Cognition and Behavior

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

Alexis Bédécarrats,[1,*] Shanping Chen,[1,*] Kaycey Pearce,[1,*] Diancai Cai,[1] and David L. Glanzman[1,2,3]

[1]Department of Integrative Biology and Physiology, University of California, Los Angeles, Los Angeles, CA 90095, [2]Department of Neurobiology, David Geffen School of Medicine at University of California, Los Angeles, Los Angeles, CA 90095, and [3]Integrative Center for Learning and Memory, Brain Research Institute, David Geffen School of Medicine at University of California, Los Angeles, Los Angeles, CA 90095

Visual Abstract
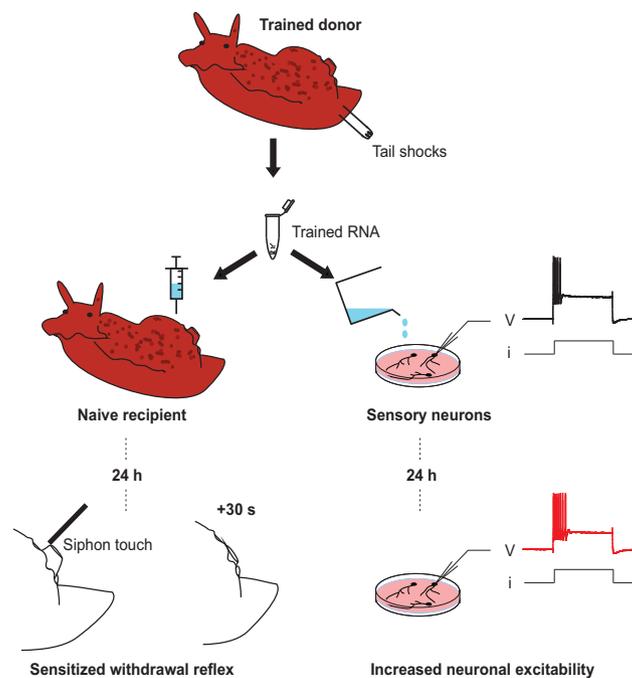
Significance Statement

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. Noncoding RNAs (ncRNAs) 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 (LTS) 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*.

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 (LTS) 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 (LTM) in *Aplysia* can be transferred by RNA. These results indicate that RNA is sufficient to generate an engram for LTS in *Aplysia* and are consistent with the hypothesis that RNA-induced epigenetic changes underlie memory storage in *Aplysia*.

Key words: Aplysia; epigenetics; learning and memory; memory transfer; RNA; sensitization

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 (Holliday, 1999; Gallistel and Balsam, 2014) 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). Noncoding RNAs (ncRNAs), which play important roles in memory formation (Rajasethupathy et al., 2009, 2012; Fiumara et al., 2015; Guven-Ozkan et al., 2016; 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 and initially housed in a 50-gallon aquarium filled with cooled (12–14°C), aerated seawater. For the experiments, the animals were placed individually into custom-built Plexiglas 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-inch coated diameter, A-M Systems). 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) connected to the platinum wires. A single posttest of the SWR, performed exactly as the pretests, was made at 48 h after the start of training. The testing and training were conducted by different experimenters, and the tester was blind to the experimental treatment of the animal.

In the experiments involving RNA injections (see Results), naïve animals were given three pretests, identical
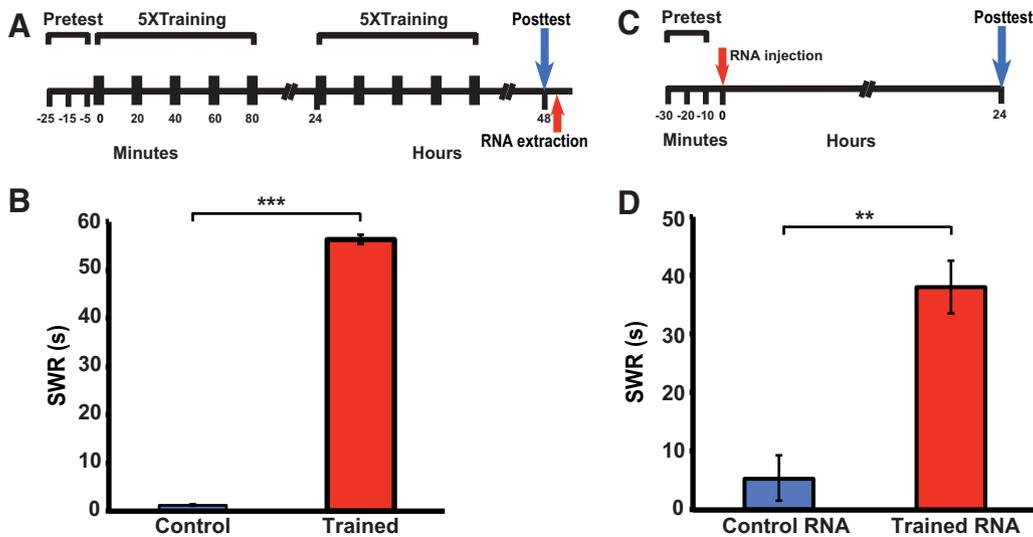
Figure 1. RNA extracted from sensitization-trained donor animals induces long-term enhancement of the SWR in recipient *Aplysia*. *A*, Experimental protocol for inducing LTS in the donor animals. *B*, Mean posttest duration of the SWR in the untrained control (1.2 ± 0.1 s, $n = 31$) and trained (56.4 ± 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 (*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, Wilcoxon 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 the control RNA group ($p > 0.2$). The bar graphs in this and the following figures display means $\pm$ SEM; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, n.s., nonsignificant.

to those that preceded the sensitization training, at 30, 20, and 10 min before the injection (Figs. 1C, 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 four to five sensitization-trained animals, or from four to five untrained
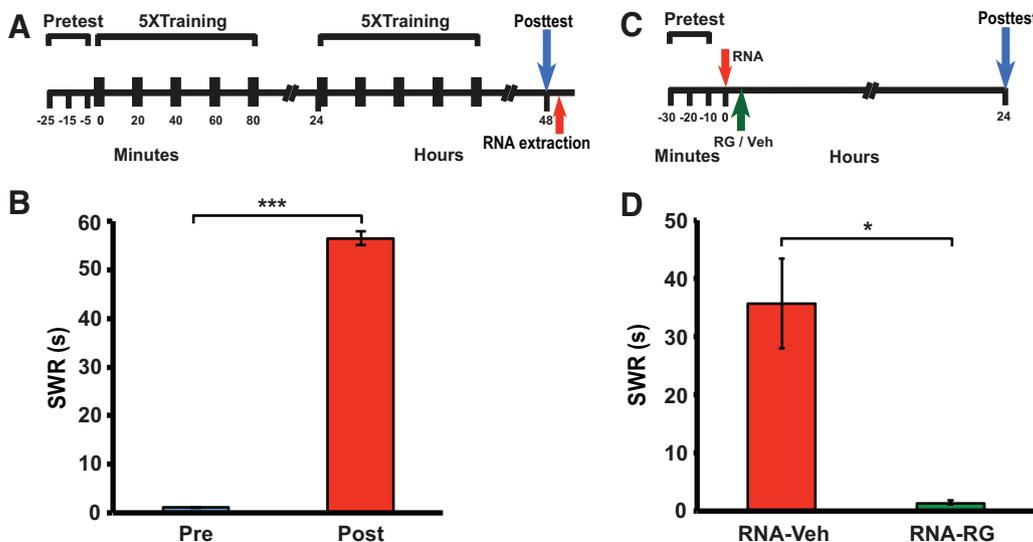
Figure 2. DNA methylation is required for RNA-induced enhancement of the SWR. *A*, Experimental protocol for inducing sensitization in the second donor group. *B*, Mean posttest duration of the SWR ($n = 38$). The training produced sensitization (mean posttest SWR = 56.4 ± 1.4 s, and mean pretest SWR = 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 postinjection 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-RG group (1.4 ± 0.3 s; $U = 27$, $p < 0.02$). Moreover, the posttest SWR was sensitized compared to the pretest reflex in the RNA-Veh group (paired t test, $p < 0.05$), but not in the RNA-RG group ($p > 0.4$).

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 12,000 × g for 15 min. The upper aqueous phase was transferred into a new tube. The sample was then centrifuged for 10 min at 4°C after addition of 500 μl isopropanol to precipitate the RNA. The resulting RNA pellets were washed with 70% ethanol and centrifuged for 2 min at 4°C. After being air-dried for 10 min, the RNA pellet from each tube was dissolved in 30 μl DIH$_2$O; 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) was dissolved in DMSO to a concentration of 25 mM. To inhibit DNMT, a volume of 100 μl/100 g of body weight of RG108 was injected intrahemocoelically into each animal (Fig. 2C).

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 (Rayport and Schacher, 1986; Lin and Glanzman, 1994). Some of the cell cultures comprised isolated neurons, either exclusively sensory or exclusively 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). During electrophysiological recording the cell cultures were perfused with 50% ASW and 50% L-15 (recording medium). The recordings from isolated neurons were made using 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 before 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 Devices), digitalized with an ITC-18 (Instrutech), and acquired and stored using Axograph software.

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

In the experiments involving sensorimotor cocultures, the amplitude of the monosynaptic EPSP evoked by a single presynaptic AP was assessed on day 1 of the experiment. For this purpose, the presynaptic sensory neuron and postsynaptic motor neuron in the coculture were impaled with sharp microelectrodes. To prevent the motor neuron from spontaneously firing during testing, the neuron's membrane potential was held at –80 to –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 Results). 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). Nonparametric tests

Table 1. Statistical table

	Data structure	Type of test	Power ($\alpha = 0.05$)
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

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 *post hoc* 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. 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. 1A). The training produced clear LTS, as indicated by the significant enhancement of the SWR 24 h after the second day of training (48-h posttest) in the trained group of animals (Fig. 1B). 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. 1C). (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. 1D). 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 (Rajasethupathy et al., 2012; Pearce et al., 2017). 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 2 d of sensitization training, which produced LTS, and afterward RNA was extracted from their central ganglia (Fig. 2A,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 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. 2C). 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. 2D). 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 (Kandel, 2001; Byrne and Hawkins, 2015). 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 of, 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 APs 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 APs evoked by a 2-s intracellular pulse of suprathreshold positive current quantified (Fig. 3A). 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 APs in sensory neurons treated with RNA from sensitized animals than in sensory neurons treated with either vehicle or RNA from control animals (Fig. 3B). 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 (Klein et al., 1986; Goldsmith and Abrams, 1992); 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. 3C,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

LTS 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 (Montarolo et al., 1986; Cai et al., 2008). There was no long-term effect of 24-h incubation with RNA from trained animals, RNA from control animals, or 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. 3A,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. 3C,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; nonetheless, because these biophysical alterations mimic those found in intact animals after LTS training (Walters, 1987; Cleary et al., 1998), they would be expected to contribute substantially to the RNA-induced sensitization.

It is interesting that the RNA from sensitization-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 an as-yet unappreciated inhomogeneity
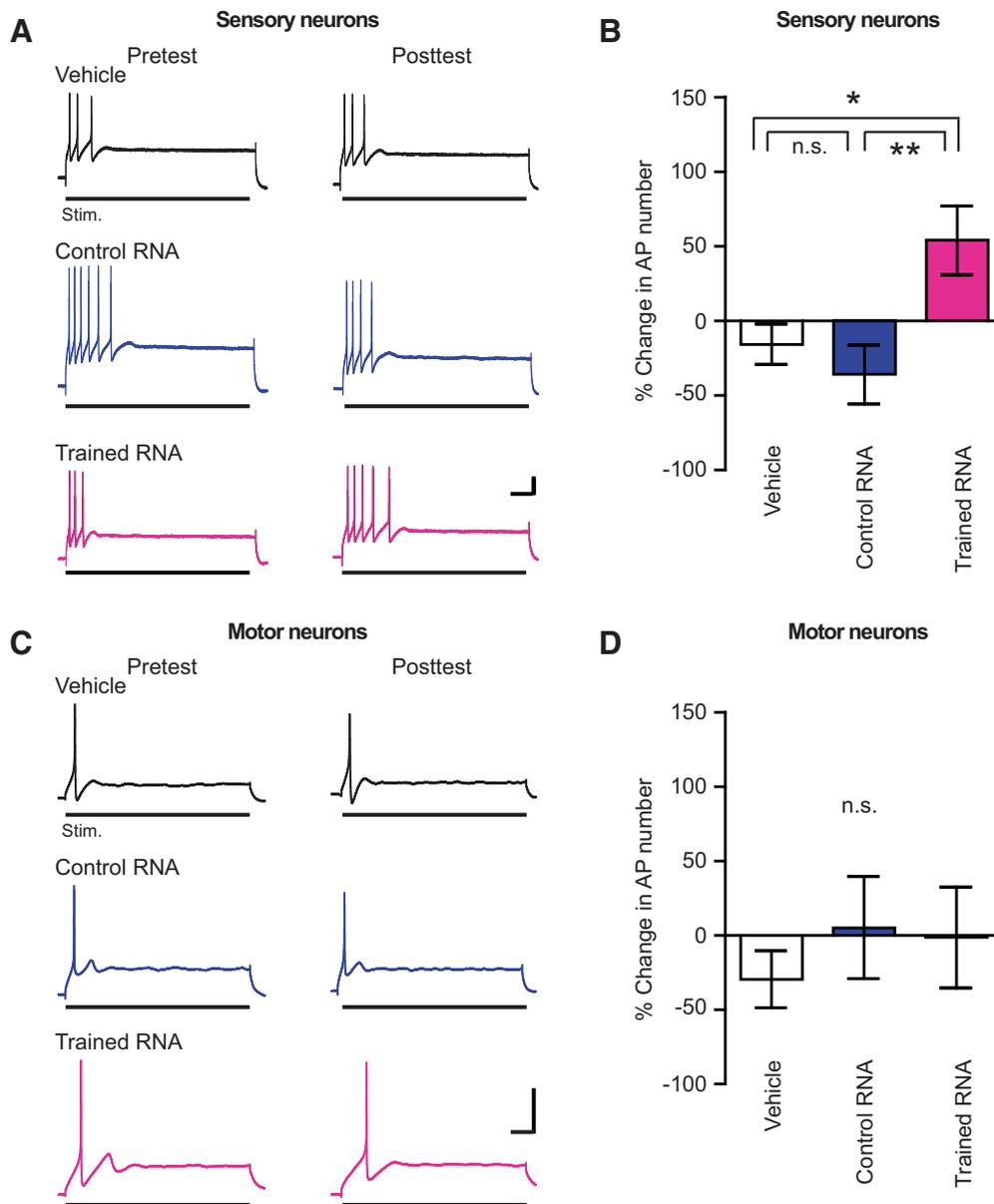
Figure 3. Treatment with RNA from trained animals increases excitability in dissociated sensory neurons but not in dissociated motor neurons. *A*, Sample electrophysiological traces from excitability tests on sensory neurons. Scale bars: 20 mV, 0.25 s. *B*, Changes in the excitability of the sensory neurons induced by RNA/vehicle treatment. The mean change in evoked APs in each group was: vehicle = −17.29 ± 12.86% (*n* = 19); control RNA = −35.76 ± 19.88% (*n* = 16); and trained RNA = 56.66 ± 22.07% (*n* = 19). The group differences were significant (Kruskal–Wallis; *H* = 11.81, *p* < 0.04). Dunn's *post hoc* tests indicated that the increased firing in the trained RNA group was greater than that in the vehicle group (*q* = 2.44, *p* < 0.05) and control RNA group (*q* = 3.25, *p* < 0.004), respectively. The difference between vehicle and control RNA groups was not significant (*p* > 0.9). *C*, Sample traces from tests of motor neuron excitability. Scale bars: 25 mV, 0.25 s. *D*, Summary of posttreatment changes in the excitability of motor neurons. The mean changes were: vehicle group = −29.28 ± 19.16% (*n* = 15); control RNA group = 5.278 ± 34.36% (*n* = 12); and trained RNA group = −1.136 ± 34.01% (*n* = 14). The group differences in excitability were insignificant (*p* > 0.7).

among the population of pleural sensory neurons and/or small siphon 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). In-
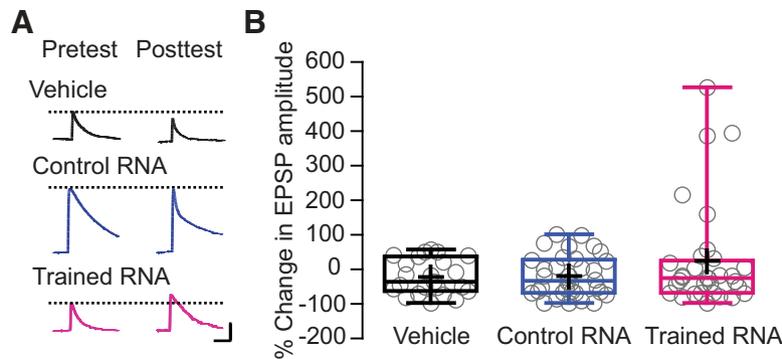
Figure 4. Exposure of *in vitro* sensorimotor synaptic connections to RNA from trained animals enhanced the strength of a subpopulation of synapses. *A*, Representative records of EPSPs evoked in motor neurons by a single presynaptic AP before and 24 h after the RNA/vehicle treatments. Scale bars: 5 mV, 0.1s. *B*, Box and whiskers plots showing the distribution of posttreatment changes in EPSP amplitude in the three experimental groups. The boxes delineate the second and third quartiles, the horizontal lines in the boxes represent the medians, and the vertical bars (whiskers) show the extent of the data spread. The crosses indicate the means, whereas individual data points are represented by circles. Mean posttreatment changes in EPSP amplitudes were: vehicle group = −23.38 ± 10.59% (n = 23); control RNA group = −21.32 ± 10.23% (n = 34); and trained RNA group = 22.71 ± 26.70% (n = 32). A Kruskal–Wallis test revealed no significant differences among the groups with respect to the mean changes in EPSP amplitude (p > 0.8). Note, however, that five of the 32 synapses treated with RNA from trained animals showed an increase of >150%, whereas none of the synapses treated with vehicle or RNA from control animals showed an increase of this magnitude. A Levene's test confirmed that the three groups displayed significantly unequal variances ($F_{(2,86)}$ = 5.883, $p < 0.005$).

jections 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 long-term training with serotonin (Liu et al., 2011, 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 ncRNAs. Note that previous results have implicated ncRNAs, notably microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs; Rajasethupathy et al., 2009, 2012; Fiumara et al., 2015), in LTM in *Aplysia*. Long ncRNAs (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., 2012, 2013). 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 vesi-

cles (Smalheiser, 2007; Valadi et al., 2007; Tkach and Théry, 2016; Ashley et al., 2018; Pastuzyn et al., 2018). 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 (Babich et al., 1965; Jacobson et al., 1965; Albert, 1966; Braud, 1970). However, these early claims have long been viewed with skepticism due to numerous failures to replicate the memory transfer effect (Hartry et al., 1964; Gross and Carey, 1965; Byrne et al., 1966; Luttges et al., 1966; Walker, 1966; Walker and Milton, 1966; McGaugh, 1967). 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 (Kandel, 2001; Kandel, 2012; Byrne and Hawkins, 2015). 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 (Landry et al., 2013; Sweatt, 2013; Fischer, 2014; Nestler, 2014; Smalheiser, 2014; Marshall and Bredy, 2016). 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 (Rajasethupathy et al., 2012; see also Pearce et al., 2017).

The discovery that RNA from trained animals can transfer the engram for LTS in *Aplysia* offers dramatic support for the idea that memory can be stored nonsynaptically (Holliday, 1999; Gallistel and Balsam, 2014; Queenan et al., 2017), and indicates the limitations of the synaptic plasticity model of LTM 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.

References

Albert DJ (1966) Memory in mammals: evidence for a system involving nuclear ribonucleic acid. Neuropsychologia 4:79–92. CrossRef

Ashley J, Cordy B, Lucia D, Fradkin LG, Budnik V, Thomson T (2018) Retrovirus-like Gag protein Arc1 binds RNA and traffics across synaptic boutons. Cell 172:262–274.e11. CrossRef

Babich FR, Jacobson AL, Bubash S, Jacobson A (1965) Transfer of a response to naive rats by injection of ribonucleic acid extracted from trained rats. Science 149:656–657. Medline

Braud WG (1970) Extinction in goldfish: facilitation by intracranial injection of RNA from brains of extinguished donors. Science 168:1234–1236. Medline

Brueckner B, Garcia Boy R, Siedlecki P, Musch T, Kliem HC, Zielenkiewicz P, Suhai S, Wiessler M, Lyko F (2005) Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases. Cancer Res 65:6305–6311. CrossRef Medline

Byrne JH, Hawkins RD (2015) Nonassociative learning in invertebrates. Cold Spring Harb Perspect Biol 7:a021675. CrossRef

Byrne WL, Samuel D, Bennett EL, Rosenzweig MR, Wasserman E (1966) Memory transfer. Science 153:658–659. Medline

Cai D, Chen S, Glanzman DL (2008) Postsynaptic regulation of long-term facilitation in *Aplysia*. Curr Biol 18:920–925. CrossRef Medline

Chen S, Cai D, Pearce K, Sun PY, Roberts AC, Glanzman DL (2014) Reinstatement of long-term memory following erasure of its behavioral and synaptic expression in *Aplysia*. Elife 3:e03896. CrossRef Medline

Cleary LJ, Byrne JH, Frost WN (1995) Role of interneurons in defensive withdrawal reflexes in *Aplysia*. Learn Mem 2:133–151. Medline

Cleary LJ, Lee WL, Byrne JH (1998) Cellular correlates of long-term sensitization in *Aplysia*. J Neurosci 18:5988–5998. Medline

Fischer A (2014) Epigenetic memory: the Lamarckian brain. EMBO J 33:945–967. CrossRef Medline

Fiumara F, Rajasethupathy P, Antonov I, Kosmidis S, Sossin WS, Kandel ER (2015) MicroRNA-22 gates long-term heterosynaptic plasticity in *Aplysia* through presynaptic regulation of CPEB and downstream targets. Cell Rep 11:1866–1875. CrossRef Medline

Frost WN, Castellucci VF, Hawkins RD, Kandel ER (1985) Monosynaptic connections made by the sensory neurons of the gill- and siphon-withdrawal reflex in *Aplysia* participate in the storage of long-term memory for sensitization. Proc Natl Acad Sci USA 82:8266–8269. Medline

Gallistel CR, Balsam PD (2014) Time to rethink the neural mechanisms of learning and memory. Neurobiol Learn Mem 108:136–144. CrossRef Medline

Goldsmith BA, Abrams TW (1992) cAMP modulates multiple K^+ currents, increasing spike duration and excitability in *Aplysia* sensory neurons. Proc Natl Acad Sci USA 89:11481–11485. Medline

Gross CG, Carey FM (1965) Transfer of learned response by RNA injection: failure of attempts to replicate. Science 150:1749. Medline

Guven-Ozkan T, Busto GU, Schutte SS, Cervantes-Sandoval I, O'Dowd DK, Davis RL (2016) MiR-980 is a memory suppressor microRNA that regulates the autism-susceptibility gene A2bp1. Cell Rep 14:1698–1709. CrossRef Medline

Hartry AL, Keith-Lee P, Morton WD (1964) Planaria: memory transfer through cannibalism reexamined. Science 146:274–275. CrossRef

Holliday R (1999) Is there an epigenetic component in long-term memory? J Theor Biol 200:339–341. CrossRef Medline

Jacobson AL, Babich FR, Bubash S, Jacobson A (1965) Differential-approach tendencies produced by injection of RNA from trained rats. Science 150:636–637. Medline

Johansson F, Jirenhed DA, Rasmussen A, Zucca R, Hesslow G (2014) Memory trace and timing mechanism localized to cerebellar Purkinje cells. Proc Natl Acad Sci USA 111:14930–14934. CrossRef Medline

Kandel ER (2001) The molecular biology of memory storage: a dialogue between genes and synapses. Science 294:1030–1038. CrossRef Medline

Kandel ER (2012) The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. Mol Brain 5:14. CrossRef Medline

Klein M, Hochner B, Kandel ER (1986) Facilitatory transmitters and cAMP can modulate accommodation as well as transmitter release in *Aplysia* sensory neurons: evidence for parallel processing in a single cell. Proc Natl Acad Sci USA 83:7994–7998. CrossRef

Landry CD, Kandel ER, Rajasethupathy P (2013) New mechanisms in memory storage: piRNAs and epigenetics. Trends Neurosci 36:535–542. CrossRef Medline

Lin XY, Glanzman DL (1994) Long-term potentiation of *Aplysia* sensorimotor synapses in cell culture: regulation by postsynaptic voltage. Proc Biol Sci 255:113–118. CrossRef Medline

Liu RY, Cleary LJ, Byrne JH (2011) The requirement for enhanced CREB1 expression in consolidation of long-term synaptic facilitation and long-term excitability in sensory neurons of *Aplysia*. J Neurosci 31:6871–6879. CrossRef Medline

Luttges M, Johnson T, Buck C, Holland J, McGaugh J (1966) An examination of "transfer of learning" by nucleic acid. Science 151:834–837. CrossRef Medline

Marshall P, Bredy TW (2016) Cognitive neuroepigenetics: the next evolution in our understanding of the molecular mechanisms underlying learning and memory? NPJ Sci Learn 1.

Mayford M, Siegelbaum SA, Kandel ER (2012) Synapses and memory storage. Cold Spring Harb Perspect Biol 4:a005751. CrossRef

McConnell JV (1962) Memory transfer through cannibalism in planarians. J Neuropsychiatry 3 [Suppl 1]:S42.

McGaugh JL (1967) Analysis of memory transfer and enhancement. Proc Am Phil Soc 111:347–351.

Meadows JP, Guzman-Karlsson MC, Phillips S, Holleman C, Posey JL, Day JJ, Hablitz JJ, Sweatt JD (2015) DNA methylation regulates neuronal glutamatergic synaptic scaling. Sci Signal 8:ra61. CrossRef Medline

Meadows JP, Guzman-Karlsson MC, Phillips S, Brown JA, Strange SK, Sweatt JD, Hablitz JJ (2016) Dynamic DNA methylation regulates neuronal intrinsic membrane excitability. Sci Signal 9:ra83. CrossRef Medline

Mercer TR, Dinger ME, Mariani J, Kosik KS, Mehler MF, Mattick JS (2008) Noncoding RNAs in long-term memory formation. Neuroscientist 14:434–445. CrossRef Medline

Montarolo PG, Goelet P, Castellucci VF, Morgan J, Kandel ER, Schacher S (1986) A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia*. Science 234:1249–1254. Medline

Nestler EJ (2014) Epigenetic mechanisms of drug addiction. Neuropharmacology 76:259–268. CrossRef

Pastuzyn ED, Day CE, Kearns RB, Kyrke-Smith M, Taibi AV, McCormick J, Yoder N, Belnap DM, Erlendsson S, Morado DR, Briggs JAG, Feschotte C, Shepherd JD (2018) The neuronal gene Arc encodes a repurposed retrotransposon Gag protein that mediates intercellular RNA transfer. Cell 172:275–288.e18. CrossRef

Pearce K, Cai D, Roberts AC, Glanzman DL (2017) Role of protein synthesis and DNA methylation in the consolidation and maintenance of long-term memory in *Aplysia*. Elife 6.

Peschansky VJ, Wahlestedt C (2014) Non-coding RNAs as direct and indirect modulators of epigenetic regulation. Epigenetics 9:3–12. CrossRef Medline

Pinsker HM, Hening WA, Carew TJ, Kandel ER (1973) Long-term sensitization of a defensive withdrawal reflex in *Aplysia*. Science 182:1039–1042. Medline

Queenan BN, Ryan TJ, Gazzaniga MS, Gallistel CR (2017) On the research of time past: the hunt for the substrate of memory. Ann NY Acad Sci 1396:108–125. CrossRef Medline

Qureshi IA, Mehler MF (2012) Emerging roles of non-coding RNAs in brain evolution, development, plasticity and disease. Nat Rev Neurosci 13:528–541. CrossRef Medline

Rajasethupathy P, Fiumara F, Sheridan R, Betel D, Puthanveettil SV, Russo JJ, Sander C, Tuschl T, Kandel E (2009) Characterization of small RNAs in *Aplysia* reveals a role for miR-124 in constraining synaptic plasticity through CREB. Neuron 63:803–817. CrossRef Medline

Rajasethupathy P, Antonov I, Sheridan R, Frey S, Sander C, Tuschl T, Kandel ER (2012) A role for neuronal piRNAs in the epigenetic control of memory-related synaptic plasticity. Cell 149:693–707. CrossRef Medline

Rayport SG, Schacher S (1986) Synaptic plasticity *in vitro*: cell culture of identified *Aplysia* neurons mediating short-term habituation and sensitization. J. Neurosci 6:759–763. Medline

Ridder K, Keller S, Dams M, Rupp AK, Schlaudraff J, Del Turco D, Starmann J, Macas J, Karpova D, Devraj K, Depboylu C, Landfried B, Arnold B, Plate KH, Höglinger G, Sültmann H, Altevogt P, Momma S (2014) Extracellular vesicle-mediated transfer of genetic information between the hematopoietic system and the brain in response to inflammation. PLoS Biol 12:e1001874. CrossRef Medline

Ryan TJ, Roy DS, Pignatelli M, Arons A, Tonegawa S (2015) Engram cells retain memory under retrograde amnesia. Science 348:1007–1013. CrossRef Medline

Savell KE, Gallus NV, Simon RC, Brown JA, Revanna JS, Osborn MK, Song EY, O'Malley JJ, Stackhouse CT, Norvil A, Gowher H, Sweatt JD, Day JJ (2016) Extra-coding RNAs regulate neuronal DNA methylation dynamics. Nat Commun 7:12091. CrossRef

Semon R (1921) The mneme. London: George Allen.

Setlow B (1997) Georges Ungar and memory transfer. J Hist Neurosci 6:181–192. CrossRef Medline

Smalheiser NR (2007) Exosomal transfer of proteins and RNAs at synapses in the nervous system. Biol Direct 2:35. CrossRef Medline

Smalheiser NR (2014) The RNA-centred view of the synapse: non-coding RNAs and synaptic plasticity. Philos Trans R Soc Lond B Biol Sci 369.

Sweatt JD (2013) The emerging field of neuroepigenetics. Neuron 80:624–632. CrossRef Medline

Takeuchi T, Duszkiewicz AJ, Morris RG (2014) The synaptic plasticity and memory hypothesis: encoding, storage and persistence. Philos Trans R Soc Lond B Biol Sci 369:20130288. CrossRef Medline

Tan MC, Widagdo J, Chau YQ, Zhu T, Wong JJ, Cheung A, Anggono V (2017) The activity-induced long non-coding RNA Meg3 modulates AMPA receptor surface expression in primary cortical neurons. Front Cell Neurosci 11:124. CrossRef Medline

Tkach M, Théry C (2016) Communication by extracellular vesicles: where we are and where we need to go. Cell 164:1226–1232. CrossRef Medline

Turchinovich A, Weiz L, Burwinkel B (2012) Extracellular miRNAs: the mystery of their origin and function. Trends Biochem Sci 37:460–465. CrossRef Medline

Turchinovich A, Samatov TR, Tonevitsky AG, Burwinkel B (2013) Circulating miRNAs: cell-cell communication function? Front Genet 4:119. CrossRef Medline

Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9:654–659. CrossRef Medline

Walker DR (1966) Memory transfer in planarians: an artifact of the experimental variables. Psychonom Sci 5:357–358. CrossRef

Walker DR, Milton GA (1966) Memory transfer vs. sensitization in cannibal planarians. Psychonom Sci 5:293–294. CrossRef

Walters ET (1987) Multiple sensory neuronal correlates of site-specific sensitization in *Aplysia*. J Neurosci 7:408–417. Medline

Xu B, Zhang Y, Du XF, Li J, Zi HX, Bu JW, Yan Y, Han H, Du JL (2017) Neurons secrete miR-132-containing exosomes to regulate brain vascular integrity. Cell Res 27:882–897. CrossRef Medline

Zovkic IB, Guzman-Karlsson MC, Sweatt JD (2013) Epigenetic regulation of memory formation and maintenance. Learn Mem 20:61–74. CrossRef Medline