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## RNA from Trained *Aplysia* Can Induce an Epigenetic Engram for Long-Term Sensitization in Untrained *Aplysia*

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1 **Manuscript Title**

2 RNA from Trained *Aplysia* Can Induce an Epigenetic Engram for Long-Term Sensitization in  
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4 **Abbreviated Title**

5 Memory Transfer in *Aplysia*

6 **List of Authors' Names and Affiliations**

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20 **Author Contributions**

21

DLG conceived and designed the research. SC, DC, KP and AB also designed the research. KP,  
22 SC, and AB performed the research. DC and AB contributed cell cultures for the research. DC  
23 and AB analyzed data. DLG wrote the paper.

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42

43 **Abstract**

44  
45 The precise nature of the engram, the physical substrate of memory, remains uncertain. Here, it  
46 is reported that RNA extracted from the central nervous system of *Aplysia* given long-term  
47 sensitization training induced sensitization when injected into untrained animals; furthermore,  
48 the RNA-induced sensitization, like training-induced sensitization, required DNA methylation.  
49 In cellular experiments, treatment with RNA extracted from trained animals was found to  
50 increase excitability in sensory neurons, but not in motor neurons, dissociated from naïve  
51 animals. Thus, the behavioral, and a subset of the cellular, modifications characteristic of a form  
52 of nonassociative long-term memory in *Aplysia* can be transferred by RNA. These results  
53 indicate that RNA is sufficient to generate an engram for long-term sensitization in *Aplysia* and  
54 are consistent with the hypothesis that RNA-induced epigenetic changes underlie memory  
55 storage in *Aplysia*.

56

57 **Significance Statement**

58 It is generally accepted that long-term memory (LTM) is encoded as alterations in synaptic  
59 strength. An alternative model, however, proposes that LTM is encoded by epigenetic changes.  
60 Non-coding RNAs can mediate epigenetic modifications. Therefore, RNA from a trained animal  
61 might be capable of producing learning-like behavioral change in an untrained animal. Here, it  
62 is demonstrated that the memory for long-term sensitization in the marine mollusk *Aplysia* can  
63 be successfully transferred by injecting RNA from sensitized into naïve animals. Moreover, a  
64 specific cellular alteration that underlies sensitization in *Aplysia*, sensory neuron  
65 hyperexcitability, can be reproduced by exposing sensory neurons in vitro to RNA from trained  
66 animals. The results provide support for a nonsynaptic, epigenetic model of memory storage in  
67 *Aplysia*.

68

69 **Introduction**

70 A major goal of modern neuroscience is to determine the identity of the engram, the physical  
71 memory trace (Semon, 1921). At present, it is widely accepted that long-term memory (LTM) is  
72 stored by learning-induced modifications of synaptic connections (Mayford et al., 2012;  
73 Takeuchi et al., 2014). But theoretical considerations (Gallistel and Balsam, 2014; Holliday,  
74 1999) and recent experimental evidence (Chen et al., 2014; Johansson et al., 2014; Ryan et al.,  
75 2015) support the idea that LTM is stored within the cell bodies of neurons. Previously, it was  
76 reported that the memory for long-term sensitization (LTS) in *Aplysia* (Pinsker et al., 1973)  
77 involves an early, protein synthesis-dependent priming component that can persist independently  
78 of memory-related behavioral and synaptic alterations; the priming component permits LTM to  
79 be reinstated following its disruption by reconsolidation blockade, or to be induced by partial  
80 training after impairment of memory consolidation by retrograde amnesia (Chen et al., 2014;  
81 Pearce et al., 2017). The molecular identity of the memory priming component is unknown, but  
82 appears to involve epigenetic modifications (Zovkic et al., 2013). Non-coding RNAs, which  
83 play important roles in memory formation (Fiumara et al., 2015; Guven-Ozkan et al., 2016;  
84 Rajasethupathy et al., 2012; Rajasethupathy et al., 2009; Tan et al., 2017), represent a major  
85 mechanism for epigenetic alterations (Peschansky and Wahlestedt, 2014; Savell et al., 2016).  
86 This raises the intriguing possibility that constituents of LTM may be transferred from a trained  
87 to an untrained animal by RNA. Here, we tested this possibility in the case of LTS in *Aplysia*.

88

89 **Materials and methods**90 **Behavioral training and testing**

91 Adult *Aplysia californica* (80-120 g) were obtained from Alacrity Marine Biological Services  
92 (Redondo Beach, CA, USA) and initially housed in a 50-gal aquarium filled with cooled (12-  
93 14°C), aerated seawater. For the experiments, the animals were placed individually into custom-  
94 built Plexiglass chambers that were continuously perfused with cooled (14°C) seawater. One day  
95 before training, each animal was implanted bilaterally with Teflon-coated platinum wires (0.008-  
96 in coated diameter, A-M Systems, Carlsborg, WA). For this procedure, the animal was  
97 anesthetized by cooling in cold seawater (4°C) for 13 min. Wires, prepared by removing the  
98 Teflon from the ends with forceps, were threaded through a 20-gauge needle, which was used to  
99 insert the wire into the animal's tail. Following this procedure, the animal was placed into the  
100 experimental chamber, where it was given 24 h to recover and acclimate to the chamber. The  
101 siphon-withdrawal reflex (SWR) was tested as follows: The siphon was lightly stimulated with a  
102 soft, flexible probe and the duration of the resulting SWR was timed. Timing of the SWR began  
103 once the siphon had retracted completely beneath the parapodia and ended as soon as the siphon  
104 reappeared. Responses were given a score of 1.0 s if the siphon did not withdraw completely  
105 into the parapodia. Three pretests were delivered once every 10 min, beginning 25 min before  
106 the start of training (**Figs. 1A, 2A**). Sensitization training comprised two rounds of training  
107 separated by 24 h. Each round of training consisted of five bouts of tail shocks delivered at 20-  
108 min intervals. During each bout of training, the animal received three trains; the intertrain  
109 interval was 2 s. Each train was 1 s in duration and consisted of shocks (10-ms pulse duration,  
110 40 Hz, 120 V) delivered to the animal's tail via a Grass stimulator (S88, Astro-Med, West

111 Warwick, RI) connected to the platinum wires. A single posttest of the SWR, performed exactly  
112 as the pretests, was made at 48 hr after the start of training. The testing and training were carried  
113 out by different experimenters, and the tester was blind to the experimental treatment of the  
114 animal.

115 In the experiments involving RNA injections (below), naïve animals were given three  
116 pretests, identical to those that preceded the sensitization training, at 30 min, 20 min and 10 min  
117 before the injection (**Figs. 1C, 2C**). A single posttest of the SWR was performed at 24 h after the  
118 injection.

119

#### 120 **RNA and drug preparation and injection**

121 To prepare a single RNA injection, the pleural-pedal and abdominal ganglia were removed from  
122 4-5 sensitization-trained animals—or from 4-5 untrained controls—immediately after the 48-h  
123 posttest. The total RNA was then extracted from the dissected ganglia. The ganglia were initially  
124 homogenized in Trizol reagent for 30 s; typically, 1 ml Trizol was used to homogenize the  
125 central ganglia from two animals. For every 1 ml Trizol reagent, 200  $\mu$ l chloroform was added  
126 and mixed by vortexing for 15 s. After incubation at room temperature for 5-10 min, the sample  
127 was centrifuged at 12,000g for 15 min. The upper aqueous phase was transferred into a new tube.  
128 The sample was then centrifuged for 10 min at 4°C after addition of 500  $\mu$ l isopropanol to  
129 precipitate the RNA. The resulted RNA pellets were washed with 70% ethanol and centrifuged  
130 for 2 min at 4°C. After being air-dried for 10 min, the RNA pellet from each tube was dissolved  
131 in 30  $\mu$ l DIH<sub>2</sub>O; then the RNA from ganglia dissected from trained animals (typically, from four  
132 animals) was combined—or the RNA from ganglia dissected from untrained animals was  
133 combined—into a single tube, and the RNA concentration was measured using Nano Drop

134 (Thermo fisher ND-1000). After the RNA concentration had been determined, 70  $\mu\text{g}$  of the  
135 combined RNA was aliquoted and ASW was added to this aliquot to attain a volume of 100  $\mu\text{l}$ ;  
136 this solution was then injected into the hemocoel of an animal via its neck. Each recipient  
137 animal therefore received 70  $\mu\text{g}$  of either RNA from trained animals or RNA from control  
138 animals.

139 The DNA methyltransferase (DNMT) inhibitor RG108 (Sigma, St Louis, MO) was  
140 dissolved in DMSO to a concentration of 25 mM. To inhibit DNMT, a volume of 100  $\mu\text{l}$  per 100  
141 g of body weight of RG108 was injected intrahemocoelically into each animal (**Fig. 2C**).

#### 142 **Cell culturing and electrophysiological measurements**

143 **Cell culturing and electrophysiological measurements**  
144  
145 Pleural sensory neurons and small siphon (LFS) motor neurons were individually dissociated  
146 from adult animals and placed into cell culture (Lin and Glanzman, 1994; Rayport and Schacher,  
147 1986). Some of the cell cultures comprised isolated neurons, either exclusively sensory or motor  
148 neurons; others comprised synaptically coupled pairs of neurons, each consisting of a single  
149 sensory neuron and a single motor neuron. The cell culture medium was composed of 50%  
150 *Aplysia* sterile hemolymph and 50% Leibowitz-15 (L-15, Sigma, St Louis, MO, USA). During  
151 electrophysiological recording the cell cultures were perfused with 50% ASW and 50% L-15  
152 (recording medium). The recordings from isolated neurons were made on dissociated neurons  
153 that had been in culture for 5 d at the start of the experiments. For the experiments on  
154 synaptically coupled pairs of neurons (sensorimotor cocultures), the neurons were in culture for 3  
155 d prior to the initial recordings. The neurons were impaled with sharp micropipettes (20-30 M $\Omega$ )  
156 filled with 1.5 M potassium acetate, 0.5 M potassium chloride and 0.01 M HEPES (pH = 7.2).  
157 The recorded voltage signals were amplified with an Axoclamp 2B amplifier (Molecular



158 Devices, Sunnyvale, CA), digitalized with an ITC-18 (Instrutech, Port Washington, NY, USA),  
159 and acquired and stored using Axograph software (axograph.com).

160         During the measurements of the biophysical properties of isolated sensory and motor  
161 neurons, the cell membrane potential was current clamped at  $-50$  mV. The action potential (AP)  
162 firing threshold was determined by injecting 2-s current pulses of incremental intensity (0.1 nA  
163 for the sensory neurons and 0.01 nA for the motor neurons). Cells were injected with a 2-s  
164 steady pulse of suprathreshold positive current for the measurements of neuronal excitability  
165 (Liu et al., 2011). In the case of the sensory neurons, current pulses of 0.5 nA, 1.0 nA, or 2.0 nA  
166 were used depending on whether the initial firing threshold was  $< 0.5$  nA,  $\geq 0.5$  nA, or  $\geq 1.0$  nA,  
167 respectively. Sensory neurons were excluded from the analysis if their resting membrane  
168 potential was more depolarized than  $-35$  mV. To test the excitability of motor neurons, positive  
169 current pulses of 0.1 nA, 0.2 nA, or 0.3 nA were used when the initial spike threshold was  $< 0.1$   
170 nA,  $\geq 0.1$  nA, or  $\geq 0.2$  nA, respectively. Motor neurons whose membrane potentials were more  
171 depolarized than  $-30$  mV were excluded. After the electrophysiological measurements were  
172 completed, the microelectrodes were removed from the neurons, and the cell cultures were  
173 treated with RNA-containing medium or vehicle solution (see below). Twenty-four hours later  
174 the neurons were reimpaled and their electrophysiological properties remeasured.

175         In the experiments involving sensorimotor cocultures, the amplitude of the monosynaptic  
176 excitatory postsynaptic potential (EPSP) evoked by a single presynaptic AP was assessed on Day  
177 1 of the experiment. For this purpose, the presynaptic sensory neuron and postsynaptic motor  
178 neuron in the coculture were impaled with sharp microelectrodes. To prevent the motor neuron  
179 from spontaneously firing during testing, the neuron's membrane potential was held at  $-80$  to  $-$   
180  $85$  mV by passing negative current (0.3–0.8 nA) into the cell via the recording microelectrode



181 using the bridge circuit of the amplifier. An initial EPSP was elicited through brief intracellular  
182 stimulation of the sensory neuron using a positive current pulse (20 ms, 0.2–0.8 nA). After the  
183 pretest, the microelectrodes were removed from the sensory and motor neurons, and the  
184 recording medium was replaced with cell culture medium. Then the coculture was treated either  
185 with RNA-containing medium or control medium (see below). The sensory and motor neurons  
186 were reimpaled with microelectrodes and the amplitude of the monosynaptic EPSP reassessed 24  
187 h later.

188

#### 189 **RNA/vehicle treatment of cell cultures**

190 Following the initial electrophysiological measurements on Day 1, the recording medium was  
191 washed out with normal cell culture medium. The cultures were then randomly assigned to  
192 treatment with RNA from trained animals (Trained RNA group), RNA from untrained animals  
193 (Control RNA group), or vehicle. For the RNA treatments, 1  $\mu$ g of RNA was added to each cell  
194 culture dish, yielding a concentration of 0.5  $\mu$ g of RNA per 1 mL of cell culture medium. The  
195 RNA from the trained animals, the RNA from the control animals, or the vehicle was added to  
196 the cell culture dish and left in the dish for 24 h, after which it was washed out with the recording  
197 medium for 30 min, and the posttest electrophysiological measurements made.

198

#### 199 **Statistical analyses**

200 The statistical analyses of the data were performed using SigmaStat (Systat Software, San Jose,  
201 CA). Nonparametric tests were used to assess the statistical significance of differences whenever  
202 necessitated due to non-normality of the data or to the violation of the assumption of  
203 homogeneity of variance among experimental groups. Mann–Whitney *U* tests were used for

204 comparisons of two independent groups. A paired *t*-test or a Wilcoxon rank-sum test was used  
205 to compare two dependent groups. When three independent groups were involved, the  
206 significance of the overall group differences was initially assessed with a one-way ANOVA or a  
207 Kruskal-Wallis test. Given that the group differences were significant, Dunn's posthoc tests  
208 were used for pairwise comparisons. Normality of the distribution were tested with a Shapiro-  
209 Wilk Test. Levene's test centered to the mean (car package) was used with R software to test for  
210 homogeneity of variance in the synaptic experiments (**Fig. 4**). All reported levels of significance  
211 represent two-tailed values. The statistical analyses are summarized in **Table 1**.

212

## 213 **Results**

### 214 **Injection of RNA from sensitization-trained donor animals causes enhancement of the** 215 **withdrawal reflex in untrained recipients**

216 To generate the RNA used for memory transfer, individual *Aplysia* were given sensitization  
217 training consisting of spaced bouts of tail shocks for two consecutive days (**Fig. 1A**). The  
218 training produced clear long-term sensitization (LTS), as indicated by the significant  
219 enhancement of the siphon-withdrawal reflex (SWR) 24 h after the second day of training (48-h  
220 posttest) in the Trained group of animals (**Fig. 1B**). Immediately after the 48-h posttest, RNA  
221 was extracted from the central nervous system (pleural, pedal and abdominal ganglia) of the  
222 Control and Trained animals. The extracted RNA was then injected intrahemocoelically into  
223 other naïve *Aplysia* (recipient animals; **Fig. 1C**). (Note that occasional batches of wild-caught  
224 *Aplysia* did not sensitize. The behavioral data from these animals were excluded from the  
225 analysis, and RNA was not extracted from them.) The duration of the SWR in the recipients was  
226 measured 24 h after the RNA injection. The SWR was significantly enhanced in the Trained

227 RNA group of animals compared to the Control RNA group (**Fig. 1D**). Furthermore, a within-  
228 group comparison indicated that the posttest duration of the reflex was significantly longer than  
229 the pretest duration in the animals that received the injection of the RNA from trained donors; by  
230 contrast, the posttest SWR was not significantly prolonged compared to the pretest SWR in  
231 animals that received the injection of RNA from the untrained donors. Thus, only the RNA from  
232 sensitized animals appeared to induce reflex enhancement in the recipient snails.

233

234 **Inhibition of DNA methylation blocks the behavioral effect of RNA from sensitized donor**  
235 **animals in the recipients.**

236 Both the consolidation and maintenance of the LTM for sensitization in *Aplysia* depend on DNA  
237 methylation (Pearce et al., 2017; Rajasethupathy et al., 2012). To determine whether the RNA-  
238 mediated behavioral enhancement similarly required DNA methylation, we examined whether  
239 inhibiting DNA methylation disrupted the sensitizing effect of the RNA from trained animals.  
240 *Aplysia* were again given two days of sensitization training, which produced LTS, and afterwards  
241 RNA was extracted from their central ganglia (**Fig. 2A, B**). The RNA was then injected into two  
242 groups of naïve snails; 5-10 min later, one of these groups (RNA-RG group) was also given an  
243 intrahemocoelic injection of the DNA methyltransferase (DNMT) inhibitor RG-108 (Brueckner  
244 et al., 2005; Pearce et al., 2017), whereas the other (RNA-Veh group) was given an injection of  
245 the vehicle solution (**Fig. 2C**). The RNA-Veh group exhibited significant enhancement of the  
246 SWR 24 h later; by contrast, the RNA-RG group did not show behavioral enhancement (**Fig.**  
247 **2D**). Therefore, DNA methylation is required for RNA-induced enhancement of the SWR, as it  
248 is for tail shock-induced LTS of the reflex (Pearce et al., 2017).

249

250 **RNA from sensitized animals induces increased excitability in sensory neurons dissociated**  
251 **from naïve animals**

252 A significant advantage of *Aplysia* as a model system for mechanistic analyses of learning and  
253 memory is the wealth of extant knowledge regarding the biological bases of sensitization in this  
254 organism (Byrne and Hawkins, 2015; Kandel, 2001). Accordingly, we tested whether RNA  
255 extracted from sensitization-trained animals caused cellular alternations that mimic those known  
256 to result from repeated tail shocks. To ascertain whether the cellular changes induced by RNA  
257 from sensitized animals mimic shock-induced cellular changes, we made use of sensory and  
258 motor neurons of the withdrawal circuit in dissociated cell culture (Lin and Glanzman, 1994).

259 In response to a prolonged pulse of depolarizing intracellular current, *Aplysia* sensory  
260 neurons exhibit spike “accommodation”: they fire at the beginning, but not throughout the  
261 current pulse (Klein et al., 1986). Long-lasting sensitization of the defensive withdrawal reflex  
262 is accompanied by a long-term increase in the excitability of the somata of central sensory  
263 neurons in the withdrawal circuit (Walters, 1987); this enhanced excitability is reflected as anti-  
264 accommodation, an increase in the number of action potentials evoked by a prolonged pulse of  
265 positive current (Cleary et al., 1998). To test whether RNA extracted from trained *Aplysia* alters  
266 sensory neuron accommodation, we used isolated sensory neurons in dissociated cell culture.  
267 The neurons were initially impaled with sharp microelectrodes and the number of action  
268 potentials evoked by a 2-s intracellular pulse of suprathreshold positive current quantified (**Fig.**  
269 **3A**). Following this pretest, the sensory neurons were treated for 24 h with RNA from trained  
270 donors or RNA from untrained donors. Other sensory neurons were treated with an equivalent  
271 amount of the vehicle alone. The next day the RNA/vehicle was washed out of the culture dishes  
272 with cell recording medium, and the neurons were reimpaled and reinjected with the same

273 suprathreshold current to measure potential changes in excitability. The current injections  
274 produced significantly more action potentials in sensory neurons treated with RNA from  
275 sensitized animals than in sensory neurons treated with either vehicle or RNA from control  
276 animals (**Fig. 3B**). There was no significant difference in excitability between the sensory  
277 neurons treated with control RNA and those treated with the vehicle. Anti-accommodation is  
278 known to result from a decrease in cyclic AMP-dependent potassium currents in *Aplysia* sensory  
279 neurons, and, in particular, to reduction of the slowly-inactivating S-type current (Goldsmith and  
280 Abrams, 1992; Klein et al., 1986); thus, the RNA from sensitization-trained animals may  
281 enhance the excitability of sensory neurons through modulation of the same current that is  
282 modulated by electrical shocks to the body wall of *Aplysia*.

283

284 **RNA from sensitized animals does not increase the excitability of dissociated motor**  
285 **neurons**

286 To ascertain the specificity of the cellular effects of the RNA treatment, we examined the effects  
287 of applying RNA from trained or control animals to isolated small siphon (LFS) motor neurons  
288 in dissociated cell culture. A previous study of LTS in *Aplysia* showed that, in contrast to the  
289 effects observed in sensory neurons, in motor neurons LTS was not accompanied by a significant  
290 increase in the number of APs evoked to intracellular injection of a prolonged pulse of  
291 suprathreshold current (Cleary et al., 1998). Thus, the induction of LTS does not produce an  
292 overall increase in the excitability of motor neurons. Similarly, we observed no effect of the  
293 RNA from sensitization-trained animals on excitability-related properties of isolated motor  
294 neurons in cell culture (**Fig. 3C, D**). This result indicates that the modulation of neuronal  
295 excitability by RNA from sensitized animals was specific to the sensory neurons.

296

297 **RNA from sensitized animals has a variable effect on synaptic strength in sensorimotor**

298 **cocultures**

299 Long-term sensitization in *Aplysia* involves long-term facilitation (LTF) of the monosynaptic  
300 connection between the sensory and motor neurons of the withdrawal circuit (Frost et al., 1985).

301 Accordingly, we examined the effects of RNA from trained and untrained donors on the strength  
302 of sensorimotor synapses in dissociated cell culture (Cai et al., 2008; Montarolo et al., 1986).

303 There was no long-term effect of 24-h incubation with RNA from trained animals, RNA from  
304 control animals, or from the vehicle on the mean EPSP evoked in the postsynaptic motor neurons

305 by a presynaptic AP (**Fig. 4**). Nonetheless, although the mean EPSPs in the three experimental

306 groups did not differ significantly, the variances among the EPSPs in the three groups were

307 significantly unequal due to the greater variance in the EPSPs for the synapses treated with RNA

308 from sensitization-trained animals. Inspection of the synaptic data revealed that the RNA from

309 trained donors produced large enhancement of a subset of the sensorimotor synapses. Such

310 enhancement was never observed for synapses treated with RNA from untrained animals or for

311 synapses treated with the vehicle.

312

### 313 **Discussion**

314 We have shown that RNA from sensitization-trained *Aplysia* contains critical components of the

315 engram for LTS, as indicated by its ability to induce sensitization-like behavioral enhancement

316 when injected into naïve recipient animals. Importantly, the RNA-induced sensitization, like the

317 LTS induced by noxious stimulation, requires DNA methylation for its consolidation (Pearce et

318 al., 2017) (**Fig. 2**). Several of our cellular and behavioral results further argue that this putative

319 transference of memory from donor animals to the recipients cannot be easily ascribed to  
320 nonspecific effects of the donor RNA. First, the control RNA (RNA extracted from untrained  
321 donors) did not produce sensitization of the SWR (**Fig. 1**). Second, the RNA from trained  
322 donors had an opposing effect on the excitability of cultured sensory neurons from that of  
323 untrained donors (**Fig. 3A, B**). Third, the changes produced by the RNA from sensitized *Aplysia*  
324 were selective for sensory neurons; the biophysical properties of motor neurons were unaltered  
325 by the RNA from sensitized donors (**Fig. 3C, D**). Admittedly, the alterations we observed in the  
326 biophysical properties of cultured sensory neurons after treatment with RNA from sensitized  
327 animals are unlikely to fully account for the behavioral changes produced in the intact recipient  
328 animals by injections of RNA from trained donors (below); nonetheless, because these  
329 biophysical alterations mimic those found in intact animals after LTS training (Cleary et al.,  
330 1998; Walters, 1987), they would be expected to contribute substantially to the RNA-induced  
331 sensitization.

332         It is interesting that the RNA from sensitized-trained animals appeared to produce strong  
333 facilitation only in a subset of sensorimotor synapses (**Fig. 4**). We do not understand the reason  
334 for the variability of the synaptic effect of the RNA from trained animals. One possibility is that  
335 there is as yet unappreciated inhomogeneity among the population of pleural sensory neurons  
336 and/or small siphon (LFS) motor neurons that were used for the sensorimotor cocultures;  
337 according to this idea, only some of the dissociated neurons had the capacity to express the long-  
338 term changes that contribute to LTF. Another possibility is that the epigenetic alterations,  
339 particularly DNA methylation, that result from treatment with the RNA from sensitized animals  
340 more reliably induce cell-wide alterations, such as changes in intrinsic neuronal excitability



341 (Meadows et al., 2016; see also Meadows et al., 2015), than synapse-specific LTF. Of course,  
342 these possibilities are not mutually exclusive.

343 Overall, the cellular changes caused by the RNA from trained animals were admittedly  
344 modest compared to the behavioral changes. But this is not unexpected; the defensive  
345 withdrawal reflexes in *Aplysia* are regulated by interneuronal neural circuits, in addition to the  
346 monosynaptic sensorimotor connections (Cleary et al., 1995). Injections of the RNA from  
347 sensitized donors may well have produced modifications of interneuronal pathways within the  
348 animals that contributed to behavioral sensitization. In addition, it is important to note that the  
349 RNA was removed from the donors 48 h after training; indeed, the RNA from trained animals  
350 produced a greater increase in the excitability of cultured sensory neurons at 48 h posttraining  
351 than did long-term training with serotonin (Liu et al., 2011; see their Fig. 6).

352 Our data indicate that essential components of the engram for LTM in *Aplysia* can be  
353 transferred to untrained animals, or to neurons in culture, via RNA. This finding raises two  
354 questions: (1) Which specific RNA(s) mediate(s) the memory transfer?, and (2) How does the  
355 naked RNA get from the hemolymph/cell culture medium into *Aplysia* neurons? Regarding the  
356 first question, although we do not know the identity of the memory-bearing molecules at present,  
357 we believe it is likely that they are non-coding RNAs (ncRNAs). Note that previous results have  
358 implicated ncRNAs, notably microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs)  
359 (Fiumara et al., 2015; Rajasethupathy et al., 2012; Rajasethupathy et al., 2009), in LTM in  
360 *Aplysia*. Long non-coding RNAs (lncRNAs) represent other potential candidate memory transfer  
361 molecules (Mercer et al., 2008). Regarding the second question, recent evidence has revealed  
362 potential pathways for the passage of cell-free, extracellular RNA from body fluids into neurons.  
363 Thus, miRNAs, for example, have been detected in many different types of body fluids,

364 including blood plasma; and cell-free extracellular miRNAs can become encapsulated within  
365 exosomes or attached to proteins of the Argonaut (AGO) family, thereby rendering the miRNAs  
366 resistant to degradation by extracellular nucleases (Turchinovich et al., 2013; Turchinovich et al.,  
367 2012). Moreover, miRNA-containing exosomes have been reported to pass freely through the  
368 blood-brain barrier (Ridder et al., 2014; Xu et al., 2017). And it is now appreciated that RNAs  
369 can be exchanged between cells of the body, including between neurons, via extracellular  
370 vesicles (Ashley et al., 2018; Pastuzyn et al., 2018; Smalheiser, 2007; Tkach and Théry, 2016;  
371 Valadi et al., 2007). If, as we believe, ncRNAs in the RNA extracted from sensitized animals  
372 were transferred to *Aplysia* neurons, perhaps via extracellular vesicles, they likely caused one or  
373 more epigenetic effects that contributed to the induction and maintenance of LTM (**Fig. 2**).

374         There have been prior reports of the successful transfer of LTM from trained donor  
375 animals to naïve recipients via cannibalism (McConnell, 1962) or RNA injection (Albert, 1966;  
376 Babich et al., 1965; Braud, 1970; Jacobson et al., 1965). However, these early claims have long  
377 been viewed with skepticism due to numerous failures to replicate the memory transfer effect  
378 (Byrne et al., 1966; Gross and Carey, 1965; Hartry et al., 1964; Luttges et al., 1966; McGaugh,  
379 1967; Walker, 1966; Walker and Milton, 1966). The negative results convinced many that the  
380 positive reports of memory transfer were attributable to lack of proper controls for training-  
381 induced factors such as stress or arousal, and/or the influence of poorly defined aspects of the  
382 experimental methods used (time between the RNA injection and behavioral testing of the  
383 recipients, specific method of RNA extraction, etc.) (McGaugh, 1967; Setlow, 1997).

384         A major advantage of our study over earlier studies of memory transfer is that we used a  
385 type of learning, sensitization of the defensive withdrawal reflex in *Aplysia*, the cellular and  
386 molecular basis of which is exceptionally well characterized (Byrne and Hawkins, 2015; Kandel,

387 2001; Kandel, 2012). The extensive knowledge base regarding sensitization in *Aplysia* enabled  
388 us to show that the RNA from sensitized donors not only produced sensitization-like behavioral  
389 change in the naïve recipients, but also caused specific electrophysiological alterations of  
390 cultured neurons that mimic those observed in sensitized animals. The cellular changes observed  
391 after exposure of cultured neurons to RNA from trained animals significantly strengthens the  
392 case for positive memory transfer in our study.

393 Another difference between our study and earlier attempts at memory transfer via RNA is  
394 that there is now at hand a mechanism, unknown 40 years ago, whereby RNA can powerfully  
395 influence the function of neurons: epigenetic modifications (Qureshi and Mehler, 2012). In fact,  
396 the role of ncRNA-mediated epigenetic changes in neural function, particularly in learning and  
397 memory, is currently the subject of vigorous investigation (Fischer, 2014; Landry et al., 2013;  
398 Marshall and Bredy, 2016; Nestler, 2014; Smalheiser, 2014; Sweatt, 2013). Our demonstration  
399 that inhibition of DNA methylation blocks the memory transfer effect (**Fig. 2**) supports the  
400 hypothesis that the behavioral and cellular effects of RNA from sensitized *Aplysia* in our study  
401 are mediated, in part, by DNA methylation (see also Pearce et al., 2017; Rajasethupathy et al.,  
402 2012).

403 The discovery that RNA from trained animals can transfer the engram for long-term  
404 sensitization in *Aplysia* offers dramatic support for the idea that memory can be stored  
405 nonsynaptically (Gallistel and Balsam, 2014; Holliday, 1999; Queenan et al., 2017), and  
406 indicates the limitations of the synaptic plasticity model of long-term memory storage (Mayford  
407 et al., 2012; Takeuchi et al., 2014). In addition, our results suggest that RNA could eventually be  
408 used to modify, either enhance or depress, memories.

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410

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562 **Figure Legends**

563

564 **Figure 1.** RNA extracted from sensitization-trained donor animals induces long-term565 enhancement of the SWR in recipient *Aplysia*. **A**, Experimental protocol for inducing LTS in the566 donor animals. **B**, Mean posttest duration of the SWR in the untrained Control ( $1.2 \pm 0.1$  s,  $n =$ 567 31) and Trained ( $56.4 \pm 2.0$  s,  $n = 34$ ) groups. The Trained group exhibited significant568 sensitization, as indicated by the comparison with Control group (Mann-Whitney test,  $U = 496$ ,  $p$ 569  $< 0.001$ ). **C**, Experimental protocol for the RNA injection experiments. The first pretest occurred570 2-3 h after the posttest for the behavioral training (see **A**). **D**, Mean duration of the SWR571 measured at  $\sim 24$  h after the injection of RNA for the Control RNA ( $5.4 \pm 3.9$  s,  $n = 7$ ) and572 Trained RNA ( $38.0 \pm 4.6$  s,  $n = 7$ ) groups. The two groups differed significantly ( $U = 30$ ,  $p <$ 573  $0.003$ ). Furthermore, Wilcoxin tests indicated that the difference between the pretest and posttest574 for the Trained RNA group was significant ( $W = 28$ ,  $p < 0.02$ ), whereas it was not significant for575 Control RNA group ( $p > 0.2$ ). The bar graphs in this and the following figures display means  $\pm$ 576 SEM. Also,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ , and n.s., non-significant.

577

578 **Figure 2. DNA methylation is required for RNA-induced enhancement of the SWR.** **A**,579 Experimental protocol for inducing sensitization in the second donor group. **B**, Mean posttest580 duration of the SWR ( $n = 38$ ). The training produced sensitization (mean posttest SWR =  $56.4 \pm$ 581  $1.4$  s, and mean pretest SWR =  $1.1 \pm 0.1$  s;  $W = 741$ ,  $p < 0.001$ ). **C**, Experimental protocol for

582 testing the effect of DNMT inhibition on RNA-induced enhancement of the SWR. RG-

583 108/vehicle was injected into animals 5-10 min after the RNA injection. **D**, Mean post-injection584 duration of the SWR in the RNA-Veh ( $n = 3$ ), and RNA-RG ( $n = 7$ ) groups. The mean duration585 of the SWR in the RNA-Veh group ( $35.7 \pm 7.7$  s) was significantly longer than that in the RNA-

586 RG group ( $1.4 \pm 0.3$  s;  $U = 27$ ,  $p < 0.02$ ). Moreover, the posttest SWR was sensitized compared  
587 to the pretest reflex in the RNA-Veh group (paired  $t$ -test,  $p < 0.05$ ), but not in the RNA-RG ( $p >$   
588 0.4) group.

589

590 **Figure 3. Treatment with RNA from trained animals increases excitability in dissociated**

591 **sensory neurons, but not in dissociated motor neurons.** *A*, Sample electrophysiological traces

592 from excitability tests on sensory neurons. Scale bars: 20 mV, 0.25 s. *B*, Changes in the

593 excitability of the sensory neurons induced by RNA/vehicle treatment. The mean change in

594 evoked action potentials (APs) in each group was: Vehicle =  $-17.29 \pm 12.86\%$  ( $n = 19$ ); mean

595 Control RNA =  $-35.76 \pm 19.88\%$  ( $n = 16$ ); Trained RNA =  $56.66 \pm 22.07\%$  ( $n = 19$ ). The group

596 differences were significant (Kruskal-Wallis;  $H = 11.81$ ,  $p < 0.04$ ). Dunn's posthoc tests

597 indicated that the increased firing in the Trained RNA group was greater than that in the Vehicle

598 group ( $q = 2.44$ ,  $p < 0.05$ ) and Control RNA group ( $q = 3.25$ ,  $p < 0.004$ ), respectively. The

599 difference between Vehicle and Control RNA groups was not significant ( $p > 0.9$ ). *C*, Sample

600 traces from tests of motor neuron excitability. Scale bars: 25 mV, 0.25 s. *D*, Summary of post-

601 treatment changes in the excitability of motor neurons. The mean changes were: Vehicle group,  $-$

602  $29.28 \pm 19.16\%$  ( $n = 15$ ); Control RNA group,  $5.278 \pm 34.36\%$  ( $n = 12$ ); and Trained RNA

603 group,  $-1.136 \pm 34.01\%$  ( $n = 14$ ). The group differences in excitability were insignificant ( $p >$

604 0.7).

605

606 **Figure 4. Exposure of *in vitro* sensorimotor synaptic connections to RNA from trained**

607 **animals does not alter synaptic strength.** *A*, Representative records of EPSPs evoked in motor

608 neurons by a single presynaptic AP before and 24 h after the RNA/vehicle treatments. Scale bars:

609 5 mV, 0.1s. **B**, Box and whiskers plots showing the distribution of posttreatment changes in  
610 EPSP amplitude in the three experimental groups. The boxes delineate the second and third  
611 quartiles, the horizontal lines in the boxes represent the medians, and the vertical bars (whiskers)  
612 show the extent of the data spread. The crosses indicate the means, whereas individual data  
613 points are represented by circles. Mean post-treatment changes in EPSP amplitudes were:  
614 Vehicle group =  $-23.38 \pm 10.59\%$  ( $n = 23$ ); Control RNA group =  $-21.32 \pm 10.23\%$  ( $n = 34$ );  
615 Trained RNA =  $22.71 \pm 26.70\%$  ( $n = 32$ ). A Kruskal-Wallis test revealed no significant  
616 differences among the groups with respect to the mean changes in EPSP amplitude ( $p > 0.8$ ).  
617 Note, however, that 5 of the 32 synapses treated with RNA from trained animals showed an  
618 increase of  $> 150\%$ , whereas none of the synapses treated with vehicle or RNA from control  
619 animals showed increases of this magnitude. A Levene's Test confirmed that the three groups  
620 displayed significantly unequal variances ( $F_{[2,86]} = 5.883$ ,  $p < 0.005$ ).  
621

622

623 **Table 1. Statistical Table**

624

	<b>Data structure</b>	<b>Type of test</b>	<b>Power (<math>\alpha = 0.05</math>)</b>
a (Fig. 1B)	Non-normally distributed	Mann-Whitney test	Not applicable
b (Fig. 1D)	Non-normally distributed	Mann-Whitney test	Not applicable
c (Fig. 1D)	Non-normally distributed	Wilcoxon test	Not applicable
d (Fig. 1D)	Non-normally distributed	Wilcoxon test	Not applicable
e (Fig. 2B)	Non-normally distributed	Wilcoxon test	Not applicable
f (Fig. 2D)	Non-normally distributed	Mann-Whitney test	Not applicable
g (Fig. 2D)	Normally distributed	Paired <i>t</i> -test	0.647
h (Fig. 2D)	Non-normally distributed	Wilcoxon test	Not applicable
i (Fig. 3B)	Non-normally distributed	Kruskal-Wallis test followed by Dunn's test	Not applicable
j (Fig. 3D)	Non-normally distributed	Kruskal-Wallis test	Not applicable
k (Fig. 4B)	Non-normally distributed	Levene's test	Not applicable
l (Fig. 4B)	Non-normally distributed	Kruskal-Wallis test	Not applicable

625









