Regulation of memory storage through epigenetic alterations: a new role for RNA

A fundamental assumption in modern psychology and neuroscience is that memory is stored as physical changes in the brain. More than a century ago, the famous neuroanatomist Ramón Y Cajal (see the article entitled “Santiago Ramón y Cajal, the ultimate scientist?” in this issue of *The Biochemist*) postulated that changes in the strength of synaptic connections between neurons were the physical substrate for memory. Extensive experimental evidence has since established the dominance of this connectionist view, referred to as the “synaptic plasticity” model. However, although the synaptic plasticity model broadly accords with the results of neurobiological studies of learning and memory, it does not fully account for the extraordinary resilience of memory despite the significant loss of synapses during such phenomena as development, trauma and ageing. Here, we will focus on the newly discovered role of small non-coding RNAs (ncRNAs) as potential master regulators of learning-induced epigenesis, neuronal plasticity and, ultimately, memory. In support of this idea, recent data from our lab indicate that RNA can promote the transfer of long-term memory from a trained to an untrained (naïve) animal.

The memory transfer experiments

The idea that RNA can mediate the biophysical neuronal changes associated with memory is not new. In the early 1960s, Swedish biologist Holger Hydén was perhaps the first to theorize that RNA is involved in learning and memory. Hydén’s visionary hypothesis found support from one of the most controversial observations made in the history of experimental psychology. In a set of experiments that would later become infamous, James McConnell and colleagues reported that they were able to induce a form of associative learning, known as Pavlovian or classical conditioning, in planarians, a type of flatworm. In this type of learning, the animal receives training in which delivery of an initially neutral stimulus, referred to as the conditioned stimulus or CS, is paired with a stimulus that produces an innate response in an animal; the latter stimulus is known as the unconditioned stimulus or US. After successful training, the CS alone will evoke a response in the animal that resembles that originally induced by the US; this post-training CS-induced response is referred to as the conditioned response or CR. As described by McConnell in a paper published in 1962, worms received training in which a flash of light, the CS, was paired with an electric shock, the US. After several bouts of training, the worms exhibited a CR – either turning or contraction – when exposed just to the light. (Control groups of worms received stimulation with either the light or the shock alone; according to McConnell, these worms did not exhibit the CR.) Then, taking advantage of planarians’ extraordinary capacity for regeneration, McConnell and his team addressed the issue of whether memory could exist outside of the brain. When individual worms that had been classically conditioned were cut into two halves – the tail and head – both halves were able to regenerate the missing part. Impressively, the two types of regenerated worms, both those from head fragments and those from tail fragments, exhibited a conditioned behavioural response when exposed to the light alone. An even more remarkable experimental result followed. In a subsequent set of experiments, the researchers cut trained worms into small
pieces and fed the pieces to naïve worms (planarians are cannibalistic.) The naïve worms that had been fed trained worms subsequently expressed greater numbers of the conditioned responses to the light CS than did naïve worms that had consumed pieces of untrained worms. These results indicated that memory could be transferred from trained to naïve worms through cannibalism.

McConnell’s research attracted a great deal of public attention, as well as significant funding from governmental agencies. Numerous laboratories attempted to replicate the phenomenon of memory transfer, not only in planarians, but also in fish and rats. In line with Hyden’s hypothesis, RNA was believed to be the agent of memory transfer; indeed, several research teams reported successful memory transfer via injection of RNA extracted from trained animals. Unfortunately for McConnell and other proponents of memory transfer, and perhaps, in retrospect, for the field of learning and memory in general, the results of these experiments could not be consistently reproduced. Because of the controversial nature of McConnell’s claim, as well as the absence of a concrete molecular basis for memory transfer, research into this phenomenon sank into oblivion.

**Molecular bases of long-term memory**

It is now widely accepted that long-term memory – memory lasting more than 24 h – requires gene expression and the synthesis of new proteins; the gene products serve to maintain persistent alterations in synaptic strength and neuronal excitability. DNA, the molecular template for the synthesis of proteins, is present in every cell type. In neurons, transcription factors – which control the conversion of DNA into RNA – such as CREB1 and CREB2, trigger or repress the expression of genes required for long-term memory. Eric Kandel, who played a major role in establishing the memory-related functions of these transcription factors in studies of the mollusc *Aplysia*, a large sea slug, won the Nobel Prize in Physiology or Medicine in 2000.

In more recent years, a handful of laboratories have investigated the role of epigenetic processes, which alter the three-dimensional structure of the DNA, in learning and memory. In particular, DNA methylation – the stable addition of a CH3 group to a cytosine base – has attracted significant attention. When DNA is methylated in promoter regions, the transcription and production of proteins is disrupted. If this process prevents the production of proteins that act as transcriptional repressors, such as CREB2, then the transcription and translation of memory-promoting proteins, such as the transcription factor CREB1, which would otherwise be inhibited by CREB2, is favoured; this is believed to facilitate the formation of a stable memory. DNA methyl transferase, the enzyme responsible for DNA methylation, is partly regulated by the presence of small ncRNAs. The relationship between DNA methylation and small ncRNAs is imperfectly understood. It appears, however, that a subclass of small ncRNAs, known as piwi-interacting RNAs or piRNAs, directly interacts with the DNA methyl transferase responsible for the addition of a methyl group to the CREB2 promoter.
RNA regulates long-term memory through DNA methylation

Can a memory truly be transferred via RNA? That is the question we addressed in experiments reported in a recent article published in eNeuro. To attempt a modern demonstration of RNA-induced memory transfer we used *Aplysia* for the following reasons. (1) *Aplysia* exhibits a robust and easily quantifiable defensive response to possibly threatening stimuli – withdrawal of the gill and siphon. When lightly touched, these external organs, which are critical for respiration, retract; the gill retracts beneath the mantle, a flap-like extension of the body wall, and the siphon retracts into the body cavity that contains the mantle and gill. (2) The duration and intensity of this behaviour can be decreased or increased for long periods of time (≥ 24 h) as a consequence of different forms of learning. (3) The neural circuit responsible for this reflex is largely known, both with respect to the individual neurons within the circuit and to the synaptic connections among them. (4) The molecular pathways involved in learning and memory in *Aplysia* are to a great extent analogous to those involved in learning and memory in vertebrates.

To induce the expression of the RNA(s) critical for memory, we first trained a group of animals using a procedure that induces long-term sensitization of the defensive withdrawal response. Here, mild electrical shocks were repeatedly delivered to the tail of the animals in a spaced training regimen. The training resulted in enhancement of siphon withdrawal 24 h later, as measured by the increased duration of the siphon's contraction in response to a light touch. Total RNA was extracted from the central nervous system of the trained donor snails, purified, and then injected directly into the hemolymph of a second group of naïve recipient animals whose siphon withdrawal was unsensitized. Twenty-four hours later the recipients exhibited an enhanced retraction of the siphon, as if they themselves had been subjected to long-term sensitization training. Animals in a second group of naïve recipients received an injection of RNA extracted from an untrained donor group of snails. The animals in this second group of recipients did not exhibit an enhanced reflex when tested 24 h after the injection, which supports the idea that the enhancement of withdrawal observed in the animals that received the RNA from the trained donors was not due to a non-specific effect of the injection. Critically, the RNA-induced long-term sensitization depends on DNA methylation, as shown by the lack of behavioural enhancement when an inhibitor of

**Figure 2. Model for RNA-induced memory in *Aplysia*.** (A) Learning induces serotonin release within the nervous system; serotonin triggers an intracellular molecular cascade involving RNA and protein synthesis that regulates the methylation status of DNA (arrows indicate bi-directional regulations). (B) Among the RNAs synthetized during learning are non-coding RNAs that indirectly regulate the methylation of DNA through the enzyme DNA methyl transferase (DNMT). This methylation silences the transcription of memory repressors (for example, CREB2) thereby promoting the production of a second wave of proteins essential for neuronal plasticity and long-term memory formation.
the DNA methyl transferase, RG-108, was injected into the recipient snails simultaneously with the RNA from trained donor snails. This result lends further credence to the notion that sensitization memory had indeed been transferred by the injected RNA, because, as we had previously showed, tail-shock-induced long-term sensitization is also blocked by RG-108.

The RNA from trained animals also produces some of the biophysical changes associated with long-term memory in *Aplysia*. We observed that, when treated with RNA from trained animals, sensory neurons that mediate the withdrawal reflex showed an increase in intrinsic excitability, similar to the increase observed in animals following training with tail shocks; by contrast, RNA from untrained animals did not induce increased excitability in sensory neurons.

RNA alone therefore appears sufficient to induce critical molecular and cellular changes that represent the physical substrate of memory.

**Which RNAs and how?**

The next step will be to identify which RNAs are responsible for producing these memory-related changes. RNA sequencing analyses performed by Eric Kandel and colleagues have identified hundreds of small ncRNAs that regulate the function of the nervous system in *Aplysia*. Particularly interesting in the context of our results is a 28-nucleotide-sized class of piRNAs. The expression of a subset of these piRNAs was found by Kandel and his fellow researchers to be upregulated by serotonin, the neuromodulatory transmitter required for long-term sensitization in *Aplysia*. Furthermore, the piRNAs were shown to facilitate the methylation of DNA in the promoter region of CREB2, thereby enhancing the induction of long-term memory.

Besides identifying the exact species of RNA that are critical for the memory transfer effect we observed, it will be necessary to determine how the critical RNAs become integrated into the cellular machinery that drives the epigenetic and downstream biophysical changes responsible for long-term memory.

The discovery that application of RNA to the nervous system induces long-term cellular changes that mediate memory in *Aplysia* suggests the potential involvement of an extracellular, RNA-mediated, signalling pathway in memory formation. Finally, the ability of RNA to directly activate molecular pathways that underlie memory opens a promising avenue for future treatments for learning-related disorders, such as dementia and post-traumatic stress disorder.