of cells. The DAPI+ gate was used as the stopping gate (10 000 events), so that a

218 set number of events were counted for each sample, allowing a more standardized comparison. Zif268+

219 cells were considered to be those that were simultaneously DAPI+, NeuN+ and Zif268+ and the

220 percentage of Zif268+ labelling each sample was calculated based on a total cell count of 10 000.

221 Western blot procedures were conducted largely as previously described (Lee and Hynds, 2013). Blots

222 were incubated first with rabbit anti-EGR1 (Cell Signalling, #4154, 1:1000 in 5% non-fat milk overnight

223 at 4°C), and then with goat anti-rabbit HRP-linked secondary antibody (Cell Signalling, #7074, 1:2000

224   in 5% non-fat milk for 60 min at RT). After enhanced chemiluminescence visualization (C-Digit, Li-

225   Cor), the HRP activity of the goat anti-rabbit secondary antibody was irreversibly quenched with 30%

226   H_2O_2 for 15 min at 37 ^{o}C (Sennepin et al., 2009). The blot was then incubated with the mouse anti-actin

227   loading control (Abcam, ab6276, 1:20000 in TBST overnight at RT), goat anti-mouse HRP-linked

228   secondary antibody (Sigma-Aldrich, A4416, 1:10000 in TBST at RT) and re-visualised with enhanced

229   chemiluminescence. The Zif268 signal-background was normalized against actin expression ([raw

230   Zif268 signal]*[mean actin signal]/[sample actin signal]) and then this figure was normalized against the

231   mean of the non-reactivated control group to generate a % control value.

232

233   *Human behavioral procedures*

234   All behavioral procedures were conducted using a visual paired-association task, run in PsychoPy

235   (Peirce, 2007) on a desktop computer in a testing cubicle. The visual images were 40 object and 40

236   scene images, randomly selected from object and scene stimulus banks (Brady et al., 2008; Konkle et

237   al., 2010). Each object stimulus was randomly associated with a scene image (with the associations

238   determined uniquely for each participant). The object image was presented directly above the scene

239   image for 4 s. During learning, the 40 paired associates were sequentially presented on a single occasion

240   each. Immediate retention of the single-trial learning was tested by presentation of the scene image alone

241   for 6 s, with the participant prompted to recall verbally the associated object image. The experimenter

242   manually recorded the response, which was subsequently coded as correct/incorrect. No feedback was

243   given.

244   48 hours after learning, the participants returned to the same testing cubicle, with the same experimenter.

245   In the experimental retrieval-10min-relearning group, participants were first presented with the scene

246   images alone (as in the immediate test after learning), and were requested to remember, but not verbalise

247 the associated object image. After a 10-min mathematical distraction task, they were then given a second

248 learning session, which was identical in nature to initial learning (but with a randomised order of paired-

249 associate presentation). Control groups (7 in total) were conducted in 3 sequential experimental cohorts,

250 with random allocation of participants to the groups within these cohorts:

251 1.   Reversal of the order of retrieval and relearning (relearning-10min-retrieval); presentation of

252 retrieval or relearning alone (followed by the distractor task); no memory experience (control group;

253 these participants simply completed the Big 5 personality test (John and Srivastava, 1999), followed by

254 the distractor task).

255 2.   Double presentation of either the retrieval (retrieval-10min-retrieval) or relearning (relearning-

256 10min-relearning) sessions, with the same distractor task between the two presentations.

257 3.   Delayed interval between relearning and retrieval, such that the second experience occurred

258 outside the putative reconsolidation window (retrieval-6hr-relearning & relearning-6hr-retrieval). The

259 distractor task was completed immediately after the first experience.

260 Another 48 hours later, all participants were tested on their paired-associate recall in an identical manner

261 to the immediate test after learning.

262

263

Results

Strengthening of contextual fear conditioning in rats

We studied the impact of a various intervals between retrieval and relearning of rodent contextual fear (Fig. 1A) as previous studies had demonstrated that intervals of 10 min and 1 hr between retrieval and extinction, but not 0 min or 6 hr, successfully and persistently diminished fear expression (Monfils et al., 2009). These conditions were split across different cohorts and so each cohort was analyzed independently, followed by an exploratory consolidated analysis of all groups. Memory strengthening was assessed at tests on days 4 & 11. Analysis of contextual freezing at these tests revealed that the retrieval-15min-relearning group displayed higher freezing compared to the unspaced retrieval-0min-conditioning control (Fig 1B). A significant main effect of group was observed ($F(1,15)=17.1$, $p<0.001$, $\eta^2_p =0.53$, $BF_{Inclusion}=16.4$), with no effect of session or group x session interaction ($F's<1.5$, $p's>0.24$, $BF_{Inclusion}<0.64$). The pattern of results at test were not due to differences in initial conditioning, as freezing on day 2 prior to footshock delivery was equivalent across groups (R-0min-C = 14.8 ± 10.4, R-15min-C = 13.1 ± 9.7; $F(1,15)=0.13$, $p=0.72$, $\eta^2_p=0.009$, $BF10=0.44$). Therefore, spacing of retrieval and conditioning resulted in greater memory strengthening. Moreover, the retrieval-1hr-conditioning group froze at higher levels than the retrieval-6hr-conditioning group (Fig 1C). A significant main effect of group was observed ($F(1,14)=9.5$, $p=0.008$, $\eta^2_p =0.41$, $BF_{Inclusion}=29.8$), with no effect of session or group x session interaction ($F's<0.98$, $p's>0.22$, $BF_{Inclusion}<0.46$). The pattern of results at test were again not due to differences in initial conditioning, as freezing on day 2 prior to footshock delivery was equivalent across groups (R-1hr-C = 18.7 ± 12.5, R-6hr-C = 18.0 ± 13.6; $F(1,14)=0.012$, $p=0.92$, $\eta^2_p =0.001$, $BF_{10}=0.43$). The exploratory analysis across all delays confirmed that greater strengthening was observed with delays of 15 min and 1 hr ($F(3,29)=9.2$, $p<0.001$, $\eta^2_p =0.49$, $BF_{Inclusion}=108$). Frequentist post-hoc comparisons ($p<0.05$) confirmed that the 0-min and 6-hr delay groups did not differ from each

other, and nor did the 15-min and 1-hr delay groups. While the 1-hr delay froze at higher levels than 0-min and 6-hr, the 15-min delay group was not significantly higher than the 6-hr group. Bayesian post-hoc tests largely supported this pattern, although there was some evidence for a difference between the 15-min and 6-hr groups (BF_{10}=4.1). So far, this pattern of results confirms that retrieval paired with reconditioning produces more substantial benefits on long-term retention when the reconditioning occurs within a critical time window opened by the preceding retrieval, and that this time window is consistent with a reconsolidation-based process.

Contextual fear strengthening is blocked by disrupting memory destabilization

If the retrieval-relearning enhancement of fear memory is mediated by a destabilization-reconsolidation process, prevention of memory destabilization should block the increase in freezing. This is a strategy that has previously been employed to conclude a role of reconsolidation in memory modification (Lee, 2008, 2010; De Oliveira Alvares et al., 2013). Given that hippocampal protein degradation at the proteasome is essential for the destabilization of contextual fear memories (Lee et al., 2008), we infused the proteasome inhibitor β-lac into the dorsal hippocampus immediately prior to memory retrieval within the retrieval-1hr-relearning condition that appeared to provide the most robust strengthening (Fig. 1D). As a control for any direct effect of β-lac upon the subsequent conditioning session, β-lac was infused in a separate group after retrieval and immediately prior to relearning. Analysis of contextual freezing at the tests revealed that the pre-retrieval β-lac group froze at lower levels than the vehicle and pre-conditioning β-lac groups (Fig 1E). A significant main effect of group was observed ($F(2,18)$=13.7, $p<0.001$, η^2_p =0.60, $BF_{Inclusion}$=173), with a significant effect of session ($F(1,18)$=13.7, $p=0.001$, η^2_p =0.44, $BF_{Inclusion}$=17.0), but less evidence for a group x session interaction ($F(2,18)$=3.11, $p=0.069$, η^2_p =0.26, $BF_{Inclusion}$=4.5). Post-hoc comparisons of the main effect of group confirmed that the pre-retrieval

β-lac group froze at a lower level than each of the other two groups ($p<0.002$, Cohen's $d>0.95$,

$BF_{10}>885$), which did not differ from each other. Given the trend towards an interaction, analysis of

simple main effects confirmed significant group differences at both tests on day 4 ($F(2,18)=15.9$,

$p<0.001$, $\eta^2_p=0.64$, $BF_{10}=215$) and day 11 ($F(2,18)=8.2$, $p=0.003$, $\eta^2_p=0.48$, $BF_{10}=14.5$), with post-hoc

comparisons revealing lower freezing in the pre-retrieval β-lac group compared to each of the other two

groups ($p<0.03$, Cohen's $d>0.63$, $BF_{10}>3.6$). Therefore, the persistent increase in freezing following

retrieval-conditioning was blocked specifically by pre-retrieval intra-hippocampal infusion of β-lac.

Contextual fear strengthening recruits Zif268 expression

This interpretation that retrieval-conditioning engages destabilization-reconsolidation to strengthen

memory expression was further explored by analysis of hippocampal Zif268 protein levels by both

western blots and flow cytometry in separate samples. Rats were initially conditioned and then subjected

to the retrieval-1hr-relearning procedure, with brains being taken 1 hr later (Fig 1F). The retrieval-

conditioning group was compared to a non-reactivation control (no behavioural session) as well as a

group that received only the retrieval session in order to determine the contribution of the initial

behavioral experience to the engagement of zif268 expression. The western blot analyses showed

evidence that retrieval-conditioning increased Zif268 expression compared to non-reactivation, with the

retrieval-only group having intermediate and non-significantly different levels of Zif268 (Fig 1G:

$F(2,8)=8.5$, $p=0.010$, $\eta^2_p=0.68$, $BF_{10}=5.3$; post-hoc $p=0.008$, $BF_{10}=8.8$ for the non-reactivation vs

retrieval-conditioning comparison). Analysis by flow cytometry revealed further evidence for an

upregulation of Zif268 expression by retrieval-conditioning (Fig 1H-I: $F(2,9)=6.8$, $p=0.023$, $\eta^2_p=0.66$,

$BF_{10}=3.5$; post-hoc $p=0.023$, $BF_{10}=3.7$ for the non-reactivation vs retrieval-conditioning comparison).

332 Therefore, the increased memory expression at test in the retrieval-conditioning groups is highly likely

333 due to a reconsolidation-mediated updating process.

334

335 *Contextual fear strengthening depends upon the nature and order of retrieval and conditioning*

336 The retrieval-conditioning groups were compared against additional groups to investigate whether the

337 nature of the sessions (i.e. retrieval vs conditioning) and the order of presentation (i.e. retrieval prior to

338 conditioning) is important for the strengthening effect. For the 15-min interval, comparison groups

339 included retrieval-retrieval and conditioning-retrieval groups (Fig 2A). A significant main effect of

340 group was observed ($F(2,21)=10.23$, $p<0.001$, $\eta^2_p =0.49$, $BF_{Inclusion}=30.8$), with no effect of session or

341 group x session interaction ($F's<2.7$, $p's>0.11$, $BF_{Inclusion}<1.8$). Post-hoc comparisons ($p<0.05$, Cohen's

342 $d>0.62$, $BF_{10}>25.9$) confirmed that the retrieval-retrieval group froze at lower levels than both retrieval-

343 conditioning and conditioning-retrieval. Therefore, spacing of retrieval and conditioning resulted in

344 greater memory strengthening that could not be attributed simply to the spaced retrieval opportunity.

345 There was no difference, however, between the retrieval-conditioning and conditioning-retrieval groups

346 ($BF_{10}=0.62$), suggesting that the order of presentation of retrieval and conditioning might not be

347 important for memory strengthening, at least for the 15-min interval.

348

349 For the 1-hr interval, we again included a conditioning-retrieval comparison, as well as a conditioning-

350 conditioning group (Fig 2B). A significant main effect of group was observed ($F(2,20)=7.3$, $p=0.004$,

351 $\eta^2_p =0.42$, $BF_{Inclusion}=9.4$), with no effect of session or group x session interaction ($F's<1.9$, $p's>0.19$,

352 $BF_{Inclusion}<0.64$). Post-hoc comparisons ($p<0.05$, Cohen's $d>0.57$, $BF_{10}'s>154$) confirmed that the

353 retrieval-conditioning and conditioning-conditioning groups differed from the conditioning-retrieval

354 group, but did not differ from each other ($BF_{10}=0.35$). Therefore, with the 1-hr interval, retrieval-

355 conditioning strengthened contextual fear memory to a similar degree as 2 spaced conditioning sessions.

356 However, retrieval after conditioning failed to strengthen memory.

357

358 Given the apparently qualitatively different effect of conditioning-1hr-retrieval compared to retrieval-

359 1hr-conditioning, we analysed Zif268 expression following conditioning-1hr-retrieval or conditioning

360 alone, comparing to the same non-reactivation control as in our previous cellular analyses. There was

361 little evidence for any difference in Zif268 expression between the groups when assessed through

362 western blots (Fig 2C; $F(2,9)=0.60$, $p=0.57$, $\eta^2_p =0.12$, $BF_{10}=0.47$). Due to the loss of samples, the

363 conditioning-retrieval group could only be compared by flow cytometry against the non-reactivation

364 group, again demonstrating little evidence for any difference (Fig 4D; $t(4)=0.58$, $p=0.59$, $d=0.47$,

365 $BF_{10}=0.62$). Therefore, it appears that conditioning-retrieval does not engage cellular mechanisms of

366 reconsolidation, at least with the 1-hr interval analysed here.

367

368 *Strengthening of paired-associate memory in humans*

369 Given the effect of retrieval-conditioning to strengthen hippocampal contextual fear memories, we

370 conducted a conceptual replication applying an analogous retrieval-relearning procedure to an

371 experimental human episodic memory paradigm. Using single-trial paired associate learning of

372 background scenes and target images, a relatively poor episodic memory was initially learned (mean

373 17.9 out of 40 associates recalled immediately after learning across all groups). This allowed for the

374 detection of quantitative memory improvements at a later test (Fig 3A; strengthening score = test

375 performance – learning performance). In an initial experiment, a retrieval-relearning group (with an

376 interval of 10 min) was compared against groups receiving individual retrieval or relearning

377 experiences, as well as the reverse relearning-retrieval order and a non-memory control (Fig 3B). One-

378 way ANOVA revealed a significant effect of group on the memory strengthening ($F_{(4,90)}=51.7$,

379 $p<0.001$, $\eta^2_p =0.70$, $BF_{10}=2.3\text{x}1019$), with planned comparisons (p's<0.05, BF_{10}'s>5.8) confirming that

380 the retrieval-relearning group improved to a greater extent than the relearning-alone, retrieval-alone and

381 control groups. Exploratory post-hoc analyses revealed, surprisingly, that the retrieval alone group had

382 no performance benefit over the control group ($p=0.55$, $BF_{10}=0.67$), and both groups in fact displayed

383 poorer memory performance at test compared to immediately after learning.

384

385 The primary conclusion from these initial results is that two experiences are more beneficial to memory

386 improvement than a single or no retrieval or relearning opportunity. It is not clear, however, whether it is

387 the different nature of the two experiences that contributes to the magnitude to memory strengthening.

388 Therefore, we tested two further conditions, in which two identical experiences were repeated –

389 retrieval-retrieval and relearning-relearning. There was a significant difference between the retrieval-

390 retrieval and relearning-relearning groups (Fig 3C: $F_{(1,36)}=103.9$, $p<0.001$, $\eta^2_p =0.74$, $BF_{10}=1.4\text{ x}109$),

391 with the retrieval-retrieval group showing no evidence of memory strengthening, in comparison to the

392 substantial improvement displayed by the relearning-relearning group. An exploratory analysis of all

393 four double-experience groups confirmed that there were equivalent levels of memory strengthening in

394 all but the retrieval-retrieval group ($F_{(3,72)}=50.4$, $p<0.001$, $\eta^2_p =0.68$, $BF_{10}=4.0\text{x}1014$; post-hoc tests,

395 p's<0.001 & BF_{10}'s$>1.2\text{x}108$ for differences to the retrieval-retrieval group, p's>0.61 & BF_{10}'s<0.57 for

396 equivalences). Therefore, it is not simply the increased number of experiences that are conducive to

397 memory strengthening, but their nature is an important factor.

398

399 Given that the combination of retrieval and relearning is important for memory strengthening, we again

400 exploited the time-dependent nature of reconsolidation updating to determine whether relearning needs

401 to be presented within the reconsolidation window (Schiller et al., 2010). We also tested whether a

402 similar temporal requirement applied to the memory strengthening observed for relearning-retrieval.

403 Therefore, retrieval-6hr-relearning and relearning-6hr-retrieval groups were compared against the

404 original relearning alone, retrieval-relearning and relearning-retrieval groups (Fig 3D). ANOVA

405 revealed a significant difference between the groups ($F_{(4,90)}=10.99$, $p<0.001$, $\eta^2_p=0.33$,

406 $BF_{10}=5.8x104$), with post-hoc comparisons demonstrating no difference between the retrieval-6hr-

407 relearning and relearning alone groups ($p=0.91$, $BF_{10}=0.55$), but greater memory strengthening in the

408 relearning-6hr-retrieval group (p's<0.02, BF_{10}'s>56). Of particular relevance was the observation that

409 the retrieval-6hr-relearning group performed more poorly than the retrieval-10min-relearning group

410 ($p<0.002$, $BF_{10}=48$), but the relearning-6hr-retrieval and relearning-10min-retrieval groups performed at

411 similarly-high levels ($p=0.56$, $BF_{10}=0.73$), These results show that when relearning was delayed until the

412 reconsolidation window had closed, there was no benefit of the prior retrieval experience, strongly

413 indicating that the retrieval-relearning effect is mediated by destabilization-reconsolidation. Moreover,

414 the preserved memory strengthening in the relearning-6hr-retrieval condition suggests that the beneficial

415 effects of relearning-retrieval are mediated by an alternative process. This interpretation is further

416 supported by an additional experiment showing that verbalised recall, which is known to prevent

417 memory destabilization in human paired associate paradigms (Forcato et al., 2009), prevented the

418 retrieval-relearning memory gain, but not that observed following relearning-retrieval (Fig. 3E).

419 ANOVA revealed a significant effect of group ($F_{(3,70)}=42.2$, $p<0.001$, $\eta^2_p=0.64$, $BF10=4.3x10^{19}$), with

420 planned comparisons (p's<0.002, BF10's>25.5) confirming that the retrieval-relearning group improved

421 to a greater extent than the retrieval-alone, but to a lesser extent than the relearning-retrieval group.

422 However, the retrieval-relearning group did not differ from the relearning-alone group ($BF10=0.72$),

423 whereas an exploratory post-hoc comparison showed that relearning-retrieval did improve test

424   performance relative to relearning-alone ($p<0.001$, BF10=708). A further exploratory comparison

425   against the retrieval-relearning group from Fig 3A revealed a weak effect of verbalising the retrieval at

426   retrieval-relearning ($t(36)=2.16$, $p=0.038$, $d=0.70$, BF10=1.85). Therefore, while both retrieval-

427   relearning and relearning-retrieval result in memory gains, they appear not to rely upon the same

428   behavioral conditions.

429

430

Discussion

The present results show that relearning within the reconsolidation window opened by retrieval improves subsequent long-term memory expression in both rodent and human hippocampal memory settings. Retrieval followed 10 - 15 min later by relearning strengthened both contextual fear memory in rats and visual paired associated memory in humans. The same benefit was present in rodents with an interval of 1h between retrieval and relearning. Critically, however, when the interval between retrieval and relearning was extended outside reconsolidation window (Nader et al., 2000; Monfils et al., 2009; Schiller et al., 2010), there was no greater strengthening observed compared to relearning alone. Furthermore, when blocking memory destabilization by preventing protein degradation in the dorsal hippocampus, the retrieval-induced strengthening effect was significantly reduced. Retrieval combined with relearning also reliably elevated the levels of hippocampal Zif268, a cellular correlate of memory destabilization. Together, these core findings strongly suggest that the memory-enhancing effects of retrieval-relearning are mediated by reconsolidation mechanisms.

On a behavioral level, the observed memory improvement is not simply a consequence of retrieval practice, as a single or double retrieval did not have beneficial effects in either setting. While this may, at first, appear to contradict the extensive literature on the retrieval practice effect in humans, it should be noted that retrieval practice is commonly implemented using several retrieval episodes, often interleaved with further learning, and taking place within the same behavioral session as initial learning (Roediger and Butler, 2011; Hulbert and Norman, 2015). The same is true for the related phenomena of test-potentiated learning (Arnold and McDermott, 2013) and the forward effect of testing (Pastotter and Bauml, 2014), where testing and learning are typically conducted within a single session. This contrasts in a number of ways with the present study, in which retrieval occurred 48 hr after learning, and on only

454   1-2 occasions, and not interleaved with relearning or with feedback. Repeated retrieval shortly after

455   learning has been shown to be greatly superior to a single retrieval opportunity (Roediger and Karpicke,

456   2006). However, a single retrieval 24 hr after learning did not improve subsequent performance *per se*

457   (Potts and Shanks, 2012), although under conditions of increased test difficulty there was evidence for a

458   retrieval practice-like effect. In our study, given the weak learning, the long 48-h interval between study

459   and retrieval practice, and the lack of feedback, the failure of retrieval in itself to produce memory

460   improvement is perhaps not unexpected, as errors in retrieval are likely to strengthen the wrong

461   associate (Roediger and Karpicke, 2006).

462

463   In rodent studies, a single or limited number of retrievals can strengthen subsequent aversive memory

464   expression in a manner that is believed to involve memory reconsolidation (Inda et al., 2011; De

465   Oliveira Alvares et al., 2013; Fukushima et al., 2014). However, in contrast, we have previously

466   demonstrated that contextual fear memory retrieval is detrimental to subsequent memory expression

467   regardless of the parameters of initial retrieval (Cassini et al., 2017). It remains unclear whether the

468   capacity for retrieval-relearning to strengthen memory is dependent upon conditions in which retrieval

469   itself does not have memory-improving effects. Perhaps it is more likely that the summative effect of

470   retrieval and relearning is magnified in weak learning settings (Hulbert and Norman, 2015).

471

472   A number of lines of evidence point towards the retrieval-relearning effect being mediated by updating

473   of memory strength via destabilization-reconsolidation. First, it should be noted that the capacity for

474   reconsolidation-mediated memory gains to be observed following post-retrieval interventions has been

475   demonstrated both pharmacologically for rodent fear memory (Lee et al., 2006; Tronson et al., 2006)

476   and also for paired-associate memory with post-retrieval presentation of negative valence pictures (Finn

477 et al., 2012). Behaviorally, we find that the memory improvement is highly robust with an interval of 15

478 min or 1h between retrieval and relearning. When shortening this interval to 0 min, or extending it to 6

479 h, the improvement was reduced by 20-30%. This temporal window of efficacy matches that shown for

480 retrieval-extinction effects that are dependent upon destabilization-reconsolidation (Monfils et al., 2009;

481 Schiller et al., 2010). With no interval between retrieval and extinction/relearning, it is likely that the

482 absence of an offset signal for the retrieval session results in the failure to trigger reconsolidation, in a

483 similar matter to the necessity for CS offset to trigger reconsolidation in crabs (Pedreira and Maldonado,

484 2003) and humans (Hu et al., 2018). With an extended interval of 6 h or more, the cellular processes of

485 reconsolidation will have proceeded to the extent that pharmacological treatment is without effect

486 (Nader et al., 2000) and behavioral intervention is unable to hijack the reconsolidating memory (Schiller

487 et al., 2010).

488

489 For our human memory data, the importance of the nature of the retrieval experience provides further

490 evidence supporting the destabilization-reconsolidation hypothesis. When retrieval preceded relearning,

491 there was a facilitative effect only when the retrieval was incomplete; that is, when the participants were

492 instructed not to verbalise the answer. With a full retrieval, including answer production, there was no

493 benefit of the retrieval. This contrast replicates conceptually the findings of Forcato et al (2009), who

494 observed that human declarative memory reconsolidation was only triggered when the reminder

495 prevented the production of the answer. Alternative explanations of our human memory strengthening,

496 including retrieval practice (Roediger and Butler, 2011), test-potentiated learning (Arnold and

497 McDermott, 2013) and the forward effect of testing (Pastotter and Bauml, 2014) are all based upon

498 studies, in which an explicit and full retrieval test is used. Therefore, none can account for the

499 dependence of the present memory strengthening upon the specific reminder structure that has

500 previously been demonstrated to be necessary to trigger memory reconsolidation (Forcato et al., 2009).

501

502 Within our rodent contextual fear experiments, the mechanistic understanding of destabilization and

503 reconsolidation allows a more direct implication of reconsolidation. First, hippocampal protein

504 degradation at the proteasome has been previously established to be necessary for destabilization (Lee et

505 al., 2008). When blocking this process specifically prior to retrieval, the memory-enhancing effects of

506 further learning were substantially reduced. A similar dependence on memory destabilization was

507 observed for cued fear memory strengthening with retrieval-relearning in a previous study (Du et al.,

508 2017). The cellular analyses of Zif268 expression further support the interpretation that retrieval-

509 relearning engages reconsolidation processes to update the existing memory. However, it should be

510 noted that our Zif268 expression data relate only to the retrieval-60min-relearning condition and so there

511 is somewhat lesser evidence that retrieval-15min-relearning similarly engages reconsolidation processes.

512 Nevertheless, there is equally no reason to suggest that the shorter interval fails to engage

513 reconsolidation, especially as the reconsolidation window has been consistently demonstrated to span 10

514 to 60 min (Monfils et al., 2009; Schiller et al., 2010; Flavell et al., 2011; Rao-Ruiz et al., 2011), and so it

515 is highly likely that a similar pattern of Zif268 expression would be observed following retrieval-15min-

516 relearning. Dorsal hippocampal Zif268 has been extensively implicated in contextual fear memory

517 reconsolidation and updating (Lee et al., 2004; Lee, 2008; Barnes et al., 2010; Lee, 2010; Cheval et al.,

518 2012; Lee and Hynds, 2013; Besnard et al., 2014; Machado et al., 2015). Here, Zif268 expression was

519 most robustly upregulated following retrieval and conditioning, which strongly supports the engagement

520 of memory reconsolidation processes for the memory strengthening effect. Somewhat surprisingly, there

521 was lesser evidence for Zif268 upregulation following retrieval alone, or conditioning alone, given that

522 retrieval alone has been shown previously to upregulate hippocampal Zif268 (Lee et al., 2004; Lee,

523 2008; Barnes et al., 2010; Lee and Hynds, 2013; Besnard et al., 2014). While we do not have an

524 explanation for this discrepancy, we would note that previous demonstrations of upregulation have used

525 stronger initial fear conditioning parameters (Lee et al., 2004; Lee and Hynds, 2013; Besnard et al.,

526 2014). The weaker initial conditioning may have contributed to the weaker engagement of Zif268 by

527 retrieval and conditioning alone.

528

529 The comparison condition, in which relearning preceded retrieval showed memory strengthening that

530 was quantitatively similar to that observed following retrieval-relearning but differed qualitatively in

531 some important ways. First, in the rodent contextual fear experiments, the strengthening effect of

532 relearning-retrieval was only observed with an interval of 15 min, but not 60 min. The latter time

533 interval is highly suited to reconsolidation effects (Monfils et al., 2009; Flavell et al., 2011), suggesting

534 that the relearning-retrieval memory strengthening is not mediated by reconsolidation. This

535 interpretation is consistent with the human paired associate memory results, which showed that the

536 memory strengthening following relearning-retrieval occurred regardless of the duration of interval

537 between relearning and retrieval, and regardless of the nature (verbalised vs non-verbalised) of the

538 retrieval. While the mechanism of the memory strengthening resulting from relearning-retrieval remains

539 unclear, it can be concluded that it is unlikely to involve memory reconsolidation.

540

541 The capacity of retrieval-relearning, and indeed relearning-retrieval, to confer substantial memory

542 improvements in hippocampal-dependent memories in both rodents and humans has potential

543 translational application across both educational and clinical settings, to maximise learning gains and

544 perhaps offset memory decline. It remains unclear at present what exactly the nature of the

545 interval/distraction between retrieval and relearning needs to be to enable memory strengthening, and so

546 it is possible even that either or both processes are engaged in everyday memory recall and endogenous

547 relearning.

548

549 **Author contributions.** KRT designed and collected and processed data for the human study; CRF

550 designed the flow cytometry analyses; LC conducted rodent behavioral experiments and the flow

551 cytometry analyses; MW designed the human study and wrote the paper; JLCL designed both studies,

552 conducted rodent behavioral experiments and western blot analyses, analysed the data and wrote the

553 paper. The authors have no competing interests.

554

555 **Materials & Correspondence.** Requests to be addressed to JLCL.

556

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671
672

Figure Legends

674 **Fig 1. Combination of retrieval and conditioning strengthened contextual fear memory via**

675 **destabilization and reconsolidation.** Previously weakly-conditioned rats were subjected to retrieval

676 and conditioning on Day 2, and tested again on Days 4 & 11 (A). With a 15-min interval between

677 retrieval and conditioning on Day 2, contextual freezing was increased at the tests compared to when

678 there was no interval (B). There was a similar increase in freezing with a 1-hr interval, but not with a 6-

679 hr interval (C). Schematic representing the infusion of β-lac into the dorsal hippocampus prior to

680 retrieval or conditioning within the retrieval-1hr-relearning procedure (D). Infusion of β-lac contextual

681 fear memory strengthening (E). Schematic of the behavioral procedures for the Zif268 expression

682 experiments (F). Retrieval-conditioning, but not retrieval alone, reliably elevated Zif268 levels

683 compared to a non-reactivated control condition, as assessed through western blots (G). Zif268

684 expression was also assessed with flow cytometry (H; image shows representative sample with events

685 plotted according to size (forward scatter, FSC) and cell granularity (side scatter, SSC), allowing the

686 isolation of cells from debris and illustrating distinct populations of labelled events (DAPI +ve (blue),

687 NeuN +ve (purple) Zif268 +ve (green) and negative/debris (black)). Flow cytometry also showed an

688 increase in Zif268 expression in retrieval-conditioning (I). Data presented as mean + SEM.

689

690

691 **Fig 2. Retrieval-conditioning strengthens contextual fear memory more reliably than other**

692 **combinations of experiences.** With a 15-min interval, both retrieval-conditioning and conditioning-

693 retrieval show greater strengthening than retrieval-retrieval (A). With a 1-hr interval retrieval-

694 conditioning strengthens contextual fear to a greater degree than conditioning-retrieval, and to an

695 equivalent degree as double conditioning (B). Conditioning-retrieval with a 1-hr interval did not

696 upregulate Zif268 expression as assessed with western blots (C) and flow cytometry (D). Data presented

697 as mean + SEM.

698

699 Fig 3. Retrieval-relearning improves human visual paired-associate memory performance.

700 Previously weakly-learned paired-associates were retrieved and/or relearned after 2 days, and tested

701 again 2 days later (A). Test performance was increased by retrieval-relearning, but also by relearning-

702 retrieval (B). When the same experience was repeated, only relearning-relearning improved memory

703 performance (C). When the interval between retrieval and relearning was increased to 6 hr, the memory

704 strengthening effect of retrieval-relearning was decreased, but that of relearning-retrieval was not (D).

705 When participants were instructed to verbalise the answer at the retrieval session there was no beneficial

706 effect of the retrieval when conducted prior to relearning (E). Data presented as mean strengthening

707 score (test performance – learning performance) + SEM.

708