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

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Manuscript Title

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Abbreviated Title

Memory Transfer in *Aplysia*

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Abstract

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

Significance Statement

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

Introduction

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

Materials and methods

Behavioral training and testing

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

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

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

RNA and drug preparation and injection

To prepare a single RNA injection, the pleural-pedal and abdominal ganglia were removed from 4-5 sensitization-trained animals—or from 4-5 untrained controls—immediately after the 48-h posttest. The total RNA was then extracted from the dissected ganglia. The ganglia were initially homogenized in Trizol reagent for 30 s; typically, 1 ml Trizol was used to homogenize the central ganglia from two animals. For every 1 ml Trizol reagent, 200 µl chloroform was added and mixed by vortexing for 15 s. After incubation at room temperature for 5-10 min, the sample was centrifuged at 12,000g for 15 min. The upper aqueous phase was transferred into a new tube. The sample was then centrifuged for 10 min at 4^{o}C after addition of 500 µl isopropanol to precipitate the RNA. The resulted RNA pellets were washed with 70% ethanol and centrifuged for 2 min at 4^{o}C. After being air-dried for 10 min, the RNA pellet from each tube was dissolved in 30 µl DIH_2O; then the RNA from ganglia dissected from trained animals (typically, from four animals) was combined—or the RNA from ganglia dissected from untrained animals was combined—into a single tube, and the RNA concentration was measured using Nano Drop

(Thermo fisher ND-1000). After the RNA concentration had been determined, 70 µg of the combined RNA was aliquoted and ASW was added to this aliquot to attain a volume of 100 µl; this solution was then injected into the hemocoel of an animal via its neck. Each recipient animal therefore received 70 µg of either RNA from trained animals or RNA from control animals.

The DNA methyltransferase (DNMT) inhibitor RG108 (Sigma, St Louis, MO) was dissolved in DMSO to a concentration of 25 mM. To inhibit DNMT, a volume of 100 µl per 100 g of body weight of RG108 was injected intrahemocoelically into each animal (**Fig. 2*C***).

Cell culturing and electrophysiological measurements

Pleural sensory neurons and small siphon (LFS) motor neurons were individually dissociated from adult animals and placed into cell culture (Lin and Glanzman, 1994; Rayport and Schacher, 1986). Some of the cell cultures comprised isolated neurons, either exclusively sensory or motor neurons; others comprised synaptically coupled pairs of neurons, each consisting of a single sensory neuron and a single motor neuron. The cell culture medium was composed of 50% *Aplysia* sterile hemolymph and 50% Leibowitz-15 (L-15, Sigma, St Louis, MO, USA). During electrophysiological recording the cell cultures were perfused with 50% ASW and 50% L-15 (recording medium). The recordings from isolated neurons were made on dissociated neurons that had been in culture for 5 d at the start of the experiments. For the experiments on synaptically coupled pairs of neurons (sensorimotor cocultures), the neurons were in culture for 3 d prior to the initial recordings. The neurons were impaled with sharp micropipettes (20-30 MΩ) filled with 1.5 M potassium acetate, 0.5 M potassium chloride and 0.01 M HEPES (pH = 7.2). 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, ≥ 0.5 nA, or ≥ 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, ≥ 0.1 nA, or ≥ 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

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

RNA/vehicle treatment of cell cultures

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

Statistical analyses

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

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

Results

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

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

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

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

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

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

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

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

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

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

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

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

Long-term sensitization in *Aplysia* involves long-term facilitation (LTF) of the monosynaptic connection between the sensory and motor neurons of the withdrawal circuit (Frost et al., 1985). Accordingly, we examined the effects of RNA from trained and untrained donors on the strength of sensorimotor synapses in dissociated cell culture (Cai et al., 2008; Montarolo et al., 1986). There was no long-term effect of 24-h incubation with RNA from trained animals, RNA from control animals, or from the vehicle on the mean EPSP evoked in the postsynaptic motor neurons by a presynaptic AP (**Fig. 4**). Nonetheless, although the mean EPSPs in the three experimental groups did not differ significantly, the variances among the EPSPs in the three groups were significantly unequal due to the greater variance in the EPSPs for the synapses treated with RNA from sensitization-trained animals. Inspection of the synaptic data revealed that the RNA from trained donors produced large enhancement of a subset of the sensorimotor synapses. Such enhancement was never observed for synapses treated with RNA from untrained animals or for synapses treated with the vehicle.

Discussion

We have shown that RNA from sensitization-trained *Aplysia* contains critical components of the engram for LTS, as indicated by its ability to induce sensitization-like behavioral enhancement when injected into naïve recipient animals. Importantly, the RNA-induced sensitization, like the LTS induced by noxious stimulation, requires DNA methylation for its consolidation (Pearce et al., 2017) (**Fig. 2**). Several of our cellular and behavioral results further argue that this putative

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

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

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

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

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

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

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

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

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

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

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

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561

Figure Legends

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

Figure 2. DNA methylation is required for RNA-induced enhancement of the SWR. ***A,*** Experimental protocol for inducing sensitization in the second donor group. ***B,*** Mean posttest duration of the SWR (n = 38). The training produced sensitization (mean posttest SWR = 56.4 ± 1.4 s, and mean pretest SWR = 1.1 ±0.1 s; $W = 741$, $p < 0.001$). ***C,*** Experimental protocol for testing the effect of DNMT inhibition on RNA-induced enhancement of the SWR. RG-108/vehicle was injected into animals 5-10 min after the RNA injection. ***D,*** Mean post-injection duration of the SWR in the RNA-Veh (n = 3), and RNA-RG (n = 7) groups. The mean duration of the SWR in the RNA-Veh group (35.7 ± 7.7 s) was significantly longer than that in the RNA-

586 RG group (1.4 ± 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 ± 12.86% ($n = 19$); mean
595 Control RNA = –35.76 ± 19.88% ($n = 16$); Trained RNA = 56.66 ± 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 ± 19.16% ($n = 15$); Control RNA group, 5.278 ± 34.36% ($n = 12$); and Trained RNA
603 group, –1.136 ± 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 ± 10.59% (n = 23); Control RNA group = –21.32 ± 10.23% (n = 34);
615 Trained RNA = 22.71 ± 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

	Data structure	Type of test	Power (α = 0.05)
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 *t*-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