

Registered Report: Transcriptional Analysis of Savings Memory Suggests Forgetting Is Due to Retrieval Failure

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Registered Report: Transcriptional Analysis of Savings Memory

Suggests Forgetting Is Due to Retrieval Failure

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Abbreviated Title:

Transcriptional Correlates of Savings Suggests Forgetting is Retrieval Failure

Author Contributions:

GG, HG, and AG collected behavioral measurements; MD and AG trained animals; TR and MN isolated RNA and conducted qPCR for quality controls; IC-J and R-CJ dissected animals, designed the study, and wrote the manuscript

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Abstract

There is fundamental debate about the nature of forgetting: some have argued that it represents the decay of the memory trace, others that the memory trace persists but becomes inaccessible due to retrieval failure. These different accounts of forgetting lead to different predictions about savings memory, the rapid re-learning of seemingly forgotten information. If forgetting is due to decay, then savings requires re-encoding and should thus involve the same mechanisms as initial learning. If forgetting is due to retrieval failure, then savings should be mechanistically distinct from encoding. In this registered report we conducted a pre-registered and rigorous test between these accounts of forgetting. Specifically, we used microarray to characterize the transcriptional correlates of a new memory (1 day after training), a forgotten memory (8 days after training), and a savings memory (8 days after training but with a reminder on day 7 to evoke a long-term savings memory) for sensitization in *Aplysia californica* ($n = 8$ samples/group). We found that the re-activation of sensitization during savings does not involve a substantial transcriptional response. Thus, savings is transcriptionally distinct relative to a newer (1-day old) memory, with no co-regulated transcripts, negligible similarity in regulation-ranked ordering of transcripts, and a negligible correlation in training-induced changes in gene expression ($r = .04$ 95% CI [-.12, .20]). Overall, our results suggest that forgetting of sensitization memory represents retrieval failure.

40

Significance Statement

41 Understanding the nature of forgetting is important because both excessive and insufficient forgetting
42 are related to profound disruptions of mental health. This registered report provides molecular data
43 indicating that forgetting of long-term sensitization in *Aplysia* represents retrieval failure, contributing
44 new evidence towards resolving a long-standing debate over the neural mechanisms of forgetting.

Registered Report: Transcriptional Analysis of Savings Memory**Suggests Forgetting Is Due to Retrieval Failure**

Long-term memory is characterized both by its duration and by its dependence on changes in gene expression (Goelet et al., 1986). Although long-term memories can last a lifetime, much of what we initially commit to long-term memory is forgotten, becoming progressively less likely to be recalled (Bahrick, 1984). Forgetting plays an essential role in memory function as both excessive and insufficient forgetting are related to profound disruptions of mental health (Ally et al., 2013; Fitzgerald et al., 2013; Mary et al., 2013; Troster et al., 1993).

Currently there is fundamental disagreement about the nature of forgetting, with one review concluding that “we do not know why or how the brain actually forgets” (p. 113, Hardt, Nadim & Nadel, 2013). Some have argued that forgetting occurs due to decay of the memory trace, and thus represents a failure of memory maintenance. In stark contrast, others have suggested that forgetting is merely a retrieval failure, and that the original memory trace persists, perhaps indefinitely (for reviews see Davis and Zhong, 2017; Wixted, 2004). For example, forgetting could be due to inhibitory processes that repress otherwise intact memory traces (Barron et al., 2017).

Here we conduct an experiment to shed light on the nature of forgetting by studying savings memory, the rapid re-acquisition of seemingly forgotten information. Ebbinghaus first characterized savings memory (1885). He learned lists of nonsense words to perfection, waited until he could no longer recall the words, and then re-learned the lists to perfection. He found that it always took less training to re-learn the lists compared to the original acquisition. Since that pioneering demonstration, savings memory has been demonstrated with multiple learning paradigms (Nelson, 1985) and in a

66 variety of species (Antzoulatos et al., 2006; Menges et al., 2015; Philips et al., 2006), suggesting that it
67 is a core feature of long-term memory.

68 Decay- and retrieval-failure accounts of forgetting make contrasting predictions about the
69 transcriptional correlates of savings memory (Figure 1). If memory traces decay, then savings is a re-
70 construction of the original information facilitated by the remnants of the original memory trace.
71 Under this account, savings is predicted to be mechanistically similar to initial memory storage and
72 should evoke a transcriptional state similar to what is observed during new learning. If, on the other
73 hand, forgetting involves only retrieval failure, then savings does not require rebuilding the original
74 memory trace. In this case, savings memory would be mechanistically and transcriptionally distinct
75 from initial memory storage.

76 In this registered report we tested the decay- and retrieval-failure accounts of savings memory
77 (Figure 2). Specifically, we used microarray to characterize the transcriptional changes that accompany
78 long-term sensitization in *Aplysia californica* for a newly stored memory (1-day post training), a
79 forgotten memory (8-days post training), and a savings memory (8 days post training but with a
80 reminder on day 7).

81 Long-term sensitization in *Aplysia* requires changes in transcription (Sutton et al., 2001) and
82 new memory storage is associated with regulation of over 1,000 transcripts within 1 day of training
83 (Conte et al., 2017). As sensitization memory is forgotten most of this transcriptional response fades;
84 7-days after training only ~7 transcripts remain regulated (Patel et al., 2018; Perez et al., 2018). Here
85 we asked if savings memory re-activates the transcriptional changes observed with new memory

86 formation (as predicted if forgetting is due to decay) or if savings has a distinct transcriptional profile
87 (as predicted if forgetting is due to retrieval failure).

88 Comparing transcriptional states with microarray can be difficult because poor signal-to-noise
89 and sampling error can produce spurious dissimilarity. These problems can be overcome in the
90 learning paradigm we selected. First, sensitization-related transcription in *Aplysia* can be characterized
91 from isolated ganglia that contain neurons known to help encode sensitization memory, providing a
92 strong learning-related transcriptional signal (Herdegen et al., 2014a). Second, learning is expressed
93 on only the trained side of the body (Scholz and Byrne, 1987), enabling a powerful within-subjects
94 comparison. Thus, microarray experiments of moderate size (8 samples/group) can attain high power
95 and convergent validity (Herdegen et al., 2014b). Third, we have previously analyzed the
96 transcriptional correlates of newly stored sensitization memory (1-day after training), providing a set of
97 benchmarks for analyzing the correlates of savings memory (Conte et al., 2017).

98 Leveraging the advantages of the sensitization paradigm we conducted a rigorous experiment
99 to provide compelling evidence about the nature of forgetting. We found that the transcriptional
100 correlates of savings memory are distinct from new memory formation suggesting that forgetting of
101 sensitization is due to retrieval failure.

102 **Materials and Methods**

103 We conducted this study as a registered report. First, we developed and publicly posted a
104 behavioral protocol, quality controls, and a behavioral analysis plan (<https://osf.io/z2uck>, 5/28/2018).
105 Then we began initial collection of behavioral data and sample preparation. Once we had enough
106 behavioral data to be confident our design was feasible, we developed a microarray analysis plan and

script and submitted a registered report proposal. After review and in-principle acceptance we publicly pre-registered the study (<https://osf.io/fqh8j>, 9/11/2019), completed behavioral data collection, and conducted the planned microarray analysis.

Open data and materials

Our pre-registration, analysis scripts, and all data for this project are available on the Open Science Framework (<https://osf.io/z2uck/>). The microarray data is also posted to NCBI's Gene Expression Omnibus (GEO: GSE152045, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE152045>) .

Animals

Animals (75-125g) were obtained from the RSMAS National Resource for *Aplysia* (Miami, FL) and maintained at 16° C in one of two 90-gallon aquariums with continuously circulating artificial sea water (Instant Ocean, Aquarium Systems Inc.).

Long-term sensitization training

A one-day long-term sensitization training protocol (Figure 2) was used (Bonnick et al., 2012) . Training consisted of 4 rounds of noxious shock applied at 30 minute intervals to one side of the body with a hand-held electrode. Each round of shock consisted of 10 pulses (60Hz biphasic) of 500ms duration at a rate of 1hz and an amplitude of 90mA. Side of training was counterbalanced. This training protocol produces memory that is strongly expressed for several days but which fades in most animals within 1 week (Perez et al., 2018).

The savings- and forgotten-memory groups received long-term sensitization training immediately after pre-tests, on the first day of the protocol. In contrast, the new-memory group

128 initially received sham training. This consisted of the same procedure but the constant-current
129 stimulator was set to deliver 0mA of current. Animals were otherwise handled in the same way and, in
130 general, were run mixed with batches of animals from the other conditions. For the new-memory
131 group, real sensitization training was finally applied after the 7-day tests, 1 day prior to harvesting
132 tissue.

133 **Reminders to elicit savings**

134 To elicit savings, animals in the savings group received a reminder shock (Perez et al., 2018;
135 Philips et al., 2006). The reminder was delivered 7 days after training, when most animals show
136 essentially no remaining sensitization memory (< 25% increase relative to pre-test). The reminder
137 consisted of two moderate shocks (60Hz biphasic DC pulse for 2s at 20ma of constant current) applied
138 to the midline of the tail with a 15-minute rest between the shocks. The reminder produces short- but
139 not long-term sensitization in naïve animals. In previously trained animals the reminder reveals a long-
140 lasting unilateral savings memory, with T-SWR durations increasing for at least 1 day after the
141 reminder but only on the previously trained side (Perez et al., 2018).

142 Animals in the forgotten-memory group received a sham reminder, where the same protocol
143 was applied but the constant-current stimulator was dialed to deliver 0mA of current.

144 Animals in the new-memory group did not receive a reminder or a sham reminder, but instead
145 received their sensitization training while the other groups received reminders.

146 **Behavioral measurement**

147 As a behavioral outcome, we measured the duration of the tail-elicited siphon-withdrawal
148 reflex (T-SWR) (see Walters and Erickson, 1986). The reflex was evoked by applying a weak shock to

one side of the tail using a hand-held stimulator (60Hz biphasic DC pulse for 500ms at 2ma of constant current). T-SWR behavior was measured as the duration of withdrawal from the moment of stimulation to the first sign of siphon relaxation.

Measurements were made blind to experimental condition. For each timepoint (pre-test, 1-day, 7-day, 20 minute savings, and 1-day savings) behavioral responsiveness was characterized by a series of 8 responses evoked on alternating sides of the body at a 10-min ISI. Scores were split by side of stimulation (trained vs. untrained) and averaged (4 responses/side for each time point characterized).

Isolation and processing of pleural ganglia RNA

We compared gene expression from pleural ganglia on the trained vs. untrained side of the animal. The pleural ganglia contain the ventro-caudal (VC) nociceptors (Walters et al., 2004) which contribute input to the T-SWR circuit as well as several T-SWR interneurons (Buonomano et al., 1992; Cleary and Byrne, 1993; Mackey et al., 1987). The VCs are essential for encoding long-term sensitization memories. Gene expression measured in whole pleural ganglia correlates strongly with expression measured from isolated VC clusters (Conte et al., 2017).

To control for lateralized gene expression, samples from two animals trained on opposite sides were pooled.

To analyze transcription, pleural ganglia RNA were isolated immediately after the long-term savings test, 8 days after protocol start. Animals were anesthetized with an injection of isotonic MgCl_2 (50% of body weight), and an incision was then made along the ventral midline to expose the CNS. As

169 dissection can alter gene expression (Alberini et al., 1994), we extracted ganglia rapidly (< 5 minutes
170 per animal) and transferred them immediately to Trizol (Invitrogen, Carlsbad CA) for homogenization.

171 Tissue was homogenized using the Bullet Blender (NextAdvance, Averill Park, NY) and RNA
172 extracted using Direct-Zol Mini RNA Kit (Zymo, Irvine, CA). Quantity and quality of RNA was assessed
173 using the NanoDrop 1000 (Thermo Scientific, Wilmington, DE).

174 **Reverse-transcription quantitative PCR (qPCR)**

175 Reverse transcription was performed using Maxima cDNA kit with DsDNase (Thermo Scientific, Carlsbad
176 CA). Quantitative PCR was conducted using Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo
177 Scientific, Carlsbad CA) and the MyIQ real time PCR system (Bio-Rad, Los Angeles CA). Primers were
178 validated for correct PCR efficiency; exact sequences are provided in Supplemental Table 1 of Patel et
179 al., 2018. qPCR samples were analyzed in duplicate or triplicate and the relative amounts of each
180 transcript were determined using the ddCT method and the Bio-rad IQ5 gene expression analysis
181 (Carlsbad CA). All qPCR expression levels were normalized to levels of histone H4, a transcript which is
182 stable during LTS training.

183 **Sample size determination**

184 We set a target of 8 biological replicates per group. This sample-size exceeds the consensus
185 recommendation of at least 5 biological replicates per group for microarray analysis (Allison et al.,
186 2006; Pavlidis et al., 2003; Tsai et al., 2003). Moreover, previous transcriptional analyses (e.g.
187 Herdegen et al., 2014b) using this learning paradigm has shown that 8 samples per group can achieve
188 very low estimated false-positive rates (1-2%) and strong convergent validity with qPCR conducted in
189 independent samples ($r^2 = 0.60$ to 0.79). Finally, we tested our analysis script with real data of known

190 levels of similarity and found that this sample-size was sufficient to distinguish between highly similar,
191 moderately similar, and orthogonal sets of regulated transcripts.

192 **Archival Data to Benchmark Similarity**

193 Our microarray analyses compared gene expression in the new-memory, forgotten-memory,
194 and savings-memory groups. To help provide context for these comparisons, we also re-analyzed gene
195 expression from a previous study examining transcriptional changes 1 day after long-term sensitization
196 (Conte et al., 2017; GEO: GSE95596). We refer to this as the “archival new-memory group”. This
197 archival data essentially replicates the new-memory group, though it did not involve as many rounds of
198 T-SWR measurement nor sham training (see Figure 2).

199 **Microarray processing**

200 We used the Aplysia Tellabs Array (ATA: GEO: GPL18666) to characterize changes in gene
201 expression due to long-term sensitization training (Herdegen et al., 2014b). This array includes 26,149
202 distinct probes representing all known sources of *Aplysia californica* ESTs and mRNAs at the time of
203 design (January 2012). Based on estimates from previous microarray designs (Moroz et al., 2006), the
204 ATA should cover >50-60% of all CNS-expressed transcripts.

205 Microarray processing was completed by Mogene Inc. (St. Louis, MO). A two-color approach
206 was used, with each array hybridized to a paired trained and untrained sample. Specifically, each of
207 the 24 arrays compared expression from the trained side of a left- and right-trained animal to
208 expression from the untrained sides of the same animals. Experimental condition (savings, new-
209 memory, forgotten-memory) was balanced across slides (n = 8/group). In addition, dye color was
210 counterbalanced across training conditions.

211 Sample integrity was determined by Bioanalyzer RNA 6000, Pico total RNA protocol. 300ng of
 212 total RNA was amplified and labeled with Cy3 or Cy5 using the Agilent Quick Amp Two-Color Labeling
 213 Kit. Dye incorporation and yield was determined by Nanodrop. Samples were hybridized to the
 214 microarray slide at 65C and 10rpm for 17 hours. Slides were scanned on an Agilent C scanner at 3um
 215 resolution. Data was extracted using Agilent Feature Extraction software, v. 11.5. All labeling and
 216 post-labeling processing was carried out in an ozone regulated environment, monitored at < 5ppb.

217 **Statistical analyses**

218 In our statistical analyses we focus on effect sizes and 95% confidence intervals (Calin-Jageman
 219 and Cumming, 2019a, 2019b). These can easily be converted to hypothesis tests. If the null hypothesis
 220 is not in the 95% confidence interval the test is significant at alpha = 0.05; otherwise the test is not
 221 significant.

222 **Behavioral Analysis.** Behavioral responses were averaged by time point and side of testing
 223 (trained or untrained). Change scores were then calculated by subtracting pre-test scores. At each
 224 time point paired comparisons were made between the average change on the trained side and the
 225 average change on the untrained side: $M_{diff} = (M_{trained_change}) - (M_{untrained_change})$. The 95% confidence
 226 interval for this contrast was then calculated. This is equivalent to estimating the interaction between
 227 training and time with a 2x2 within-subjects ANOVA. Along with raw-score effect sizes we report
 228 standardized effect size estimates (Cohen's d). These are corrected for bias (Hedges, 1981) and
 229 calculated so that positive values represents a stronger increase in response on the trained side
 230 (sensitization).

231 **Microarray.** Microarray data was analyzed using limma (M. E. Ritchie et al., 2015; Smyth, 2005)
 232 from the Bioconductor suite of tools (Gentleman et al., 2004) for R (Ihaka and Gentleman, 1996).
 233 Median expression values were analyzed (Zahurak et al., 2007). These were corrected for background
 234 using the normexp+offset algorithm recommended for Agilent microarrays by Ritchie et al. (Ritchie et
 235 al., 2007). Expression was then normalized using the loess function (Smyth and Speed, 2003). Where
 236 multiple probes were used to measure the same EST or mRNA, these were averaged (Holmes et al.,
 237 2014).

238 **Identification of regulated transcripts.** Within each experimental group, trained and control
 239 expression was compared by computing a log fold change (*LFC*) score indicating the ratio of expression
 240 from the trained to control sides (base 2). Changes in expression were flagged by using the treat
 241 function from limma (McCarthy and Smyth, 2009) to test for regulation significantly greater than 10%
 242 in either direction (an interval null from -10% to 10%) with an empirical Bayes-moderated *t*-test
 243 (Smyth, 2004). Benjamini-Hochberg correction was used to maintain a 5% overall false-discovery rate
 244 (Benjamini and Hochberg, 1995). All the confidence intervals reported for individual transcripts reflect
 245 the same correction for multiple comparisons.

246 **Check for completeness of gene lists.** We estimated the proportion of true nulls in each
 247 condition using the propTrueNull function (Ritchie et al., 2015) using the convex decreasing densities
 248 approach developed by Langaas et al. (2005). We then calculated the false negative rate as $1 -$
 249 $\frac{\%regulated}{\%truenull}$. Based on previous analyses we established a criterion of false negative rates
 250 less than 4% for subsequent comparisons of transcription to be considered valid.

251 **Degree of overlapping regulation.** For the genes regulated in the new-memory group (1 day
 252 after training) we examined the proportion (P) also regulated in the forgotten-memory, savings-
 253 memory, and archival new-memory groups. We then estimated the difference in overlap as a memory
 254 is re-activated during savings: $P_{\text{diff}} = P_{\text{savings_overlap}} - P_{\text{forgotten_overlap}}$.

255 To follow up on this analysis of overlap we tested formally for differences in regulation
 256 between the forgotten- and savings-memory groups among the transcripts regulated 1 day after
 257 training. This is equivalent to testing each transcript for a training x condition interaction. We again
 258 used an interval null of +/- 10%.

259 **Similarity of ranked transcript lists.** We used the `OrderedList` package for R (Yang et al., 2019)
 260 to calculate similarity scores based on overlap across ordered ranks of transcripts. For each condition,
 261 transcripts were first ranked by strength of evidence (p value) and sign of regulation (up or down). We
 262 then computed and assessed overlap across the top and bottom ~1000 transcripts in each list.

263 **Correlations in training-evoked expression.** We also examined correlations in LFC scores. The
 264 critical question to examine was similarity to the new-memory group, so we first restricted down to
 265 the transcripts flagged as clearly regulated in this group. Then we calculated the correlation in log-fold
 266 change to the savings ($r_{\text{new_savings}}$), forgotten-memory ($r_{\text{new_forgotten}}$), and archival new-memory groups
 267 ($r_{\text{new_archivalnew}}$). We calculated each relationship with a simple Pearson's r and with correction for
 268 potential measurement error using the `genuine association of gene-expression profiles` function
 269 (`genas`) in `limma` (Ritchie et al., 2015).

270 As our primary outcome we examined if the savings- and forgotten-memory groups differed in
 271 similarity to the new-memory group. To do this we calculated the difference in correlations across

these groups ($r_{\text{diff}} = r_{\text{new_forgotten}} - r_{\text{new_savings}}$) using the paired.r function from the psych package in R (Revelle, 2018). We expected that if savings re-activates transcriptional mechanisms of memory storage then it should show stronger similarity to the new-memory group, leading to a positive value of r_{diff} . We pre-established the following interpretations based on analysis of previous data: strong increase in similarity if $r_{\text{diff}} \geq 0.5$, moderate increase in similarity if $r_{\text{diff}} \geq .25$ but $< .50$, little-to-no increase in similarity if $r_{\text{diff}} < 0.25$.

Data Collection and Quality Controls

We collected data from 98 *Aplysia*. With pairing left- and right-trained animals this provided 49 RNA samples (15 assigned to the savings-memory group, 16 in the new-memory group, and 18 in the forgotten-memory group). Making a fair test between decay and retrieval-failure accounts of forgetting requires that the transcriptional analysis proceeds with samples that exemplify each memory state. Therefore, prior to data collection we established a strong set of quality controls and posted them publicly to the Open Science Framework (<https://osf.io/z2uck/wiki/Experimental%20Protocol/>, posted on 05/28/2018).

First, we checked behavioral data to ensure each sample selected for microarray exemplified the desired memory state:

- To ensure training effectiveness we required all animals to show robust but unilateral long-term sensitization on measures taken 1 day after their training (>30% increase in T-SWR duration on the side of training and less than a 30% change on the untrained side). All but 2 animals met this criteria; the samples they were part of were discarded (1 from the savings-memory group, 1 from the forgotten-memory group).

- 293 • There is some variation in *Aplysia* in forgetting. To ensure that the savings and forgotten-
294 memory groups truly represent a forgotten-memory state we required that animals in these
295 groups show negligible behavioral sensitization by the 7-day tests, indicated by T-SWR
296 durations that have returned to within 25% of pre-test. Two animals from the forgotten-
297 memory group did not meet this criterion (they showed lingering sensitization), so the 2
298 samples they were part of were discarded.
- 299 • We required animals in the savings group to show savings memory after the reminder, defined
300 as having T-SWR durations during the savings test that had increased over baseline more on the
301 previously-trained side than on the previously-untrained side. All animals assigned to the
302 savings group met this criterion.
- 303 • We checked to ensure that habituation from repeated testing did not contaminate our
304 comparison, requiring that T-SWR measures on the 7-day test were within 30% of baseline on
305 the untrained side. Animals from 1 sample in the forgetting condition did not meet this
306 criterion. However, in coding this criterion we accidentally applied it to the trained side (which
307 all samples passed) and this error was not detected until after this sample had been included in
308 the microarray analysis. As reported in the exploratory analysis section, excluding this sample
309 did not impact the results of the study.

310 We also checked the quantity and quality of isolated RNA for each sample, discarding any
311 samples with a very low or uneven yield, poor quality, and/or genomic contamination. Based on this
312 we discarded an additional 12 samples (6 from the new-memory group and 6 from the forgotten-
313 memory group).

314 Finally, to ensure samples had been properly processed, we used quantitative real-time PCR to
315 check for up-regulation of well-defined transcriptional markers of sensitization training:

- 316 • For the new-memory group, we checked for up-regulation of the transcript encoding ApBiP
317 (GenBank: NM_001204652; Kuhl et al., 1992). This transcript is strongly and consistently up-
318 regulated 1-day after sensitization training (Conte et al., 2017). As expected, there was strong
319 up-regulation of ApBiP in samples from the new-memory group (log fold change (*LFC*) = 1.49
320 95% CI[0.79, 2.18], *n* = 10). However, one sample unexpectedly showed lower expression on
321 the trained side. We discarded this sample.
- 322 • We have not previously examined transcriptional correlates of savings, but we reasoned that
323 transcripts which remain persistently regulated during forgetting should still be regulated
324 during savings. Thus, we checked the savings-memory group for regulation of the transcript
325 encoding the peptide neurotransmitter Phe-Met-Arg-Phe NH₂ (FMRFa; GenBank: M11283.1;
326 Schaefer et al., 1985). This transcript is strongly up-regulated within 1-day of sensitization
327 training and continues to be up-regulated for more than 1 week (Patel et al., 2018). As
328 expected, we observed strong up-regulation of FMRFa in this group (*LFC* = 1.28 95% CI[0.86,
329 1.69], *n* = 14). We found two samples, though, with decreased FMRFa expression on the
330 trained side; these were discarded.
- 331 • We did not specify a positive control for the forgotten-memory group but as expected there
332 was upregulation of FMRFa in this group as well (*LFC* = 0.61 95% CI[0.01, 1.21], *n* = 9).

333 For each group we selected 8 valid samples, leaving 1-4 samples/group for potential use with qPCR
334 validation.

Results

First we report the behavioral data, which was collected primarily before pre-registration to ensure the samples would adequately represent the different stages of memory. Then we report our pre-registered microarray analysis; all and only planned analyses are reported. Finally, we report additional exploratory analyses that were not part of our pre-registered plan.

Behavioral Validation

We confirmed that samples selected for microarray showed the expected trends in sensitization memory. In the savings group ($n = 16$ animals to provide 8 samples; Figure 3A), training produced long-term sensitization, expressed as a large but unilateral increase in T-SWR duration when tested 1 day later. T-SWR responses increased by 5.4 seconds on the trained side (95% CI [4.7, 6.1]) but showed no change on the untrained side ($M_{\text{untrained_change}} = 0.0\text{s}$ 95% CI [-0.4, 0.4]). Thus, comparing changes on the trained and untrained side indicated a very large training effect ($M_{\text{diff}} = 5.4\text{s}$ 95% CI [4.7, 6.1], $d = 4.7$ 95% CI [3.7, 6.5]). Although initial learning was strong, sensitization was then forgotten, as by the 7-day post-tests responses were slightly below pretest on both sides ($M_{\text{trained_change}} = -0.2\text{s}$ 95% CI [-0.7, 0.2]; $M_{\text{untrained_change}} = -0.6\text{s}$ 95% CI [-1.0, -0.2]), so there was only a weak residual training effect ($M_{\text{diff}} = 0.3\text{s}$ 95% CI [-0.2, 0.9], $d = 0.4$ 95% CI [-0.2, 1.1]). Despite this apparent forgetting, all animals showed robust savings memory, as a reminder evoked a long-term re-expression of sensitization on the previously untrained side. Specifically, one day after the reminder (1-day savings test) T-SWR responses were moderately increased on the previously trained side ($M_{\text{trained_change}} = 2.0\text{s}$ 95% CI [1.7, 2.4]) but continued to be slightly below pre-test on the previously untrained side; $M_{\text{untrained_change}} = -1.0\text{s}$ 95% CI [-1.3, -0.6]), reinstating a relatively large training effect indicative of savings memory (M_{diff}

356 = 3.0s 95% CI [2.6, 3.5], $d = 4.3$ 95% CI [3.3, 6.0]). Savings memory was also evident when normalized
357 to 7-day post-test scores ($M_{\text{diff}} = 1.9\text{s}$ 95% CI [1.2, 2.5], $d = 1.3$ 95% CI [0.9, 1.8]).

358 In the forgotten-memory group ($n = 16$ animals to provide 8 samples; Figure 3B) sensitization
359 training also produced robust but unilateral increases in T-SWR duration (1-day post-tests: $M_{\text{diff}} = 7.2\text{s}$
360 95% CI [6.0, 8.5], $d = 3.1$ 95% CI [2.3, 4.2]). Sensitization was largely, but perhaps not completely
361 forgotten, within 1 week (7-day post-tests: $M_{\text{diff}} = 0.9\text{s}$ 95% CI [-0.1, 1.9], $d = 0.4$ 95% CI [0.0, 0.8]). The
362 sham reminder did not produce a lasting change in behavior; long-term savings tests showed only
363 weak behavioral expression of sensitization memory ($M_{\text{diff}} = 0.8\text{s}$ 95% CI [0.0, 1.6], $d = 0.5$ 95% CI [0.0,
364 1.1]).

365 Finally, in the new-memory group ($n = 16$ animals to provide 8 samples; Figure 3C) sham
366 training did not alter behavior, so there was no training effect at the 1-day post-tests ($M_{\text{diff}} = 0.0\text{s}$ 95%
367 CI [-0.7, 0.7], $d = 0.0$ 95% CI [-0.7, 0.7]) nor at the 7-day post-tests ($M_{\text{diff}} = 0.0\text{s}$ 95% CI [-0.6, 0.6], $d = 0.0$
368 95% CI [-0.5, 0.4]). Real sensitization training administered after the 7-day post-tests produced the
369 expected unilateral sensitization, with a large training effect on measures taken the next day (savings
370 post-test: $M_{\text{diff}} = 6.4$ 95% CI [5.1, 7.6], $d = 3.3$ 95% CI [2.5, 4.6]).

371 Overall, animals selected for microarray exhibited clear and consistent behavioral patterns
372 representative of new, forgotten, and savings stages of sensitization memory.

373 **Planned Microarray Analysis**

374 The decay account of forgetting predicts that savings recapitulates most of the transcriptional
375 response required to store a memory (savings will be similar to the new-memory group). The retrieval-
376 failure account of forgetting predicts that savings will have distinct transcriptional mechanisms (savings

will not be similar to the new-memory group). To test these predictions we measured the similarity of microarray results between the groups. We assessed similarity in three different ways: 1) as the degree of overlap among transcripts flagged as regulated, 2) as the consistency of rank-order gene lists, and 3) as the linear correlation between log-fold changes in expression.

How similar is savings memory to new memory? Overlapping regulation approach. As an initial way to characterize similarity we flagged clearly-regulated transcripts at each epoch of memory (new, forgotten, and savings) and then calculated the overlap in flagged transcripts across conditions. We defined clearly-regulated transcripts as those that showed significantly more than a 10% change in expression, with adjustment to maintain a 5% overall false-discovery rate. This is a stringent criterion likely to miss some regulated transcripts, but our goal for this initial analysis was to compare results without noise from potentially negligible changes in expression (see below for more sensitive comparisons and also for exploratory analyses based on less stringent criteria). Table 1 gives the counts of transcripts flagged in each group. Extended Data Table 1-1 gives full results for each transcript in each condition and is also posted to the Open Science Framework (<https://osf.io/z2uck/>).

In the new-memory group there were 148 transcripts that were clearly regulated. The log-fold change for each of these transcripts is plotted by condition in Figure 4. As expected, nearly all these transcripts had been previously linked with the maintenance phase of sensitization memory (Conte et al., 2017; Liu et al., 1997), and represented predicted proteins with diverse functions, including signaling (ApTBL-1, GenBank: U57369.1, $LFC = 0.45$ 95%CI [0.09, 0.79]), protein production (an EIF2 subunit, GenBank: EB232654.1; $LFC = 0.57$ 95%CI [0.40, 0.74]), the unfolded protein response (GCN-1 like, GenBank: EB230807.1, $LFC = 0.53$ 95% CI [0.34, 0.72]), cytoskeletal function (septin-7-like, GenBank: EB260579.1; $LFC = 0.58$ 95% CI[0.29, 0.87]), and transport (a Dynein β chain component,

399 GenBank: GD206216.1, $LFC = -0.37$ 95% CI[-0.63, -0.12]. Flagged transcripts also correctly included the
 400 positive control, ApBiP ($LFC = 0.79$ 95% CI [0.43, 1.16]).

401 Transcriptional regulation dissipated over time, with no transcripts flagged as clearly regulated
 402 in the forgotten-memory condition. This can be seen in Figure 4. where most transcripts regulated in
 403 the new-memory condition collapsed towards 0 in the forgotten-memory condition. Thus, defined in
 404 terms of overlapping regulation, the forgotten and new phases of memory showed no similarity (Table
 405 1, column showing proportion of overlap). This was expected, as previous studies have also shown
 406 that the transcriptional response to sensitization training mostly fades over time, and that the very few
 407 transcriptional changes that persist are difficult to detect with an array-wide screen (Patel et al., 2018;
 408 Perez et al., 2018). Indeed, even though we had confirmed up-regulation of FMRFa via qPCR in these
 409 samples (see methods), for the microarray analysis this transcript did not meet the threshold for being
 410 flagged as clearly regulated ($LFC = 0.41$ 95% CI [0.19, 0.72], $p = .02$ before correction, but $p = 1$ after
 411 correction).

412 The critical question was if re-activating the memory during savings would reinstate the
 413 transcriptional regulation observed with a new memory. It did not. In the savings-memory group
 414 there were also no transcripts flagged as clearly regulated. This missed at least some transcripts, as
 415 even the positive control, FMRFa, did not make the cutoff ($LFC = 0.67$ 95% CI[0.48, 0.86], $p = .0001$
 416 before correction, but $p = 1$ after correction). Still, this indicates that among unequivocally-regulated
 417 transcripts there was no overlap with the new-memory group. In Figure 4 this is shown as the lack of
 418 perturbation from the forgetting to the savings conditions. Thus, on the basis of this (admittedly
 419 crude) measure of similarity, the savings condition produced no re-activation of transcription, a finding
 420 that supports the retrieval-failure account of forgetting.

421 This was not due to an inability to detect similarity with this approach, as we could detect
422 strong overlap between samples given similar treatments. Specifically, 141 of the 148 transcripts
423 clearly regulated in the new-memory group were also flagged as clearly regulated in the archival new-
424 memory group, which had also been harvested 1 day after sensitization training (Conte et al., 2017).

425 We also conducted a formal condition by training interaction analysis, but did not find any
426 transcripts showing a statistically significant change in training effect from the forgotten to savings
427 stages of memory.

428 **How similar is savings memory to new memory? Gene ranking approach.** Overlap of gene
429 lists is not always a sensitive measure of similarity, as it depends on somewhat arbitrary significance
430 classifications. Indeed, our stringent criteria clearly did not capture all regulated transcripts.

431 As a more sensitive way to measure similarity in transcriptional states we compared rankings of
432 transcripts across conditions using the using the OrderedList package in R (Yang et al., 2019). This
433 allowed us to evaluate similarity in gene rankings across the 1000 most up- and down-regulated
434 transcripts in each condition regardless of statistical significance status. We compared the
435 transcriptional state in the new-memory group to the forgotten-memory and savings-memory groups
436 and benchmarked against the archival new-memory group.

437 Analysis of similarity by ranking also indicated that transcriptional regulation fades as a
438 sensitization memory is forgotten. Comparing the new-memory to the forgotten-memory conditions
439 showed only very weak similarity. The left part of Figure 5A shows the observed levels of similarity
440 relative to what is expected based on a random shuffle of gene lists. As can be seen, similarity tracks
441 only slightly higher than the average expected by chance. The right part of Figure 5A then compares

the similarity score observed with the distribution of scores created with the random shuffles. This shows that the level of similarity is marginal (similarity score = 449.0, $p = .06$). Thus, as forgetting progresses, the pattern of regulation shown during encoding is largely lost (though perhaps not completely).

Again, our critical question is what happens as sensitization memory is re-expressed via savings? Figure 5B shows this result, comparing the new-memory and savings groups. The savings group actually showed weaker similarity to the new-memory group (similarity score = 319.9, $p = .41$). Note that although the scale of the similarity scores is arbitrary, it can be meaningfully compared across these analyses. Based on gene rankings, savings does not seem to appreciably re-activate the pattern of regulation observed as a memory is stored, a result more consistent with the retrieval-failure account of forgetting. Again, this was not due to a lack of sensitivity of our approach, as we could detect similarity between samples treated with similar protocols. Specifically, Figure 5C compares the new-memory group with the archival new-memory group; this shows very strong similarity in gene list rankings (similarity score = 10,873, $p < .0001$).

How similar is savings-memory to new memory? Correlational approach. One weakness of quantifying similarity based on rankings is that it omits the magnitude of regulation in the calculation of similarity. Thus, we made a third and final set of similarity measurements by examining correlations between changes in gene expression across conditions. Because we anticipated this to be the most sensitive and complete measure of sensitivity, we pre-registered this measure as our primary outcome.

To calculate correlations we first subsetting to the 148 transcripts clearly regulated in the new-memory group. We then examined the correlation in log-fold changes in these transcripts in the new-

memory and savings-memory groups. We did this using a direct linear correlation (Pearson's r) and with correction for possible measurement error using the genuine association (*genas*) function in the limma package for R.

Measuring similarity via correlation also indicated that the transcriptional changes that accompany encoding are largely (but not entirely) dissolved as forgetting proceeds: $r = .23$ 95% CI [.07, .38]; $r_{\text{corrected}} = .31$, $p = .03$, Figure 6A. This data is compatible with weak to modest levels of shared variance.

The critical question was if savings would re-activate encoding-related transcriptional changes. By this metric the answer was again no. The correlation in regulation between the new and savings conditions was very weak when calculated on raw data ($r = .04$ 95% CI [-.13, .20], Figure 6B) and only marginal when corrected for possible measurement error ($r_{\text{corrected}} = 0.36$, $p = .09$). Calculated in raw scores, there was actually a decrease in correlation strength from forgetting to savings ($r_{\text{savings-forgotten}} = -.19$, $p = .05$). Calculated with correction for measurement error, there was a negligible increase in correlation strength ($r_{\text{savings-forgotten}} = .05$, $p = .30$). This finding is most consistent with forgetting as retrieval failure. This was not due to lack of sensitivity, as we observed a strong correlation among samples treated similarly. Specifically, the correlation between the new-memory and archival new-memory groups was $r = .95$ 95% CI [.93, .96] from raw scores and even higher ($r_{\text{corrected}} = .99$, $p < .001$) when corrected for possible measurement error (Figure 6C).

Exploratory Analyses

Comparison with previous results. We had previously characterized changes in gene expression that occur 1 day after sensitization training, identifying 1,259 clearly-regulated transcripts

484 (the archival new-memory group drawn from Conte et al., 2017). We explored the degree to which
485 these transcripts were similarly regulated in this new study. Specifically, we tested for regulation of
486 just these putative memory-related transcripts in the new-memory condition, which was also
487 harvested 1 day after sensitization training (though with additional pre-testing and sham training). We
488 considered transcripts similarly regulated if they showed a statistically significant change in expression
489 (null of no change), with correction to maintain a 5% false-discovery rate.

490 We found strong consistency of results, with 77% of previously-identified transcripts qualifying
491 as regulated in this focused test (972 of the 1,259). Moreover, there was a strong correlation in
492 regulation ($r = .93$ 95% CI[.92, .94] across log-fold change scores, $N = 1,258$). This is consistent with the
493 planned analyses reported above, which showed high similarity between these data sets. It is notable,
494 though, that for the current study only 148 of these transcripts were flagged when screened over the
495 whole array. Part of this discrepancy is due to the aggressive corrections required for a larger screen
496 (whole array of 26,091 transcripts vs. a focused test of 1,198 transcripts). In addition, we noticed a
497 general increase in within-group variation (noise) in the current study. Specifically, 70% of the
498 transcripts identified in the archival new-memory group showed higher variance in the current study
499 (larger standard deviation in log-fold change across samples). Overall, variance increased by 30% 95%
500 CI[27%, 34%], raising thresholds for flagging a transcript as clearly regulated. This increase in noise did
501 not seem to be due to an outlier or bad microarray sample (see below). It may have been due to the
502 longer duration of the protocol in the current study, which included sham training and three additional
503 rounds of behavioral measurements (compare new-memory to archival new-memory protocols in
504 Figure 2). Consistent with this possibility, there was modest habituation evident in these animals just
505 prior to training (see Figure 3).

506 Figure 7 shows how fate of the transcripts flagged in the archival new-memory group, plotting
 507 log-fold changes in the new, forgotten, and savings conditions. This graphically shows the strong
 508 consistency of expression between the archival and current new-memory conditions. Even with this
 509 broader set of transcripts, it is clear that regulation collapses during forgetting (most transcripts
 510 decline towards 0 in the forgotten-memory condition) and that savings does not strongly perturb gene
 511 expression (most transcripts remain near 0 in the savings-memory condition).

512 We have also previously examined changes in gene expression 7 days after sensitization
 513 training (Patel et al., 2018; Perez et al., 2018), finding via microarray and qPCR that there is persistent
 514 regulation in 7 transcripts, all of which are also regulated during initial memory maintenance (1 day
 515 after training). To explore consistency with these previous results we tested for regulation just in these
 516 transcripts in both the forgotten- and savings-memory conditions (both of which were harvested 8
 517 days after training). For this analysis we considered a transcript to be consistently regulated if there
 518 was a statistically significant change in expression (null of no change) with correction for multiple
 519 comparisons. Table 2 summarizes the results and compares them with previous findings. Overall,
 520 there was fair consistency, with 4 of 7 transcripts showing regulation in the forgotten-memory
 521 condition, and 2 of 7 in the savings-memory condition. None of these had been flagged in the array-
 522 wide screen reported above due to the lower-power of this analysis, but these focused tests again
 523 confirm that some transcriptional changes persist beyond the behavioral expression of sensitization
 524 memory. This focused test did not, however, reveal a strong change in regulation with savings
 525 memory.

526 **How robust are results to analysis parameters?** As the above considerations make clear,
 527 microarray results can depend critically on how the analysis is conducted. Although our plan was

vetted through peer review and pre-registered it still represents only one reasonable analysis approach out of many. Thus, to examine the analysis generalizability of our results we conducted an exploratory multiverse analysis.

- To check for outsized influence of outliers we varied the inclusion of each the 32 microarray samples.
- As the new-memory group formed the focal point for comparisons, we ensured results would generalize by varying whether comparisons were made to the new-memory group or to the archival new-memory group.
- To ensure our results were not incomplete due to our discovery-based microarray approach we ran the analysis not only broadly on all 26,091 unique transcripts on the array but also narrowly on 1,198 transcripts we have previously identified as regulated by sensitization.
- Finally, we varied the stringency with which we flagged regulated transcripts, varying both the use of corrections for multiple comparison and the stringency of the null hypothesis (interval null of +/- 10% vs. a standard null of no change).

These variations provided 528 different analysis specifications. We examined how results varied across these different analysis specifications.

Similarity based on overlap consistently supported forgetting as retrieval failure. The proportion of overlap between savings and the new-memory conditions was always modest, ranging from 0 up to .12. Moreover, overlap was always similar to or weaker than in the forgotten-memory conditions: $P_{diff} \leq .006$ in all specifications.

548 Similarity based on ordered lists was also very stable, with no specifications indicating a statistically
549 significant positive association in regulation between the new and savings phases of memory.

550 Similarity based on correlation also consistently supported forgetting as retrieval failure. The
551 correlation in regulation between the savings- and new-memory condition was never more than
552 moderate (r values ranged from $-.15$ to $.26$). Moreover, the relationship was never substantially
553 stronger than what was observed at forgetting: $r_{\text{savings-forgotten}}$ was negative in 319 of 528 analyses,
554 between 0 and $.1$ in 200 analyses, and between $.1$ and $.21$ in 9 analyses. Thus, no analysis specification
555 yielded an increase in similarity that met our pre-registered criteria of at least $.25$ to indicate modest
556 support for forgetting as decay.

557 Finally, leaving out each sample did not reveal any outlier that produced a strong or consistent
558 influence on measures of similarity. In particular, we examined the effect of dropping the one sample
559 from the forgotten-memory condition which had shown some habituation 1 week after training. This
560 did not systematically alter any of the similarity measurements. Overall, our multiverse analysis
561 suggests that our findings are robust to a variety of reasonable analysis specifications.

562 Discussion

563 Sensitization training produces complex waves of transcriptional change. This transcriptional
564 response is essential for creating a long-term memory, as blocking transcription during training
565 abolishes long-term memory (Sutton et al., 2001), the long-term facilitation that mediates sensitization
566 (Castellucci et al., 1989; Montarolo et al., 1986), and the structural correlates of long-term memory
567 (Bailey et al., 1992) Moreover, several of the transcripts initially activated by sensitization training are
568 essential for inducing the long-term facilitation thought to contribute to the behavioral expression of

569 sensitization (e.g. C/EBP: Alberini et al., 1994; CREB1: Dash et al., 1990). This does not establish that
570 transcriptional changes *maintain* long-term sensitization memory, but it is clear that transcriptional
571 changes are required to activate maintenance mechanisms.

572 As sensitization is forgotten, the transcriptional changes produced by training also fade away. Here
573 we find that sensitization memory can be persistently re-activated (savings memory) without re-
574 activating storage-related transcriptional changes. Savings memory seems mechanistically distinct
575 from maintaining a new memory—few transcripts are co-regulated at both phases of memory and
576 overall transcriptional states show only negligible similarity.

577 One limitation of this study is that power to detect clearly-regulated transcripts was lower than
578 anticipated. This means that our finding of no transcriptional changes during savings is tenuous;
579 savings may activate some transcriptional changes that were missed.

580 This shortcoming did not limit our ability to compare transcriptional states. First, we planned for
581 assessments of similarity based on global patterns of regulation, not just on the statistical-significance
582 status of transcripts. We found that our measures of similarity could detect when samples had been
583 treated alike, always showing very strong similarity between the new memory and archival new-
584 memory conditions. In addition, our findings are robust to a range of reasonable analysis
585 specifications, including ones which more aggressively classify transcripts as regulated.

586 Overall, our results are not consistent with decay models of forgetting. If the sensitization memory
587 trace substantially decays during forgetting, savings would require at least partly rebuilding it. We
588 found no evidence for this: long-term savings does not reactivate the transcriptional changes observed

589 with an initial long-term memory. This suggests that the memory trace for sensitization remains
590 reasonably intact and that forgetting of sensitization in *Aplysia* is due to retrieval failure.

591 This conclusion may seem puzzling: how could the memory trace be intact if there are almost no
592 transcriptional changes that persist during forgetting and savings? The answer may be that the
593 memory trace is molecularly sparse. For example, a recent screen found only 2 clear transcriptional
594 changes in the hippocampus 1 week after fear conditioning, a time-point when behavioral expression
595 of the memory would still be quite strong (Mizuno et al., 2020). Thus, even though learning initially
596 produces widespread transcriptional changes, these may be refined to a relatively small core for
597 maintenance. It is also possible that the maintenance mechanisms are not transcriptional at all, but
598 merely require transcription to be initiated. For example, one (controversial) possibility is that the
599 memory trace for sensitization is epigenetic, and requires transcriptional changes only for behavioral
600 expression (Pearce et al., 2017). We are now working to determine if the long-term expression of
601 savings requires any transcriptional changes by injecting a transcriptional inhibitor just prior to the
602 reminder shock used to evoke savings.

603 In retrieval-failure accounts of forgetting, memory traces persist yet gradually become decoupled
604 from behavior. How might that happen in long-term sensitization? We have previously found that the
605 transcriptional response to sensitization includes not only changes likely to promote memory but also
606 changes likely to limit its behavioral expression. Specifically, we have found that sensitization training
607 produces a robust and very long-lasting increase in the mRNA encoding FMRFa (Conte et al., 2017;
608 Patel et al., 2018). In *Aplysia* FMRFamide is an inhibitory neuromodulator. It inhibits the VC neurons
609 that help encode sensitization memory, depresses synapses, and decreases the strength of the T-SWR
610 response (Abrams et al., 1984; Fioravante et al., 2006). This suggests the possibility that sensitization

611 training not only builds the memory trace but also promotes inhibitory processes that can impair
612 retrieval, a form of active forgetting (Davis & Zhong, 2017). If this is correct, it should be possible to
613 manipulate the forgetting of sensitization memory by altering FMRFa signaling; we are now working to
614 test this hypothesis.

615 Retrieval-failure accounts of forgetting propose that the memory trace is enduring, but that
616 accessibility to retrieval is highly dynamic. Learning is thought to produce an initially accessible
617 memory, time and new learning then decrease accessibility, but a variety of experiences can increase
618 accessibility for short or long durations (context re-exposure, brief re-training, reminders, etc.). These
619 different dynamics suggest that storage and retrieval are organized at different levels of neuronal
620 function. Multiple lines of research indicate that encoding a long-term memory activates at least two
621 distinct storage mechanisms: changes in synaptic efficacy and changes in connectivity. For example, in
622 *Aplysia* long-term sensitization produces morphological changes related to increased synaptic strength
623 (increased active zone size and vesicle complement) and additional changes related to increased
624 connectivity, such as new synaptic varicosities and active zones (Bailey and Chen, 1988). These decay
625 at different rates, with changes in synaptic strength decaying over time while changes in connectivity
626 endure (Bailey and Chen, 1989). In the mammalian visual system, changes in connectivity have
627 specifically been shown to persist beyond forgetting (Hofer et al., 2009; Linkenhoker et al., 2005). One
628 intriguing possibility, then, is that the memory trace is represented by enduring changes in connectivity
629 while retrieval is made possible by more labile changes in synaptic strength. One testable prediction of
630 this dual-process account of memory expression is that a generalized increase in activity could re-
631 potentiate synapses and re-express a seemingly forgotten sensitization memory.

632 One critical question is how forgetting is related to other forms of memory disruption, such as the
633 amnesia induced by disruptions of consolidation or reconsolidation. At the behavioral level, there are
634 strong similarities, including the fact that memories which seem lost due to an amnestic intervention
635 can often be re-expressed through a reminder or a brief retraining (Riccio and Richardson, 1984). On
636 the other hand, current evidence indicates induced amnesia may be due to storage failure (Haubrich et
637 al., 2020). Amnestic agents tend to degrade the physical correlates of memory. In contrast to our
638 findings with forgetting, memory recovery after an amnestic agent tends to refresh the physical
639 correlates of memory (Haubrich et al., 2020). Thus, forgetting and memory impairment through
640 disrupted consolidation may be quite distinct. One difficulty with interpreting these results is that it is
641 not yet possible to demarcate which brain correlates of memory are related to storage and which are
642 related to accessibility. Still, it seems possible that induced amnesia and forgetting are mechanistically
643 distinct. It would be useful to directly compare these processes in the same system.

644 Grand debates in science are never settled by one study. Our results strongly implicate retrieval
645 failure as the mechanism of forgetting for sensitization in *Aplysia*, but a definitive account of forgetting
646 will require additional studies across organisms and learning paradigms. The strategy of monitoring
647 neuronal changes across multiple memory states seems likely to be fruitful for better resolving how
648 and why forgetting occurs.

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- 797
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Figure Captions

Figure 1 – Savings memory according to decay and retrieval-failure accounts of forgetting. Top: Savings memory. Repeated rounds of training (lightning bolts) increase strength of recall, but in the absence of additional practice, forgetting occurs, indicating by a decline in the strength of recall towards zero. Nevertheless, a brief reminder can re-instate recall; this is known as savings memory. Middle: In decay theories of forgetting, initial learning changes synaptic connectivity and strength forming a memory trace. Over time, though, these changes decay away, leading to forgetting (reduced recall). During savings memory, the memory trace must be almost entirely rebuilt. Savings is thus predicted to utilize the same transcriptional mechanisms that initially created the memory trace. Bottom: In retrieval-failure theories of forgetting, forgetting is due not to decay but to interference from other memories. For example, additional learning could inhibit (dark circles) the otherwise intact memory trace. In this framework savings involves repairing retrieval mechanisms (e.g. down-regulating inhibition). Thus, savings is predicted to be transcriptionally distinct from initial memory storage.

Figure 2 – Long-term sensitization in *Aplysia*. A) Overview of the behavioral paradigm. Sensitization is an increase in responsiveness due to noxious stimulation. To produce long-term sensitization in *Aplysia* animals were exposed to 4 rounds of painful shock to one side of the body (training site: lightning bolt). The effect of training was monitored by measuring the duration of the T-SWR, a defensive withdrawal of the siphon evoked by an innocuous stimulus to the left or right side of the tail (test site). To document savings, a reminder was administered to the midline of the tail (reminder site, wide arrow). In naïve animals this reminder produces short- but not long-term sensitization. B) Behavioral Protocols. Behavioral measures were the same in all experimental groups: T-SWR

821 responses were evoked on the left and right tail (red and black hash lines). Responses were measured
 822 at pre-test, 1-day after training, 7-days after training, 20 minutes after the reminder, and 1 day after
 823 the reminder (day 8 from training). Animals differed in their experimental treatments. For the
 824 savings-memory group, animals received standard sensitization training after pre-test measures (4
 825 strong shocks, 30 minutes apart, lightning bolts). Then, 7-days after training animals received the
 826 reminder (wide arrow) to evoke savings memory. For the forgotten-memory group the treatment was
 827 the same except animals were given sham reminder (grey arrow crossed out), leaving the sensitization
 828 memory dormant. Finally, for new-memory group animals initially received sham sensitization training
 829 (gray lightning bolts crossed out) and then received real sensitization training after the 7-day post-
 830 tests. All animals were harvested for microarray analysis after the 1-day savings tests (day 8 from
 831 start). Thus, all animals received the same behavioral testing, but when harvested were expressing
 832 different states of sensitization memory: new (1 day since training), forgotten (8 days since training
 833 with no reminder), or savings (8 days since training and 1-day since the reminder). In addition, we
 834 analyzed an archival data set from Conte et al. (2018) which roughly replicates the new-memory group
 835 (harvested 1 day after sensitization training). We used this archival data to benchmark assessments of
 836 similarity.

837 **Figure 3 – Behavioral changes in the savings, forgetting, and new-memory conditions.** This figure
 838 shows T-SWR duration as a % change from pre-test on both the trained (red) and untrained (black)
 839 sides. Dark lines with dots represent group means; shading indicates 95% confidence interval of the
 840 mean. Individual animals are represented by the light lines. A) Savings-memory group, which received
 841 real training (lightning bolts) after pre-tests and a reminder (yellow arrow) after the 7-day tests. All
 842 animals were expressing a long-term savings memory when harvested on Day 8. B) Forgotten-memory

group, which received real training after pre-tests but a sham reminder (crossed-out arrow) after the 7-day tests. All animals showed apparent forgetting when harvested. C) New-memory group, which received sham training (crossed-out lightning bolts) after pre-tests and then real-training after the 7-day tests. All animals were expressing a new (1-day old) memory when harvested.

Figure 4 – Fate of transcripts regulated during initial learning. This graph shows mean log-fold change (trained vs. untrained) for each of the 148 transcripts flagged as clearly regulated in the new-memory group, tracking their expression during forgetting and savings. The dashed line at 0 represents no change in expression (when trained and untrained expression are the same, their ratio is 1, which gives a log-fold change of 0). Although these transcripts were clearly regulated 1 day after sensitization (new memory) none were clearly regulated during forgetting or savings.

Figure 5 – Similarity of conditions measured by rank-ordering of transcripts. These figures show similarity in the transcriptional states between the new-memory group and the forgotten-memory group (A), the savings-memory group (B), and the archival new-memory group (C). Panels on left compare observed similarity by gene-list length (black line) relative to the range of similarity observed with random shuffles of gene lists (yellow bars). Panels on right show overall similarity score for top ~1000 genes against the distribution of scores from random shuffles.

Figure 6 – Similarity of conditions measured by correlation in log-fold changes. These figures show the correlation in log-fold change between the new-memory group and the forgotten-memory group (A), the savings-memory group (B), and the archival new-memory group (C). Only the 148 transcripts clearly regulated in the new-memory group are shown. The black dots are individual transcripts; the

863 blue line is the line of best fit; the diagonal line shows a 1:1 relationship that would occur with perfect
864 similarity.

865 **Figure 7 – Exploratory analysis of fate of transcripts previously identified as regulated 1 day after**
866 **sensitization training.** This graph shows mean log-fold change (trained vs. untrained) for 1,198
867 transcripts which showed clear regulation in the archival new-memory condition (Conte et al. 2017),
868 tracking their expression in the current new, forgetting, and savings groups. The dashed line at 0
869 represents no change in expression. Comparing the archival new-memory group to the current new-
870 memory group shows some regression to the mean but that most transcripts show similar regulation.
871 These transcriptional changes fade during forgetting (most transcripts collapse towards 0), and there is
872 no obvious perturbation in expression when savings memory is induced.

873

874 **Table Descriptions**

875 **Table 1 – Counts of Clearly-Regulated Transcripts and Overlap of Regulation**

876 Note: A complete table of microarray results is provided in Extended Data Table 1-1.

877 **Table 2 – Transcriptional changes after forgetting of sensitization**

878 Note: This table shows results for a set of 7 transcripts previously identified via microarray and qPCR as
 879 being regulated after forgetting of sensitization. The previous result column is taken from Patel et al.
 880 (2018); it is qPCR data from pleural ganglia harvested 7 days after training, when sensitization had
 881 been forgotten. The Forgetting and Savings columns microarray are from the current study, both of
 882 which were harvested 8 days after training. Numeric results are log-fold changes in expression +/- the
 883 95% margin of error (expanded for multiple comparisons).

884 **Extended Data Table 1-1 – Complete table of microarray results.**

885 Note: This table provides microarray results for each transcript in each condition. The "Transcript"
 886 column provides the unique identifier for the transcript probe. The "BestAnnotation" column provides
 887 an annotation (if available) for that transcript. The "Previous_finding" column indicates if that
 888 transcript was previously identified as regulated after long-term sensitization training. The "LFC"
 889 columns report the mean log-fold change for that transcript (trained vs. control) by condition (d1 for
 890 new, w1 for forgetting, sav for savings). The "adj.MoE" columns report the 95% margins of error for
 891 these mean log-fold changes, adjusted to maintain a 5% false-discovery rate. The "adj.p" values report
 892 the p values for a test for regulation (against an interval null of +/- 10%), adjusted to maintain a 5%
 893 false-discovery rate. Note that where the adjusted p value is 1, the corresponding adjusted margin of

894 error cannot be calculated and is listed as NA. Finally, the "MoE" columns report the raw 95% margins
895 of error.

896

Table 1 – Counts of Clearly-Regulated Transcripts and Overlap of Regulation

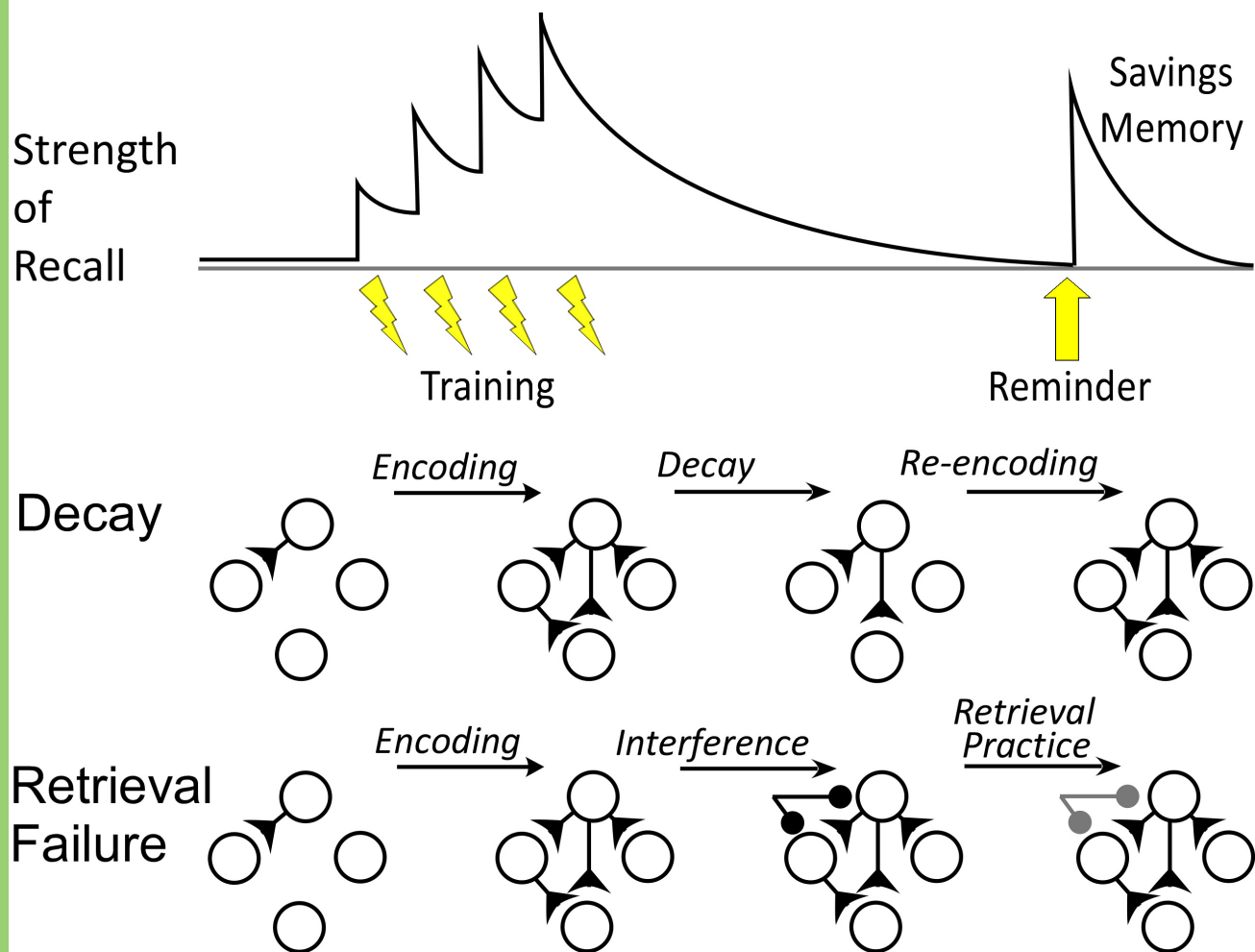
Group	Up-regulated	Down-regulated	Estimated False Negative Rate	Proportion of New Memory Transcripts Co-Regulated
New Memory	131	17	<1%	
Forgotten Memory	0	0	<1%	0.00 95% CI[0.00, 0.03]
Savings Memory	0	0	<1%	0.00 95% CI[0.00, 0.03]
Archival New Memory	798	463	<1%	0.95 95% CI[0.91, 0.98]

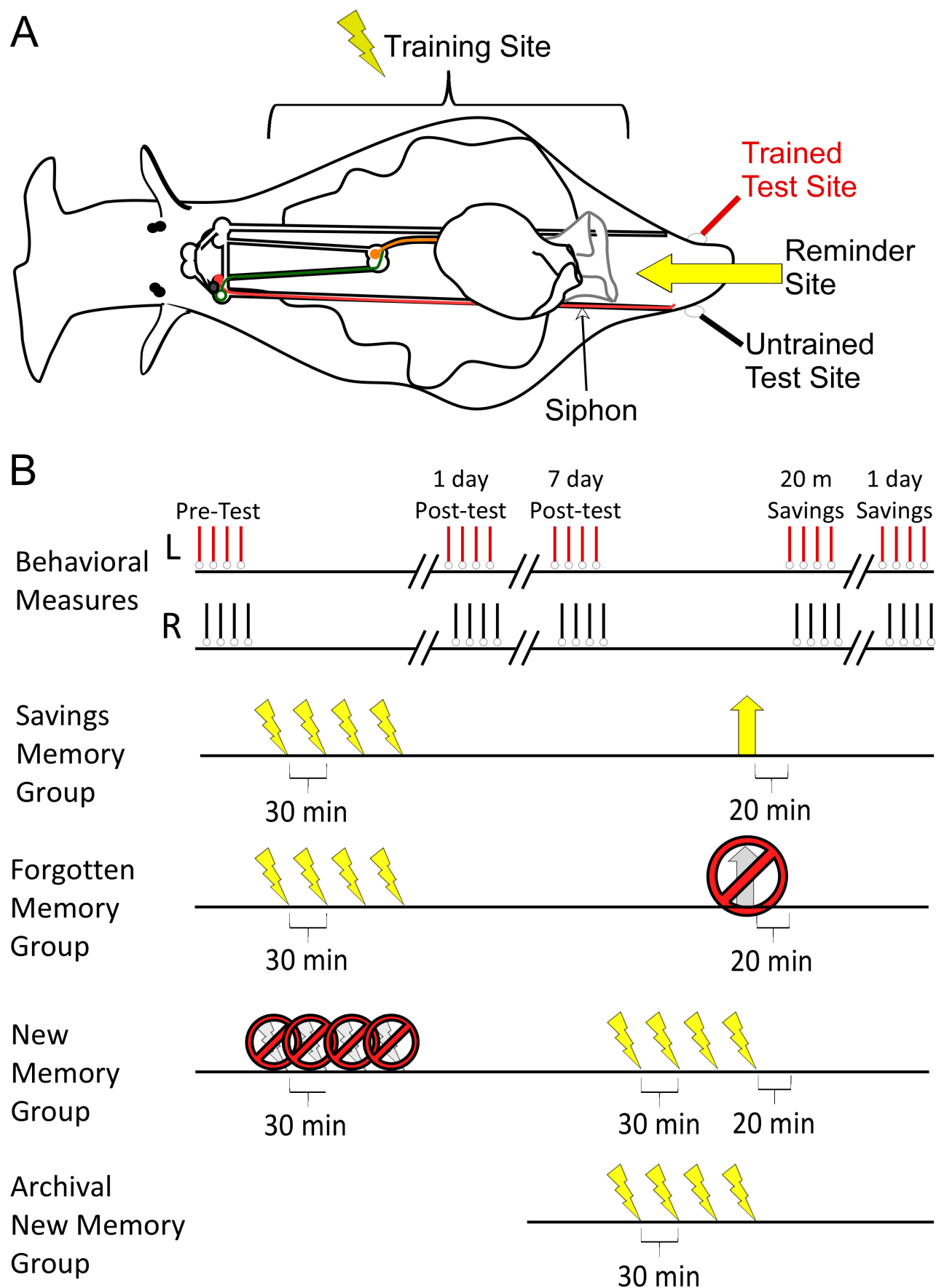
Note: A complete table of microarray results is provided in Extended Data Table 1-1.

Table 2 – Transcriptional changes after forgetting of sensitization

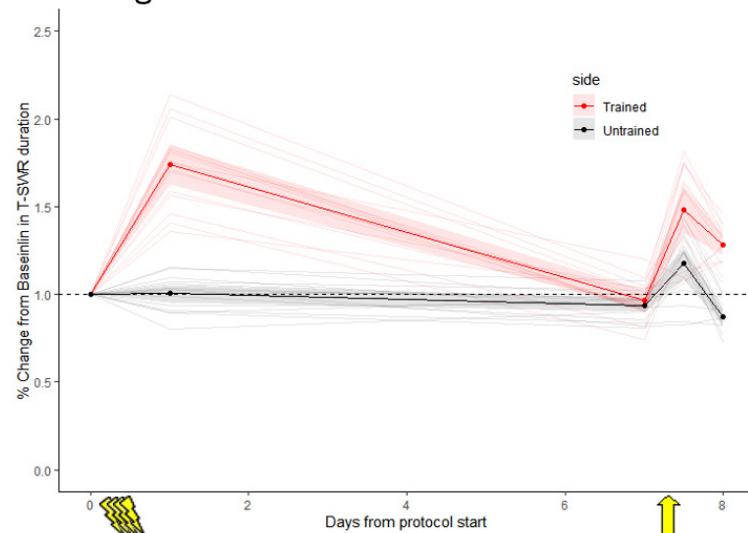
Transcript	Description	Previous Result	Forgetting	Savings
Z15041.1	ApBiP	0.63 +/- 0.46	-0.04 +/- 0.71	0.07 +/- 0.31
M11283.1	FMRFa	0.53 +/- 0.51	0.41 +/- 0.26	0.67 +/- 0.18
EB257711.1	LOC101857556	0.52 +/- 0.46	0.26 +/- 0.24	0.11 +/- 0.32
EB254334.1	Transcribed locus	0.51 +/- 0.50	0.73 +/- 0.31	0.19 +/- 0.58
FF066943.1	LOC106013098	0.36 +/- 0.42	0.57 +/- 0.13	0.49 +/- 0.14
EB243511.1	Transcribed locus	-0.23 +/- 0.50	0.00 +/- 0.22	-0.21 +/- 0.24
EB342172.1	Transcribed locus	-0.62 +/- 0.35	-0.17 +/- 0.23	0.08 +/- 0.25

Note: This table shows results for a set of 7 transcripts previously identified via microarray and qPCR as being regulated after forgetting of sensitization. The previous result column is taken from Patel et al. (2018); it is qPCR data from pleural ganglia harvested 7 days after training, when sensitization had been forgotten. The Forgetting and Savings columns microarray are from the current study, both of which were harvested 8 days after training. Numeric results are log-fold changes in expression +/- the 95% margin of error (expanded for multiple comparisons).

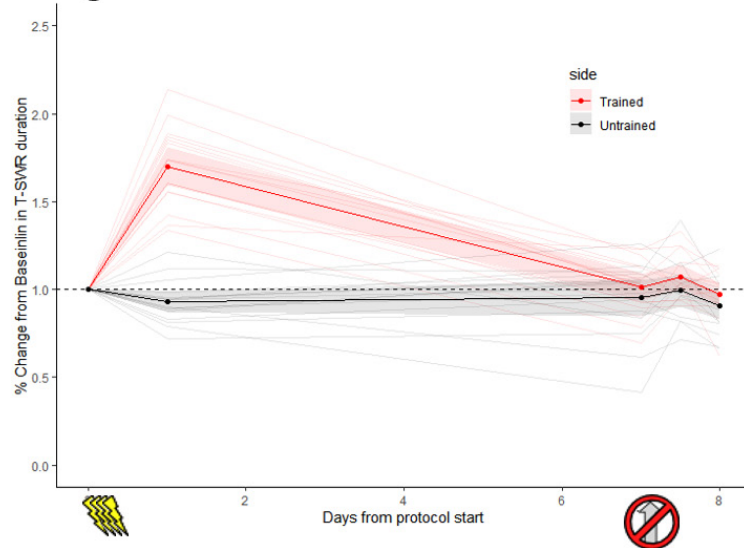




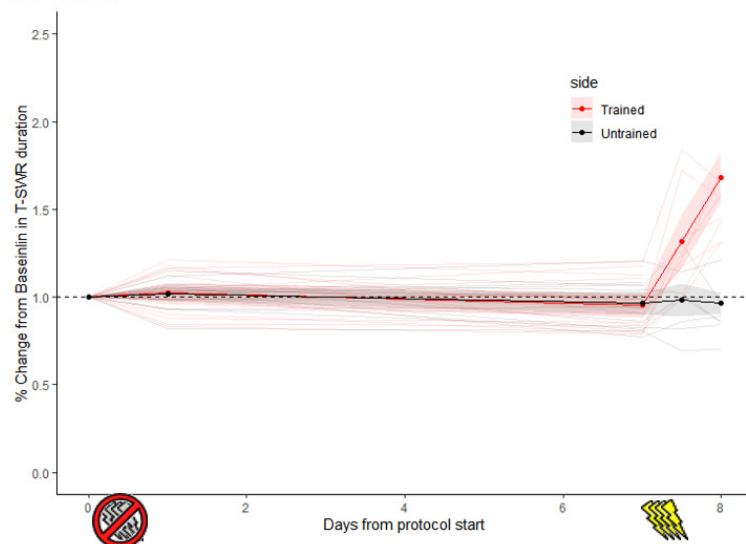
A. Savings

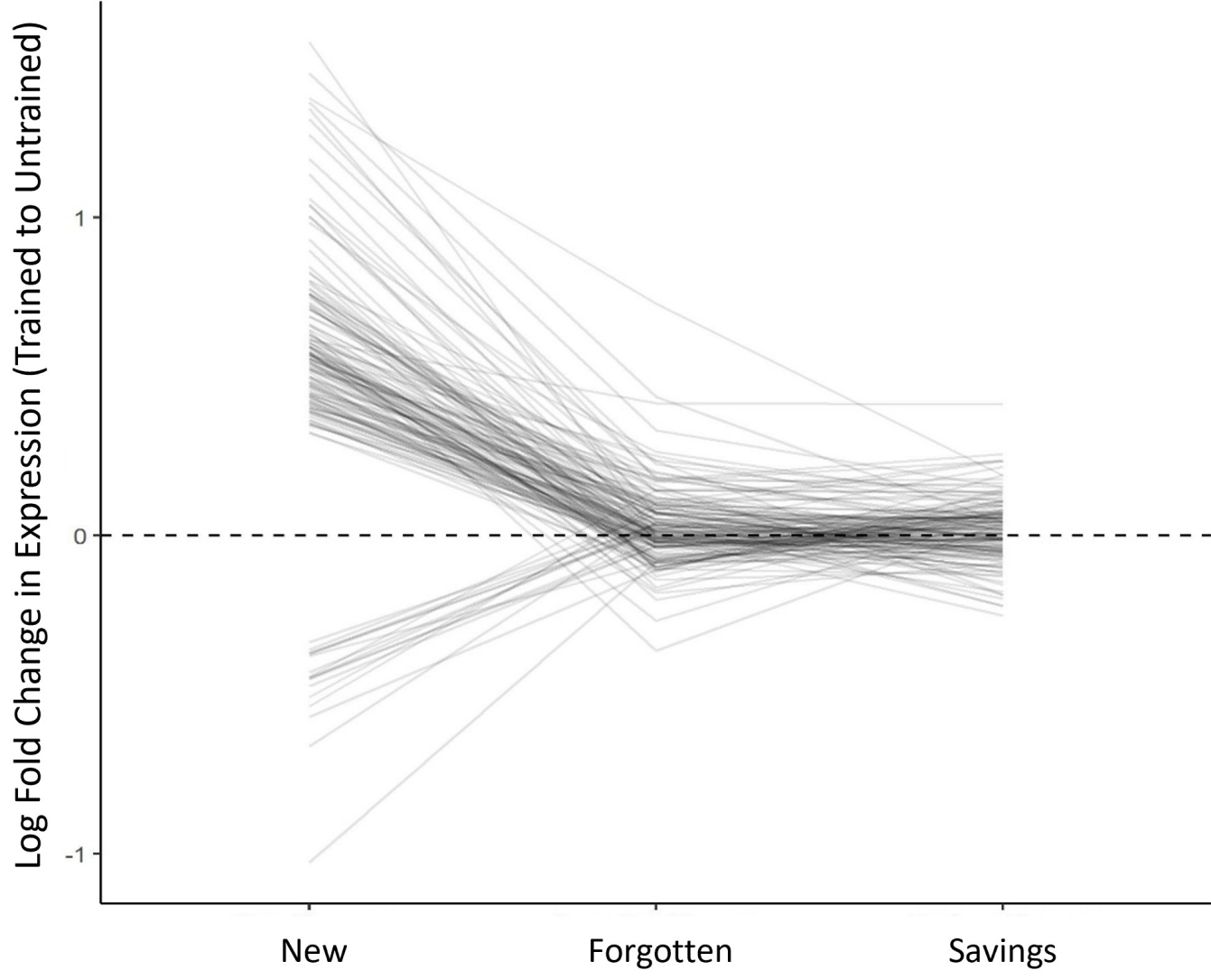


B. Forgotten

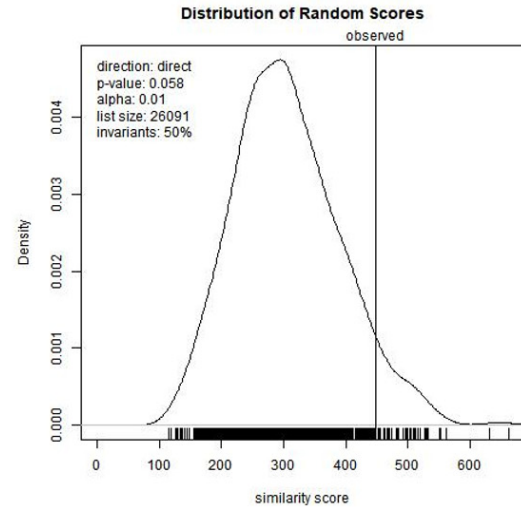
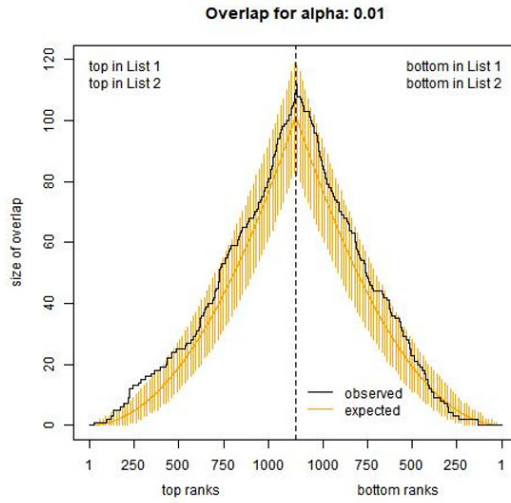


C. New

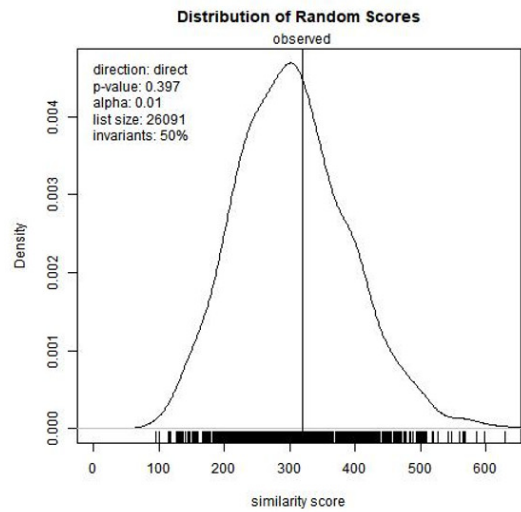
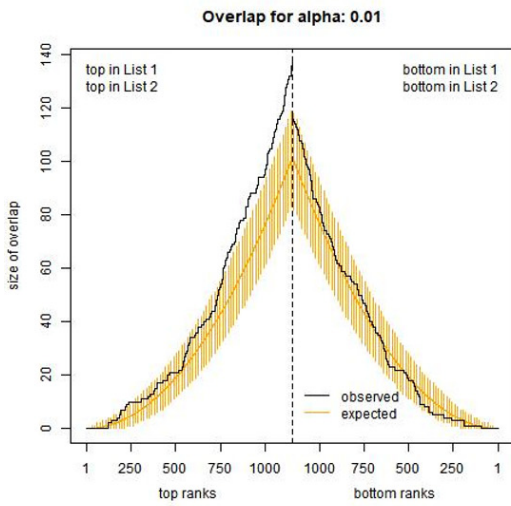




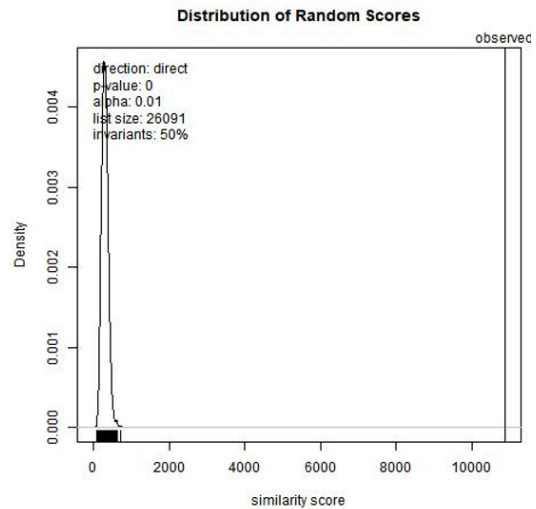
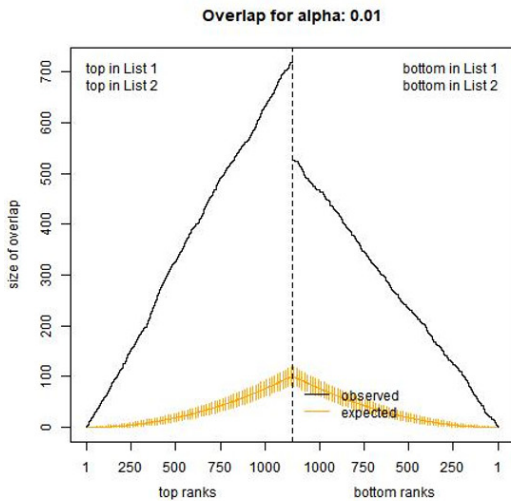
A. Compare New to Forgotten



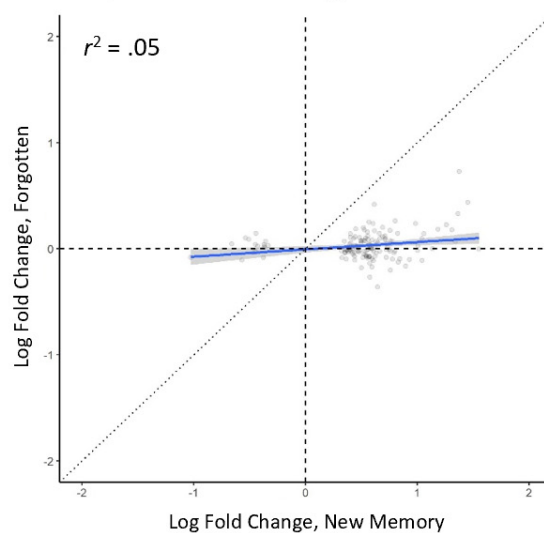
B. Compare New to Savings



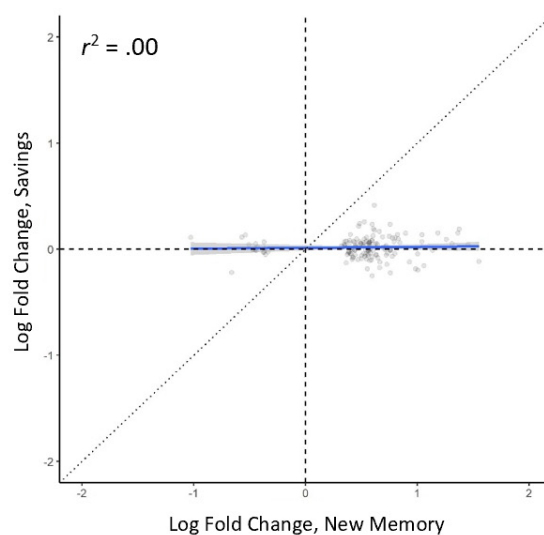
C. Compare New to Archival New



A. Compare New to Forgotten



B. Compare New to Savings



C. Compare New to Archival New

