Savings memory is accompanied by transcriptional changes that persist beyond the decay of recall

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Most long-term memories are forgotten. What happens, then, to the changes in neuronal gene expression that were initially required to encode and maintain the memory? Here we show that the decay of recall for long-term sensitization memory in *Aplysia* is accompanied both by a form of savings memory (easier relearning) and by persistent transcriptional regulation. A behavioral experiment ($N = 14$) shows that sensitization training produces a robust long-term sensitization memory, but that recall fades completely within 1 wk. This apparent forgetting, though, is belied by persistent savings memory, as we found that a weak reminder protocol reinstates a long-term sensitization memory only on the previously trained side of the body. Using microarray ($N = 8$ biological replicates), we found that transcriptional regulation largely decays along with recall. Of the transcripts known to be regulated 1 d after training, 98% (1172/1198) are no longer significantly regulated 7 d after training. Still, there is a small set of transcripts which remain strongly regulated even when recall is absent. Using qPCR ($N = 11$ additional biological replicates) we confirmed that these include the peptide transmitter FMRFamide, a transcript encoding a putative homolog of spectrin beta chain (Genbank: EB255259), a transcript encoding a protein with a predicted EF-hand calcium-binding domain (Genbank: EB257711), and eight uncharacterized transcripts. To our knowledge, this is the first work to show that transcriptional changes evoked by learning can outlast recall. The small set of transcriptional changes that persist could mediate the rapid relearning of the memory (savings), or the decay of recall, or both, or neither.

[Supplemental material is available for this article.]

Most long-term memories fade away, becoming progressively less likely to be recalled. There is long-standing controversy over what this means about the underlying memory trace (Wixted 2004): some have argued it decays; others that it merely becomes inaccessible. One important clue is the persistence of savings memory: the fact that even when previously learned information is beyond recall it can still be easier to relearn (Ebbinghaus 1885). Savings memory occurs across the animal kingdom (Wickelgren 1972), suggesting that apparent forgetting belies the persistence of at least some aspects of the long-term memory trace. What, exactly, persists, though, and how does this mediate savings? We know surprisingly little about these topics (Hardt et al. 2013).

One promising way forward is to track learning-induced changes in the nervous system over the course of a memory (Davis and Zhong 2017). Forming a long-term memory triggers profound changes in neuronal transcription (Alberini 2009), and these changes seem to be essential for both the encoding (Chew et al. 1995) and initial maintenance (Igaz et al. 2002; Lefer et al. 2012) of long-term memory. Thus, tracking neuronal changes in transcription over time provides one level of analysis that could help elucidate the nature of forgetting. If memory traces fully dissolve as recall decays, then transcriptional changes should also fade away. If memory traces simply become inaccessible or disrupted, then some aspects of transcriptional regulation might persist beyond the decay of recall, perhaps in support of savings memory. Arbitrating between these possibilities poses a significant technical challenge, as apparent decay could be conflated with a lack of sensitivity/power to detect subtle transcriptional changes.

Here we characterize the transcriptional changes accompanying recall decay and savings memory for long-term sensitization in the marine mollusk *Aplysia californica*. Sensitization is an increase in reflex responsiveness due to exposure to a noxious stimulus. It is a ubiquitous form of nonassociative memory from which more complex forms of memory may have evolved (Walters and Moroz 2009). Sensitization in *Aplysia* is attractive for study because (a) it can be induced unilaterally (Scholz and Byrne 1987; Herdegen et al. 2014b), allowing powerful within-subjects designs (Fig. 1A) and (b) previous work has identified 1198 transcripts strongly and persistently regulated 1 d after sensitization training (Conte et al. 2017), providing a rich set of targets to track as recall fades.

First, we documented recall decay and savings memory for long-term sensitization using a preregistered protocol and analysis plan (https://osf.io/dhcye/). To induce a long-term sensitization memory, *Aplysia* received a series of four noxious shocks applied to one side of the body (Fig. 1B; protocol as reported in Conte et al. 2017). This produced a unilateral long-term sensitization memory (Fig. 1B), expressed as a persistent increase in the duration of a defensive reflex (the tail-elicited siphon-withdrawal reflex; measured as reported in Conte et al. 2017) on the side of training (comparing change from baseline to 1-d post tests on the trained versus untrained side: $M_{diff} = 74\%$, 95% CI [59, 88], $d = 3.5$ [2.2, 5.1], $t_{(14)} = 10.9$, $P < 0.001$). Although robustly expressed 1 d after training, recall of the sensitization memory decayed over the course of a week. To document savings, animals were selected to meet preregistered criteria for showing no sign of recall 7 d after

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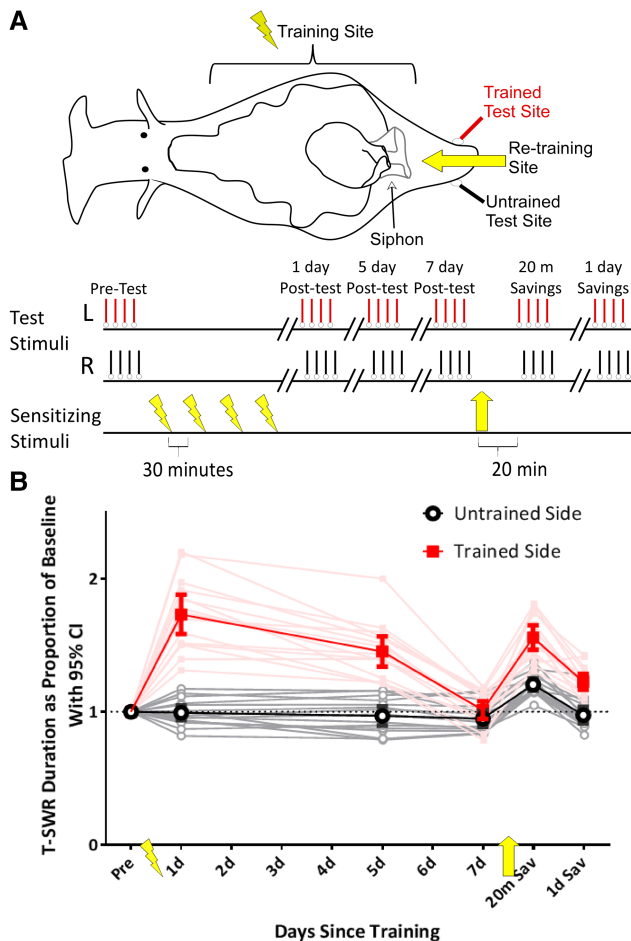
Figure 1. Savings memory persists after the decay of recall for long-term sensitization memory. (*A*) Experimental protocol. The duration of the tail-elicited siphon withdrawal reflex (T-SWR) was measured by applying weak shocks (2 mA, 0.5 sec) to the *left* or *right* tail (test sites). After pretests, long-term sensitization was induced by four strong shocks (90 mA, 0.5 sec pulses for 10 sec) to one side of the body (training site). Reflex duration was then monitored 1, 5, and 7 d after training. On the seventh day, a weak retraining protocol (2 × 15 mA, 2 sec) was applied to the *middle* of the tail (retraining site). The effect of retraining was monitored 20 min and 1 d later. (*B*) Changes in reflex duration on the trained and untrained side. LTS training evoked robust unilateral sensitization. At 7 d, animals were selected which showed no signs of recall ($N = 14$). Nevertheless, retraining revealed robust long-term savings memory only on the trained side (same results were obtained when normalized to 7-d post-tests).

training (<20% change from baseline in either direction at 7-d test; $N = 14$ animals selected; average change from baseline = 1% at 7-d test). Still, savings memory persisted and was revealed by a weak retraining protocol applied to the middle of the tail (administered as in Philips et al. 2006). At 20 min after the retraining, there was an increase of 20% in reflex duration on the previously untrained side ($t_{(14)} = 9.51$, $P < 0.001$), reflecting short-term sensitization. On the previously trained side, though, there was a 55% increase in reflex duration ($t_{(14)} = 13.8$, $P < 0.001$), indicating an even stronger short-term response to the same stimulus due to the previous training ($M_{diff} = 35\%$, [26, 45], $d = 2.5$ [1.5, 3.8], $t_{(14)} = 8.0$, $P < 0.001$). A day after the reminder, there was no long-term sensitization on the untrained side, with reflex duration reduced by 3% relative to baseline ($t(14) = 0.9$, $p = 0.78$). On the trained side, reflex durations were still increased by 23% ($t_{(15)} = 7.9$, $P < 0.001$), a significant change compared with the untrained side ($M_{diff} = 25\%$, [18, 32],

$d = 2.2$ [1.2, 3.3], $t_{(14)} = 7.5$, $P < 0.001$). Thus, the reminder rekindled a long-lasting memory on the previously trained side, but failed to produce a long-term memory on the previously untrained side. These results are consistent with prior reports of savings memory (Antzoulatos et al. 2006; Philips et al. 2006) for sensitization in *Aplysia*.

Next, we used microarray analysis ($N = 8$ biological replicates) to characterize changes in gene expression (trained versus untrained) 7 d after training, a time-point when recall has decayed but savings persists (see also Supplemental Fig. 1A). Pleural ganglia were analyzed; these contain the VC nociceptors that help mediate the expression of long-term sensitization memory (Cleary et al. 1998) as well as several types of interneurons in the reflex circuit. Each sample pooled tissue from one left-trained animal and one right-trained animal to control for lateralized gene expression. Gene expression was analyzed using the Aplysia Tellabs Array (ATA: GEO: GPL18666), which contains probes for 26,149 distinct ESTs and is thought to represent >50%–60% of all neuronally expressed transcripts (full details on array design in Herdegen et al. 2014b). Analysis of array data was as described in Conte et al. 2017; our analysis script is posted to the Open Science Framework. Specifically, array data were corrected for background, normalized, and then averaged across condition. Expression on the trained and untrained sides was then compared with an empirical Bayes moderated *t*-test to identify transcripts with a statistically significant change in expression >10% in either direction (McCarthy and Smyth 2009). Correction for multiple comparisons was made to maintain a 5% overall false-discovery rate (Benjamini and Hochberg 1995).

To identify transcripts regulated by training, we used three approaches. First, we tested only the 1198 transcripts known to be regulated 1-d after training (Conte et al. 2017). This focused analysis increased statistical power by making fewer comparisons and thus requiring less correction for multiple comparisons. Transcripts identified by this approach are marked "Persistent" in Supplemental Table 1, as they are transcripts which were regulated at 1-d which continue to be regulated 7-d after training. Next, we tested all the remaining transcripts on the microarray. Transcripts identified in this second screen are marked "Unique" in Supplemental Table 1, as they are transcripts not previously known to be regulated. As a final approach, we collected an additional four microarray samples, but isolated only the VC nociceptors from the pleural ganglion, which are thought to make a major contribution to expressing long-term sensitization memory. Analysis of VC neurons was as previously described (Herdegen et al. 2014a). We have found that microarray results from VC neurons is well-correlated with results from the whole ganglia (Conte et al. 2017), but used this approach to increase power and to potentially screen for any transcripts uniquely regulated in the VC neurons. The one transcript further identified via this approach is marked "VC Cluster" in Supplemental Table 1.

We found that transcriptional regulation largely decays along with recall. Of the transcripts known to be regulated 1 d after training, 98% (1172/1198) were no longer significantly regulated 7 d after training. These null results are not likely to be due to poor sensitivity, as our paired design and sample size conferred an estimated false negative rate of only 1.4% (Langaas et al. 2005). In addition, examining the correlation in gene expression changes across time points supports the same conclusion: very little of the pattern of regulation evident 1 d after training is still evident after recall has decayed ($r^2 = 0.038$ [.03, 0.04], $N = 25,091$, $P < 0.001$; Fig. 2).

Not all of the transcriptional changes associated with maintenance faded: the microarray analysis revealed 35 very persistently regulated transcripts (Supplemental Table 1). Due to the high risk of false positives with such a short list, we sought verification using
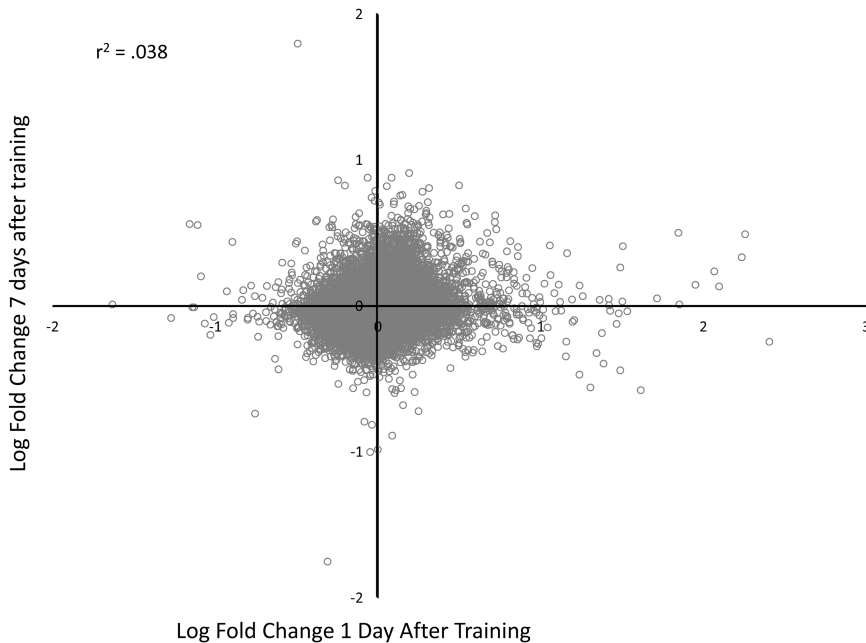
$r^2 = .038$

Log Fold Change 7 days after training

Log Fold Change 1 Day After Training

Figure 2. Very little of the pattern of regulation evident 1 d after training is preserved 7 d after training. This scatter plot compares microarray data from 7 d after training (y axis) to results from a previous analysis completed 1 d after training (x axis; data from Conte et al. 2017). Each axis shows the log-fold change in gene expression comparing trained to untrained side (0 represents no change, positive values represent up-regulation, negative values represent down-regulation). Each dot is a single transcript out of the 25,091 measured on the array. This plot was created using the genas function (genuine association) for limma.

qPCR in independent samples ($N = 11$ additional biological replicates; protocol as described in Conte et al. 2017; see Supplemental Fig. 1B). As expected, a high proportion (18 of 29) did not reach statistical significance in the validation set. We did, however, confirm that 11 transcripts exhibit persistent regulation that outlasts the recall of sensitization memory (Fig. 3, listed in ascending order by average log-fold change). These include a transcript encoding the peptide transmitter Phe-Met-Arg-Phe NH2 (FMRFamide, GenBank: M11283 Schaefer et al. 1985), a transcript encoding a putative homolog of spectrin beta chain (Genbank:
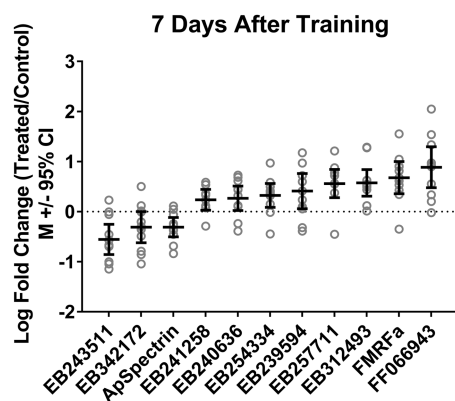

7 Days After Training

Log Fold Change (Treated/Control) M +/- 95% CI

EB243511 EB342172 ApSpectrin EB241258 EB240636 EB254334 EB239594 EB257711 EB312493 FMRFa FF066943

Figure 3. Gene expression is regulated after recall decay. qPCR from an independent set of samples ($n = 11$) indicates continued differential expression of these 11 transcripts one week after long-term sensitization training, a point at which recall has completely decayed but savings memory is still evident. Transcripts are shown in rank order from most down-regulated to most up-regulated.

EB255259), a transcript encoding a protein with a predicted EF-hand calcium-binding domain (Genbank: EB257711), and eight uncharacterized transcripts. Note that the regulation of FMRF-amide was detected in microarray samples from pleural ganglia ($P_{corrected} = 0.006$, $n = 8$) but not from VC clusters ($P_{corrected} = 0.72$, $n = 4$). This is consistent with the fact that the VCs do not express FMRF-amide.

The mechanisms that mediate savings memory are likely to be complex and to involve multiple levels of nervous system function. One key finding is that learning can induce structural plasticity that persists after recall decays (Linkenhoker et al. 2005; Hofer et al. 2009). This work shows that transcriptional regulation induced by learning can also outlast recall. As these transcripts are regulated in tandem with both the decay of recall and the persistence of savings memory, they could potentially be related to forgetting (recall decay), latent memory (savings memory), or both, or neither. Functional experiments are required to clarify the significance of these long-lasting transcriptional changes.

The extended persistence of some transcriptional aspects of the putative memory trace suggests that decay theories of forgetting are not entirely correct. Still, we observed substantial degradation of transcriptional regulation. A challenge for future work is determining how different components of a memory trace persist in a way that is ineffective for recall but still sufficient for savings memory. In this light, it is notable that we observed long-lasting up-regulation of FMRFamide, a peptide transmitter that can function as a memory suppressor by antagonizing the expression of sensitization memory (Fioravante et al. 2006). In addition, the down-regulation of a putative spectrin beta chain homolog is intriguing, as gene silencing via DNA methylation may play a critical role in the maintenance of sensitization memory (Pearce et al. 2017). Both of these transcriptional changes could potentially produce the increased lability in the reflex circuit required for savings memory.

All data for this project is posted to the Open Science Framework (https://osf.io/zyj3w/). The microarray data are also posted to NCBI's Gene Expression Omnibus (Geo: GSE99792).

Acknowledgements

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