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51. The work summarized here reflects outstanding contributions from many highly gifted associates who have worked in our laboratory. I would particularly like to mention A. C. Nairn, who has been a close colleague and friend for more than 20 years. This work has also benefited enormously from collaborations with excellent scientists at several other universities. Our work on regulation of ion pumps was carried out in collaboration with A. Aperia at the Karolinska Institute. We continue to collaborate with R. L. Huganir, who was at The Rockefeller University, and is now at The Johns Hopkins University School of Medicine and with E. J. Nestler, who was at the Yale University School of Medicine and is now at the University of Texas Southwestern Medical Center. Much of our electrophysiological work has been done in collaboration with D. J. Surmeier at Northwestern University. The work of our research group has been very generously supported for over 30 years by the National Institutes of Health, including the National Institute of Mental Health, the National Institute on Drug Abuse, and the National Institute on Aging.

REVIEW: NEUROSCIENCE

The Molecular Biology of Memory Storage: A Dialogue Between Genes and Synapses

Eric R. Kandel*

One of the most remarkable aspects of an animal's behavior is the ability to modify that behavior by learning, an ability that reaches its highest form in human beings. For me, learning and memory have proven to be endlessly fascinating mental processes because they address one of the fundamental features of human activity: our ability to acquire new ideas from experience and to retain these ideas over time in memory. Moreover, unlike other mental processes such as thought, language, and consciousness, learning seemed from the outset to be readily accessible to cellular and molecular analysis. I, therefore, have been curious to know: What changes in the brain when we learn? And, once something is learned, how is that information retained in the brain? I have tried to address these questions through a reductionist approach that would allow me to investigate elementary forms of learning and memory at a cellular molecular level—as specific molecular activities within identified nerve cells.

I first became interested in the study of memory in 1950 as a result of my readings in psychoanalysis while still an undergraduate at Harvard College. Later, during medical training, I began to find the psychoanalytic approach limiting because it tended to treat the brain, the organ that generates behavior, as a black box. In the mid-1950s, while still in medical school, I began to appreciate that during my lifetime the black box of the brain would be opened and that the problems of memory storage, once the exclusive domain of psychologists and psychoanalysts, could be investigated with the methods of modern biology. As a result, my interest in memory shifted from a psychoanalytic to a biological approach. As a postdoctoral fellow at the National Institutes of Health (NIH) in Bethesda from 1957 to 1960, I focused on learning more about the biology of the brain and became interested in knowing how learning produces changes in the neural networks of the brain.

My purpose in translating questions about the psychology of learning into the empirical language of biology was not to replace the logic of psychology or psychoanalysis with the logic of cellular molecular biology, but to try to join these two disciplines and to contribute to a new synthesis that would combine the mentalistic psychology of memory storage with the biology of neuronal signaling. I hoped further that the biological analysis of memory might carry with it an extra bonus, that the study of memory storage might reveal new aspects of neuronal signaling. Indeed, this has proven true.

A Radical Reductionist Strategy to Learning and Memory

At first thought, someone interested in learning and memory might be tempted to tackle the problem in its most complex and interesting form. This was the approach that Alden Spencer and I took when we joined forces at NIH in 1958 to study the cellular properties of the hippocampus, the part of the mammalian brain thought to be most directly involved in aspects of complex memory (*1*). We initially asked, rather naïvely: Are the electrophysiological properties of the pyramidal cells of the hippocampus, which were thought to be the key hippocampal cells involved in memory storage, fundamentally different from other neurons in the brain? With study, it became clear to us that all nerve cells, including the pyramidal cells of the hippocampus, have similar signaling properties. Therefore, the intrinsic signaling properties of neurons would themselves not give us key insights into memory storage (*2*). The unique functions of the hippocampus had to arise not so much from the intrinsic properties of pyramidal neurons but from the pattern of functional interconnections of these cells, and how those interconnections are affected by learning. To tackle that problem we needed to know how sensory information about a learning task reaches the hippocampus and how information processed by the hippocampus influences behavioral output. This was a formidable challenge, since the hippocampus has a large number of neurons and an immense number of interconnections. It seemed unlikely that we would be able to work out in any reasonable period of time how the neural networks, in which the hippocampus was embedded, participate in behavior and how those networks are affected by learning.

To bring the power of modern biology to bear on the study of learning, it seemed necessary to take a very different approach—a radically reductionist approach. We needed

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to study not the most complex but the simplest instances of memory storage, and to study them in animals that were most tractable experimentally. Such a reductionist approach was hardly new in 20th-century biology. One need only think of the use of *Drosophila* in genetics, of bacteria and bacteriophages in molecular biology, and of the squid giant axon in the study of the conduction of nerve impulses. Nevertheless, when it came to the study of behavior, many investigators were reluctant to use a reductionist strategy. In the 1950s and 1960s many biologists and most psychologists believed that learning was the one area of biology in which the use of simple animal models, particularly invertebrate ones, was least likely to succeed. They argued that only higher animals exhibit interesting forms of learning and that these forms require neuronal organizations and neuronal mechanisms qualitatively different from those found in simple animals.

It was my belief, however, that concerns about the use of a simple experimental system to study learning were misplaced. If elementary forms of learning are common to all animals with an evolved nervous system, there must be conserved features in the mechanisms of learning at the cell and molecular level that can be studied effectively even in simple invertebrate animals.

A Simple Learned Behavior in an Invertebrate

After an extensive search for a suitable experimental animal, I settled on the giant marine snail *Aplysia* (Fig. 1A) because it offers three important advantages: Its nervous system is made up of a small number of nerve cells; many of these are gigantic; and (as became evident to me later) many are uniquely identifiable (*3*, *4*). Whereas the mammalian brain has a trillion central nerve cells, *Aplysia* has only 20,000, and the simplest behaviors that can be modified by learning may directly involve less than 100 central nerve cells. In addition to being few in numbers, these cells are the largest nerve cells in the animal kingdom, reaching up to 1000 μm in diameter, large enough to be seen with the naked eye. One can record from these large cells for many hours without any difficulty, and the same cell can be returned to and recorded from over a period of days. The cells can easily be dissected out for biochemical studies, so that from a single cell one can obtain sufficient mRNA to make a cDNA library. Finally, these identified cells can readily be injected with labeled compounds, antibodies, or genetic constructs, procedures which opened up the molecular study of signal transduction within individual nerve cells.

Irving Kupfermann and I soon delineated a very simple defensive reflex: The withdrawal of the gill upon stimulation of the siphon, an action that is like the quick withdrawal of a hand from a hot object. When a weak tactile stimulus is applied to the siphon, both the siphon and gill are withdrawn into the mantle cavity for protection under the mantle shelf (Fig. 1A) (*5*). Kupfermann, Harold Pinsker, and later Tom Carew, Robert Hawkins, and I found that this simple reflex could be modified by three different forms of learning: habituation, sensitization, and classical conditioning (*5*–*7*). As we examined these three forms of learning, we were struck by the resemblance each had to corresponding forms of learning in higher vertebrates and humans. As with vertebrate learning, memory storage for each type of learning in *Aplysia* has two phases: a transient memory that lasts minutes and an enduring memory that lasts days. Conversion of short-term to long-term memory storage requires spaced repetition—practice makes perfect, even in snails (Fig. 1B) (*6*–*8*).

We focused initially on one type of learning. *Sensitization* is a form of learned fear in which a person or an experimental animal learns to respond strongly to an otherwise neutral stimulus (*5*, *6*, *8*). For example, if a person is suddenly exposed to an aversive stimulus, such as a gunshot going off nearby, that person will be sensitized by the unexpected noise. As a result, that person will be frightened and will now startle to an otherwise innocuous stimulus like a tap on the shoulder. Similarly, on receiving an aversive shock to a part of the body such as the tail, an *Aplysia* recognizes the stimulus as aversive and learns to enhance its defensive reflex responses to a variety of subsequent stimuli applied to the siphon, even innocuous stimuli (Fig. 1A) (*9*). The animal remembers the shock, and the duration of this memory is a function of the number of repetitions of the noxious experience (Fig. 1B). A single shock gives rise to a memory lasting only minutes; this short-term memory does not require the synthesis of new protein. In contrast, four or five spaced shocks to the tail give rise to a memory lasting several days; this long-term

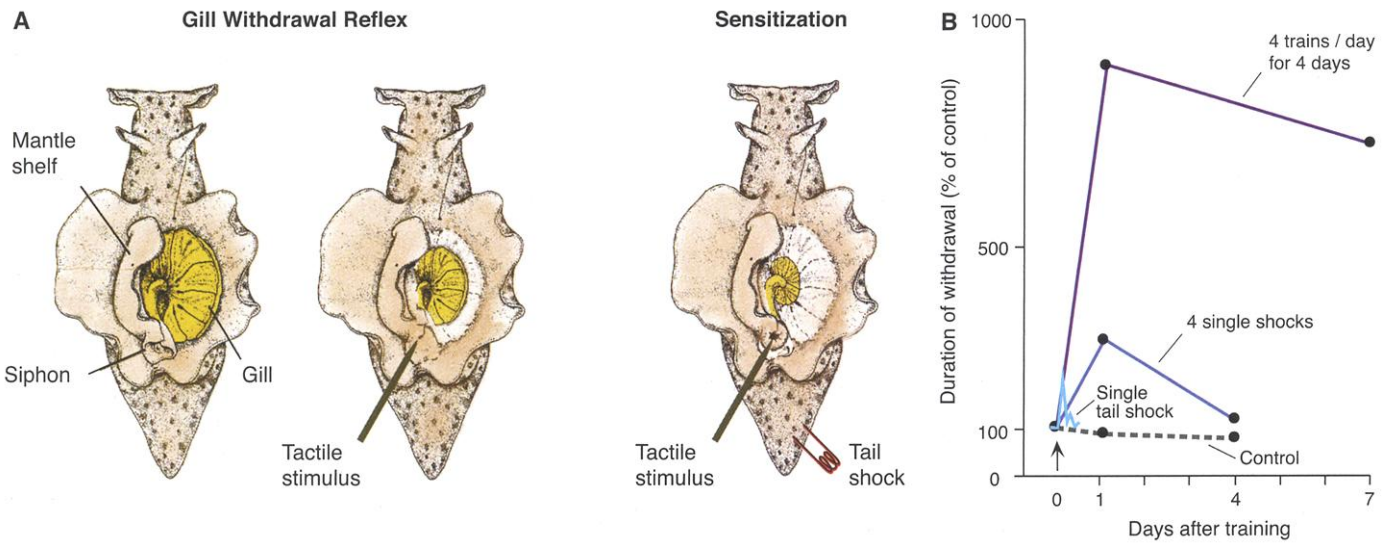

Fig. 1. A simple learned behavior. (**A**) A dorsal view of *Aplysia* showing the gill, the animal's respiratory organ. A light touch to the siphon with a fine probe causes the siphon to contract and the gill to withdraw. Here, the mantle shelf is retracted for a better view of the gill. Sensitization of the gill-withdrawal reflex, by applying a noxious stimulus to another part of the body, such as the tail, enhances the withdrawal reflex of both the siphon and the gill. (**B**) Spaced repetition converts short-term memory into long-term memory in *Aplysia*. Before sensitization training, a weak touch to the siphon causes only a weak, brief siphon and gill withdrawal reflex. Following a single noxious, sensitizing, shock to the tail, that same weak touch produces a much larger siphon and gill reflex withdrawal response, an enhancement that lasts about 1 hour. More tail shocks increase the size and duration of the response. [Modified from (*79*)]

memory does require the synthesis of new protein. Further training, four brief trains a day for four days, gives rise to an even more enduring memory lasting weeks, which also requires new protein synthesis. Thus, just as in complex learning in mammals (*10*, *11*), the long-term memory for sensitization differs from the short-term memory in requiring the synthesis of new proteins. This was our first clear evidence for the conservation of biochemical mechanisms between *Aplysia* and vertebrates.

Kupfermann, Castellucci, Carew, Hawkins, John Byrne, and I worked out significant components of the neural circuit gill-withdrawal reflex (Fig. 2). The circuit is located in the abdominal ganglion and has 24 central mechanoreceptor sensory neurons that innervate the siphon skin and make direct monosynaptic connections with 6 gill motor cells (Fig. 2C) (*12*–*14*). The sensory neurons also made indirect connections with the motor cells through small groups of excitatory and inhibitory interneurons (*15*, *16*). In addition to being identifiable, individual cells also have surprisingly large effects on behavior (Fig. 2B) (*4*, *14*, *17*). As we examined the neural circuit of this reflex, we were struck by its invariance. In every animal we examined, each cell connected only to certain target cells and not to others (Fig. 2C). This also was true in the neural circuitry of other behaviors in *Aplysia* including inking, control of the circulation, and locomotion (*4*, *18*). This raised a key question in the cell biological study of learning: How can learning occur in a neural circuit that is so precisely wired?

In 1894, Santiago Ramón y Cajal proposed a theory of memory storage according to which memory is stored in the growth of new connections (*19*). This prescient idea was neglected in good part for half a century as students of learning fought over newer competing ideas. First, Karl Lashley, Wolfgang Köhler, and a number of Gestalt psychologists proposed that learning leads to changes in electric fields or chemical gradients, which they postulated surround neuronal populations and are produced by the aggregate activity of cells recruited by the learning process. Second, Alexander Forbes and Lorente de Nó proposed that memory is stored dynamically by a self-reexciting chain of neurons. Donald Hebb later championed this idea as a mechanism for short-term memory. Finally, Holger Hyden proposed that learning led to changes in the base composition of DNA or RNA. Even though there was much discussion about the merits of each of these ideas, there was no direct evidence to support any of them (*2*).

Kupfermann, Castellucci, Carew, Hawkins, and I addressed these alternative ideas directly by confronting the question of how learning can occur in a circuit with fixed neuronal elements. To address this question, we examined the neural circuit of the gill-withdrawal reflex while the animal underwent sensitization, classical conditioning, or habituation. Our studies provided clear evidence for the idea proposed by Ramón y Cajal, that learning results from changes in the strength of the synaptic connections between precisely interconnected cells (*12*, *20*). Thus, while the organism's developmental program assures that the connections between cells are invariant, it does not specify their precise strength. Rather, experience alters the strength and effectiveness of these preexisting chemical connections. Seen in the perspective of these three forms of learning, synaptic plasticity emerged as a fundamental mechanism for information storage by the nervous system, a mechanism that is built into the very molecular architecture of chemical synapses (*21*).

Molecular Biology of Short- and Long-Term Memory Storage

What are the molecular mechanisms whereby short-term memory is established, and how is it converted to long-term memory? Initially, we focused on short-term sensitization. In collaboration with James H. Schwartz, we found that the synaptic changes, like short-term behavior, were expressed even when protein synthesis was inhibited. This finding first suggested to us that short-term synaptic plasticity might be mediated by a second messenger system such as cyclic AMP (*22*). Following up on this idea, Schwartz, Howard Cedar, and I found in 1972 that stimulation of the modulatory pathways

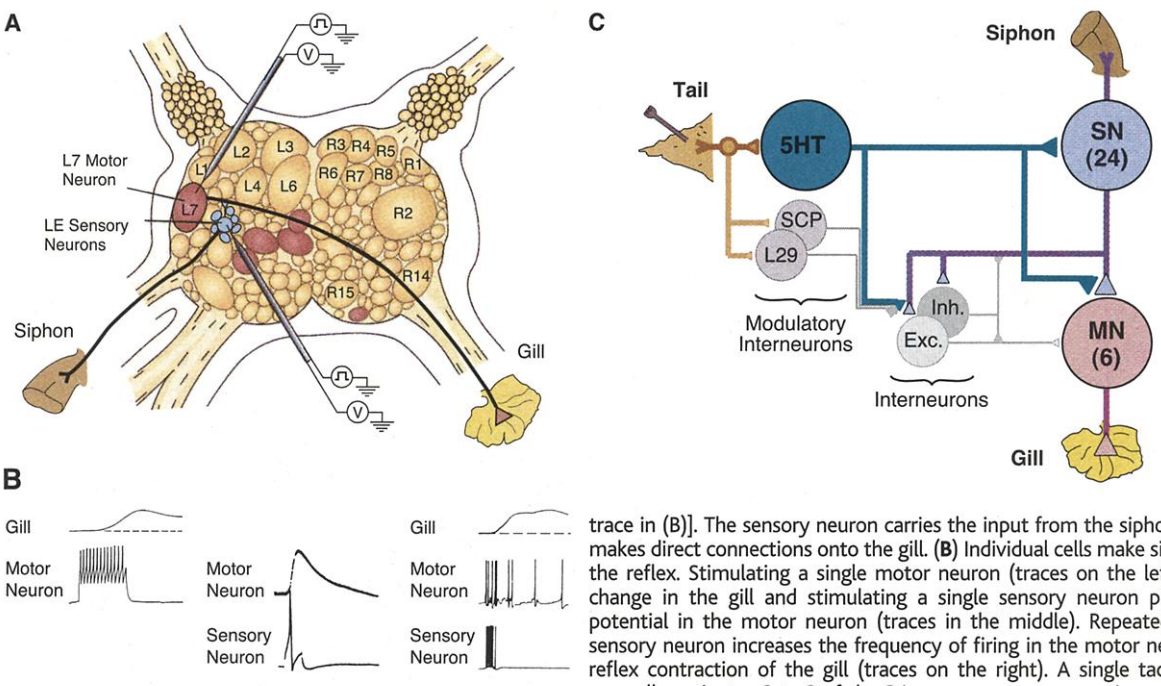

Fig. 2. The neural circuit of the *Aplysia* gill-withdrawal reflex. (**A**) In this dorsal view of the abdominal ganglion, the six identified motor cells to the gill are brown and the seven sensory neurons are blue. A sensory neuron that synapses on gill motor neuron L7 is stimulated electrically with an intracellular electrode and a microelectrode in the motor neuron records the synaptic potential produced by the action potential in the sensory neuron [see middle trace in (B)]. The sensory neuron carries the input from the siphon skin; the motor neuron makes direct connections onto the gill. (**B**) Individual cells make significant contributions to the reflex. Stimulating a single motor neuron (traces on the left) produces a detectable change in the gill and stimulating a single sensory neuron produces a large synaptic potential in the motor neuron (traces in the middle). Repeated stimulation of a single sensory neuron increases the frequency of firing in the motor neuron, leading to a visible reflex contraction of the gill (traces on the right). A single tactile stimulus to the skin normally activates 6 to 8 of the 24 sensory neurons, causing each to fire 1 to 2 action potentials. The repetitive firing of 10 action potentials in a single sensory neuron, designed to simulate the firing of the total population (trace on the right) simulates the reflex behavior reasonably well. (**C**) Diagram of the circuit of the gill-withdrawal reflex. The siphon is innervated by 24 sensory neurons that connect directly with the six motor neurons. The sensory neurons also connect to populations of excitatory and inhibitory interneurons that in turn connect with the motor neurons. Stimulating the tail activates three classes of modulatory interneurons (serotonergic neurons, neurons that release the small cardioactive peptide, and the L29 cells) that act on the terminals of the sensory neurons as well as on those of the excitatory interneurons. The serotonergic modulatory action is the most important; blocking the action of these cells blocks the effects of sensitizing stimuli. [From (*25*)]

recruited during heterosynaptic facilitation led to an increase in cAMP in the abdominal ganglion (*23*). Cedar and Schwartz found that the neurotransmitter candidates serotonin and dopamine could simulate this action of electrical stimulation and increase levels of cAMP (*24*). Later, Hawkins, Castellucci, David Glanzman, and I delineated the modulatory system activated by a sensitizing stimulus to the tail (*16*, *25*, *26*), and confirmed that it contains serotonergic interneurons.

We next found that serotonin acts on specific receptors in the presynaptic terminals of the sensory neuron to enhance transmitter release. In 1976, Marcello Brunelli, Castellucci, and I injected cAMP directly into the presynaptic cells and found that it too produced presynaptic facilitation (*27*, *28*). This provided the most compelling evidence then available that cAMP is involved in controlling synaptic strength and gave us our first insight into one molecular mechanism of short-term memory—the regulation of transmitter release (Fig. 3).

How does cAMP enhance transmitter release? Serotonin, or injected cAMP, leads to increased excitability and a broadening of the action potential by reducing specific K^+ currents, allowing greater Ca^{2+} influx into the presynaptic terminal with each action potential (*29*). The greater Ca^{2+} influx could contribute to the enhanced transmitter release. Following the lead of Paul Greengard, who had proposed that cAMP produces its action in the brain through the cAMP-dependent protein kinase (PKA), Marc Klein and I suggested that cAMP may cause phosphorylation of this K^+ channel by activating PKA (*29*). In collaborative experiments with Paul Greengard in 1980, Castellucci, Schwartz, and I found that the active catalytic subunit of PKA by itself produced broadening of the action potential and enhancement of glutamate release (*30*). Conversely, the specific peptide inhibitor of PKA (PKI) blocked the actions of serotonin. These findings provided direct evidence for the role of PKA in short-term presynaptic facilitation (*31*, *32*).

In an elegant series of experiments, Steven Siegelbaum, Joseph Camardo and Michael Schuster identified a novel K^+ channel, the S-type K^+ channel, and showed that it too could be modulated by cAMP (*33*) and that PKA could act on the S-type K^+ channel directly (*34*). Later, Byrne showed that serotonin also modulates a delayed-rectifier K^+ (*32*). The S-type channel mediated the increase in excitability with a minor contribution to broadening, whereas the delayed-rectifier K^+ channel contributed little to excitability but had a major role in spike broadening. Finally, Hochner, Klein, and I—and independently Jack Byrne and his colleagues—showed that, in addition to spike broadening, serotonin also enhanced release by an as-yet-unspecified action on the release machinery. Thus, serotonin leads to an increase in presynaptic cAMP, which activates PKA and leads to synaptic strengthening through enhanced transmitter release produced by a combination of mechanisms (Fig. 3) (*32*).

CREB-1 mediated transcription. By substituting puffs of serotonin, the transmitter released by tail shocks, for the tail shocks themselves, Samuel Schacher, Pier Giorgio Montarolo, Philip Goelet, and I modeled sensitization in a culture dish consisting of a single sensory cell making synaptic connections with a single motor cell (*35*). We were able to induce both short- and long-term facilitation and found, as with the intact animal, that the long-term process differed from the short-term process in requiring the synthesis of new proteins.

We used this cell culture to ask: What genes are activated to convert the short-term to the long-term process, and what genes are essential for the maintenance of the long-term process? We found that five spaced puffs of serotonin (simulating five spaced shocks to the tail) activate PKA, which in turn recruits the mitogen-activated protein kinase (MAPK). Both translocate to the nucleus, where they activate a transcriptional cascade beginning with the transcription factor CREB-1, the cAMP response element binding protein−1, so called because it binds to a cAMP response element (CRE) in the promoters of target genes (Fig. 3). The first clue to the importance of CREB in long-term memory was provided in 1990 by Pramod Dash and Binyamin Hochner (*36*). They injected, into the nucleus of a sensory neuron in culture, oligonucleotides carrying the CRE DNA element, thereby titrating out CREB. This treatment blocked long-term but not short-term facilitation (Fig. 3). Later, Dusan Bartsch cloned *Aplysia* CREB-1a (ApCREB-1a) and showed that injection of the phosphorylated form of this transcription factor by itself could initiate the long-term memory process. Downstream from ApCREB (*37*), Cristina Alberini and Bartsch

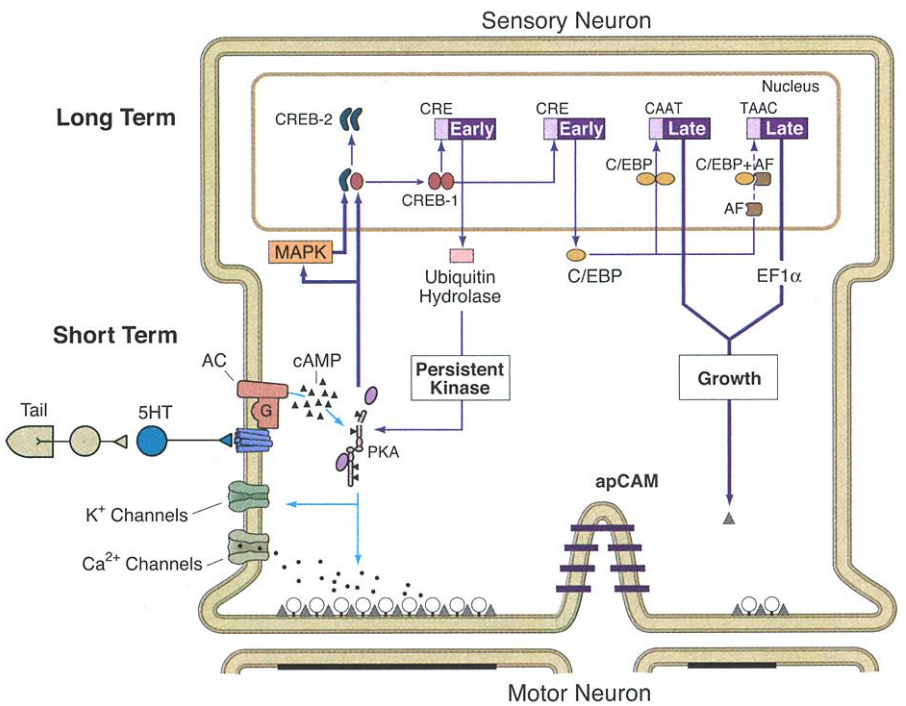

Fig. 3. Effects of short- and long-term sensitization on the monosynaptic component of the gill-withdrawal reflex of *Aplysia*. In short-term sensitization (lasting minutes to hours) a single tail shock causes a transient release of serotonin that leads to covalent modification of preexisting proteins. The serotonin acts on a transmembrane serotonin receptor to activate the enzyme adenylyl cyclase (AC), which converts ATP to the second messenger cyclic AMP. In turn, cAMP recruits the cAMP-dependent protein kinase A (PKA) by binding to the regulatory subunits (spindles), causing them to dissociate from and free the catalytic subunits (ovals). These subunits can then phosphorylate substrates (channels and exocytosis machinery) in the presynaptic terminals, leading to enhanced transmitter availability and release. In long-term sensitization, repeated stimulation causes the level of cAMP to rise and persist for several minutes. The catalytic subunits can then translocate to the nucleus, and recruit the mitogen-activated protein kinase (MAPK). In the nucleus, PKA and MAPK phosphorylate and activate the cAMP response element-binding (CREB) protein and remove the repressive action of CREB-2, an inhibitor of CREB-1. CREB-1 in turn activates several immediate-response genes, including a ubiquitin hydrolase necessary for regulated proteolysis of the regulatory subunit of PKA. Cleavage of the (inhibitory) regulatory subunit results in persistent activity of PKA, leading to persistent phosphorylation of the substrate proteins of PKA. A second immediate-response gene activated by CREB-1 is C/EBP, which acts both as a homodimer and as a heterodimer with activating factor (AF) to activate downstream genes [including elongation factor 1α (EF1α)] that lead to the growth of new synaptic connections.

found two additional positive transcription regulators, the CAAT box enhancer binding protein (ApC/EBP) and activation factor (Ap/AF) (*38*, *39*). CREB-1 activates this set of immediate response genes, which in turn act on downstream genes, to give rise to the growth of new synaptic connections (Fig. 3) (*36*, *40*–*46*). As first shown by Craig Bailey and Mary Chen, long-term memory endures by virtue of the growth of new synaptic connections, a structural change that parallels the duration of the behavioral memory (*45*–*48*). As the memory fades, the connections retract over time. A typical sensory neuron in the intact *Aplysia* has about 1200 synaptic varicosities. Following long-term sensitization, the number more than doubles to about 2600; with time the number returns to about 1500.

Inhibitory constraints. In 1995 Bartsch found that positive regulators are only half the story—there are also inhibitory constraints on memory (*49*). Long-term synaptic facilitation requires not only activation of memory-enhancer genes, but also inactivation of memory-suppressor genes (Fig. 3). One of these, the transcription factor ApCREB-2, can repress ApCREB-1a mediated transcription; relieving this repression lowers the threshold for the long-term process.

Thus, during long-term memory storage, a tightly controlled cascade of gene activation is switched on, with memory-suppressor genes providing a threshold or checkpoint for memory storage, presumably to ensure that only salient features are learned. Memory suppressors may allow for the modulation of memory storage by emotional stimuli, as occurs in "flashbulb memories," memories of emotionally charged events that are recalled in detail, as if a complete picture had been instantly and powerfully etched in the brain.

Synapse-Specificity of Long-Term Facilitation

The finding of a transcriptional cascade explained why long-term memory requires new protein synthesis immediately after training, but it posed a new cell-biological problem. A single neuron makes hundreds of contacts on many different target cells. Short-term synaptic changes are synapse-specific. Since long-lasting synaptic changes require transcription and thus the nucleus, is long-term memory storage a cell-wide process, or are there cell-biological mechanisms that maintain the synapse specificity of long-term facilitation?

To examine these questions, Kelsey Martin cultured one *Aplysia* sensory cell with a bifurcating axon with two motor neurons, forming two widely separated synapses (Fig. 4A). In this culture system, a single puff of serotonin applied to one synapse produces transient facilitation at that synapse only, as expected (*50*, *51*). Five puffs of serotonin applied to one branch produces long-lasting facilitation (72 hours), also restricted to the stimulated synapse (Fig. 4B). This long-lasting synapse-specific facilitation requires CREB and also leads to structural changes. Thus, despite recruitment of nuclear processes, long-term changes in synaptic function and structure are confined only to those synapses stimulated by serotonin.

How does this come about? Martin, Andrea Casadio, Bailey, and I found that five puffs of serotonin send a signal to the nucleus to activate CREB-1, which then appears to send proteins to all terminals; however, only those terminals that have been marked by serotonin can use these proteins productively for synaptic growth. Indeed, one puff of serotonin to the previously unstimulated synapse is sufficient to mark that synapse so that it can capture a reduced form of the long-term facilitation induced at the other site by five puffs of serotonin (Fig. 4B).

These results gave us a new and surprising insight into short-term facilitation. The stimulus that produces the short-term process has two functions (Fig. 4C). When acting alone, it provides a selective, synapse-specific enhancement of synaptic strength, which contributes to short-term memory, lasting minutes. When acting in conjunction with the activation of CREB initiated by a long-term process in either that synapse or in any other synapse on the same neuron, the stimulus locally marks those synapses at which it occurs. The marked synapse can then utilize the proteins activated by CREB for synaptic growth to produce a persistent change in synaptic strength. Thus, the logic for the long-term process involves a long-range integration that is different from the short-term

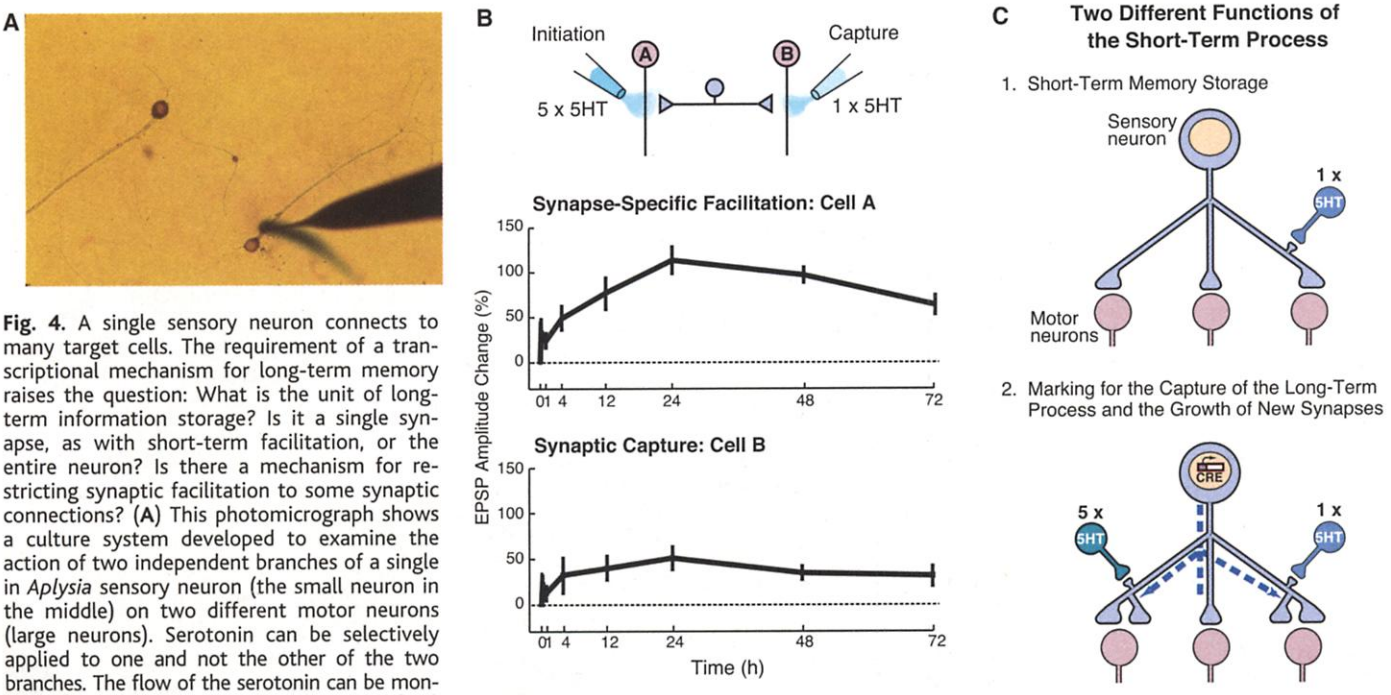

Fig. 4. A single sensory neuron connects to many target cells. The requirement of a transcriptional mechanism for long-term memory raises the question: What is the unit of long-term information storage? Is it a single synapse, as with short-term facilitation, or the entire neuron? Is there a mechanism for restricting synaptic facilitation to some synaptic connections? (**A**) This photomicrograph shows a culture system developed to examine the action of two independent branches of a single in *Aplysia* sensory neuron (the small neuron in the middle) on two different motor neurons (large neurons). Serotonin can be selectively applied to one and not the other of the two branches. The flow of the serotonin can be monitored with the dye, fast green. [From (*50*)] (**B**) Long-term facilitation is synapse-specific and can be captured at another branch by the stimulus that initiates the short-term process. Five puffs of serotonin applied at the initiation site (cell A) produce a synapse-specific facilitation shown in (B). This synapse-specific facilitation is not evident at the synapse of cell B unless that synapse is itself primed with a single puff of serotonin. [From (*50*)] (**C**) Two effects of short-term facilitation: short-term memory storage when acting by itself and marking of the specific synapse to which it is applied for subsequent capture of the proteins necessary for long-term facilitation and growth when applied in conjunction with five pulses to another set of terminals.

process. In the long-term, the function of a synapse is not only determined by the history of usage of that synapse. It is also determined by the state of the transcriptional machinery in the nucleus.

How does one puff of serotonin mark a synapse for long-term change? For structural changes to persist, local protein synthesis is required (*51*). Oswald Steward's important work in the early 1980s had shown that dendrites contain ribosomes, and that specific mRNAs are transported to the dendrites and translated there (*52*). Our experiments showed that one function of these locally translated mRNAs was to stabilize the synapse-specific long-term functional and structural changes.

Neurotransmitter regulation of local protein synthesis. These studies thus revealed a new, fourth type, of synaptic action mediated by neurotransmitter signaling (Fig. 5). Three of these four have emerged, at least in part, from the study of learning and memory. First, in 1951, Katz and Fatt opened up the modern study of chemical transmission with their discovery of ionotropic receptors that regulate ion flux through transmitter-gated ion channels to produce fast synaptic actions, lasting milliseconds (*53*). Second, in the 1970s, metabotropic receptors were found to activate second-messenger pathways, such as the cAMP-PKA pathway, to produce slow synaptic activity lasting minutes (*54*). As we have seen in *Aplysia*, this slow synaptic action can regulate transmitter release, thereby contributing to short-term memory for sensitization. Third, an even more persistent synaptic action, lasting days, results from repeated action of a modulatory transmitter such as serotonin. With repeated applications of serotonin, second-messenger kinases translocate to the nucleus, where they activate a cascade of gene induction leading to the growth of new synaptic connections. This of course raises the problem of synapse specificity that we have considered above. Our experiments, in the bifurcated culture system, revealed a novel fourth action of neurotransmitters, the marking of the synapse and the regulation of local protein synthesis, which contributes to the establishment of synapse-specific long-term facilitation.

Explicit Memory

I have so far considered only the simplest cases of memory storage—those involving reflexes—a form called implicit or procedural memory. Implicit memory is memory for perceptual and motor skills and is expressed through performance, without conscious recall of past episodes. In contrast, the memories we hold near and dear are called explicit (or declarative) memories. These memories require conscious recall and are concerned with memories for people, places, objects, and events. Explicit memory involves a specialized anatomical system in the medial temporal lobe, and a structure deep to it, the hippocampus (Fig. 6A) (*21*, *55*, *56*). How is explicit memory stored? Louis Flexner, Bernard Agranoff, Sam Barondes, and Larry Squire had shown that explicit memory, like implicit memory, has a short-term phase that does not require protein synthesis and a long-term phase that does (*55*). Are these two components of memory storage also represented at the cellular level? What rules govern explicit memory storage?

A decade ago, when I reached my 60th birthday, I gathered up my courage and returned to the hippocampus. Mario Capecchi and Oliver Smithies, by achieving targeted gene ablation in mouse embryonic stem cells, provided a superb genetic system for relating individual genes to synaptic plasticity, on the one hand, and to complex explicit memory storage on the other. Mice have a medial temporal lobe system, including a hippocampus, that resembles that of humans, and they use their hippocampus much as we do to store memory of places and objects (Fig. 6A).

Although we still do not know much about how information is transformed as it gets into and out of the hippocampus, it is well established that the hippocampus contains a cellular representation of extrapersonal space—a cognitive map of space—and lesions of the hippocampus interfere with spatial tasks (*57*). Moreover, in 1972, Terje Lømo and Tim Bliss discovered that the perforant path, a major pathway within the hippocampus, exhibits activity-dependent plasticity, a change now called long-term potentiation (LTP) (Fig. 6B). In the CA1 region of the hippocampus, LTP is induced postsynaptically by activation of an NMDA receptor to glutamate. In the late 1980s Richard Morris found that blocking the NMDA receptor pharmacologically not only interfered with LTP but also blocked memory storage (*58*, *59*).

This earlier work on LTP in hippocampal slices had focused on the response to one or two trains of electrical stimuli. But in *Aplysia* we had found that long-term memory emerges most effectively with repeated stimuli (Fig. 1B). So when Uwe Frey, Yan-You Huang, Peter Nguyen, and I turned to the hippocampus, we examined whether LTP changed with repeated stimulation (*60*–*62*) and found that hippocampal LTP has phases, much like facilitation in *Aplysia*. The early phase of LTP, produced by a single train of stimuli, lasts only 1 to 3 hours and does not require new protein synthesis (*62*); it involves covalent modifications of preexisting proteins that lead to the strengthening of preexisting connections, similar in principle to short-term facilitation in *Aplysia*. By contrast, repeated trains of electrical stimuli produce a late phase of LTP, which has properties quite different from early LTP and similar to long-term facilitation in *Aplysia* (Fig. 6B). The late phase of LTP persists for at least a day and requires both translation and transcription. The late phase of LTP, like long-term storage of implicit memory, requires PKA, MAPK, and CREB and appears to lead to the growth of new synaptic connections (Fig. 6C) (*60*–*69*).

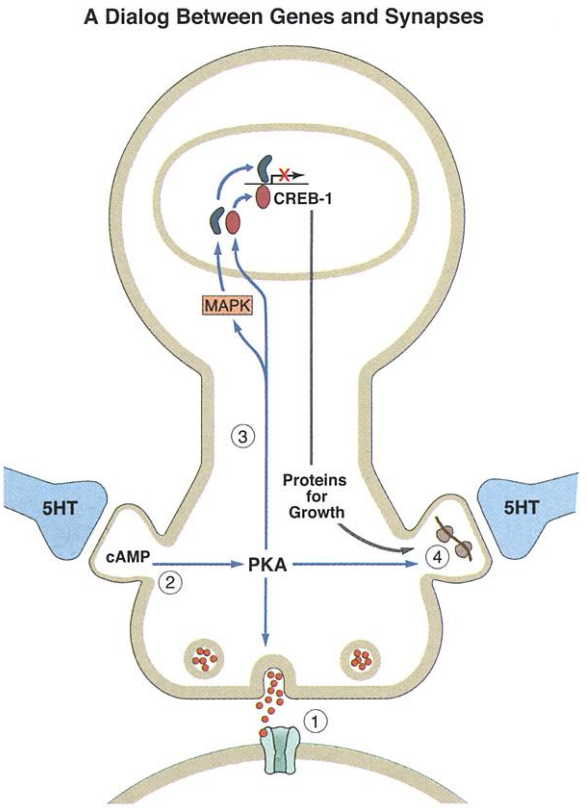

Fig. 5. Four consequences of the action of neurotransmitters. **1.** Transmitter activation of a ligand-gated ion channel leads to a rapid synaptic action lasting milliseconds. **2.** Transmitter activation of a seven transmembrane receptor and a second messenger kinase leads to a more enduring synaptic action lasting minutes. **3.** Repeated transmitter activation of a seven transmembrane receptor leads to the translocation of the kinase to the nucleus and to activation of transcription, producing a persistent synaptic action. **4.** Transmitter activation of local protein synthesis to stabilize the synapse-specific facilitation.

The late phase of LTP and explicit memory. To explore further the specific role of PKA and late LTP in memory storage, Ted Abel, Mark Barad, Rusiko Bourtchouladze, Peter Nguyen, and I generated transgenic mice that express R(AB), a mutant form of the regulatory subunit of PKA that inhibits enzyme activity (*70*). In these R(AB) transgenic mice, the reduction in hippocampal PKA activity was paralleled by a significant decrease in late LTP, while basal synaptic transmission and early LTP remained unchanged. Most interesting, this deficit in the late phase of LTP was paralleled by behavioral deficits in hippocampus-dependent long-term memory for extrapersonal space, whereas learning, and short-term memory, are unimpaired (Fig. 7, A and B). Thus, in the storage of explicit memory of extrapersonal space in the mammalian hippocampus, PKA plays a critical role in the transformation of short-term memory into long-term memory, much as it does in the storage of implicit memory in *Aplysia* and *Drosophila*.

Using the R(AB) mice we could now ask: Why do animals with compromised PKA signaling have difficulty with space (*70*)? We were influenced by the classic studies of John O'Keefe and John Dostrovsky, who in 1971 discovered that the pyramidal cells of the hippocampus—the cells one examines artificially by using electrically stimulating the Schaffer collateral pathway while studying LTP—are "place cells;" they actually encode extrapersonal space in the animal (*71*). A given pyramidal cell will fire only when the head of the mouse is in a certain part of an enclosed space—the cell's place field. When placed in a new environment, within minutes an animal develops an internal representation of the space (by the coordinated firing of a population of place cells), which is normally stable for days. The same cell will have the same firing field each time the animal is reintroduced to that environment. When now placed in a second environment, a new map is formed—again in minutes—in part from some of the cells that made up the map of the first environment and in part from pyramidal cells that had been silent previously (*71*).

It struck me that the formation of a new map resembled a learning process. The map develops with time as the animal familiarizes itself with the space, and once learned, the map of space is retained for days and weeks. To first test whether the molecular pathways underlying the late phase of LTP were important for the long-term stabilization of this map, Cliff Kentros, Robert Muller, Hawkins, and I simply blocked LTP pharmacologically with an NMDA receptor antagonist (*72*). When placed in a new environment, the animals with blocked NMDA receptors formed a good spatial map that was still stable 1 hour later. However, by 24 hours, most pyramidal cells no longer retained the representation of the field they had initially. This suggested that activation of NMDA receptors—perhaps a step in modifying the strength of the synapse—is required for the long-term stabilization of a place cell map, a result consistent with the role for the late phase of LTP in the stabilization of a place cell map.

We next asked whether a selective deficit that affects only the late phase of LTP, causes a selective abnormality in the long-term stability of place cells. Since only the late phase of LTP requires PKA, Alex Rotenberg, Muller, Abel, Hawkins, and I returned to the R(AB) transgenic mice with diminished PKA activity and a diminished form of late LTP (*73*). If reduced activity of PKA affected the stability of place cells, R(AB) mice should be able to form a stable map of space in a novel environment, as in normal animals, that is stable for at least 1 hour. However, the cell field should be unstable when recorded 24 hours later. This is precisely what we found (Fig. 7C). The fact that long-term instability in the spatial map and the deficit in long-term memory paralleled the deficit in the late phase of LTP suggested that PKA-

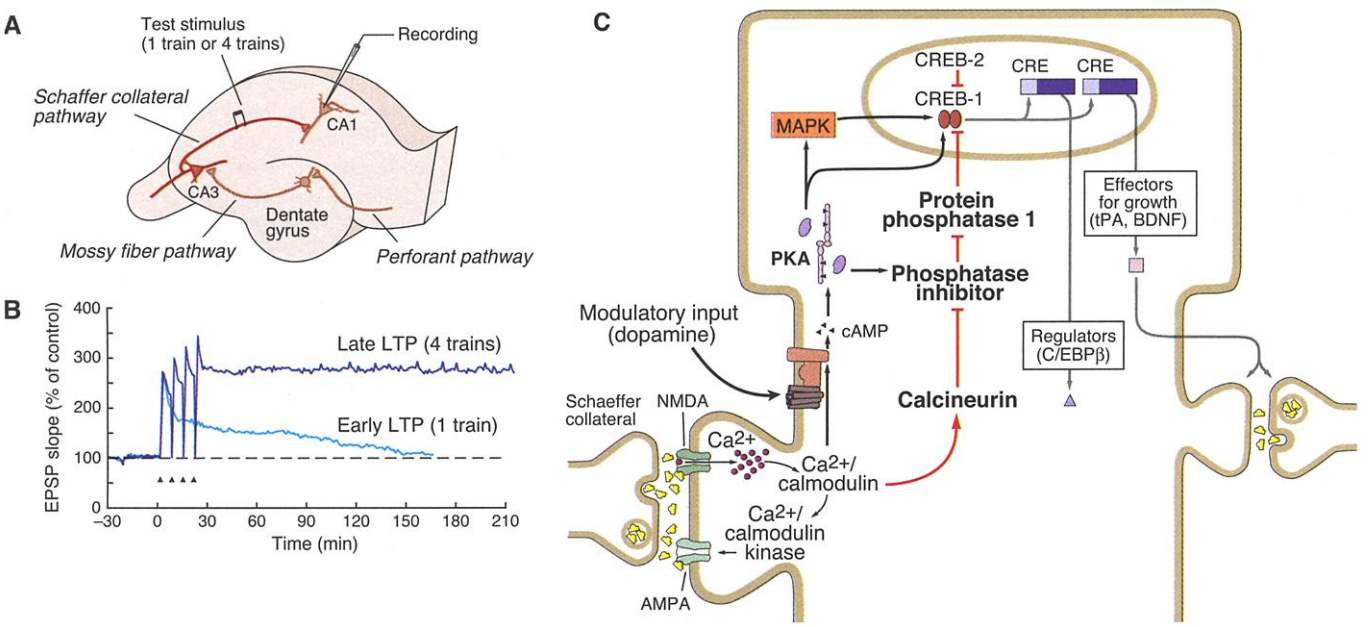

Fig. 6. Long-term potentiation (LTP) in the hippocampus. (**A**) Three major pathways, each of which gives rise to LTP. The *perforant pathway* from the subiculum forms excitatory connections with the granule cells of the dentate gyrus. The *mossy fiber pathway*, formed by the axons of the granule cells of the dentate gyrus, connects the granule cells with the pyramidal cells in area CA3 of the hippocampus. The *Schaffer collateral pathway* connects the pyramidal cells of the CA3 region with the pyramidal cells in the CA1 region of the hippocampus. (**B**) The early and late phases of LTP in the Schaffer collateral pathway. A single train of stimuli for one second at 100 Hz elicits an early LTP, and four trains at 10-minute intervals elicit the late phase of LTP. The early LTP lasts about 2 hours, the late LTP more than 24 hours. (**C**) A model for the late phase of LTP in the Schaffer collateral pathway. A single train of action potentials initiates early LTP by activating NMDA receptors, Ca^{2+} influx into the postsynaptic cell, and the activation of a set of second messengers. With repeated trains of action potentials (illustrated here) the Ca^{2+} influx also recruits an adenylyl cyclase (AC), which activates the cAMP-dependent protein kinase. The kinase is transported to the nucleus where it phosphorylates CREB. CREB in turn activates targets (C/EBPB, EPA, BDNF) that are thought to lead to structural changes. Mutations in mice that block PKA or CREB reduce or eliminate the late phase of LTP. The adenylyl cyclase can also be modulated by dopamine signals and perhaps other modulatory inputs. In addition, there are constraints (in red) that inhibit L-LTP and memory storage. Removal of these constraints lowers the threshold for L-LTP and enhances memory storage.

mediated gene activation and the synthesis of new protein might be essential for the stabilization of the spatial map. Naveen Agnihotri, Kentros, Hawkins, and I tested this idea, and found that inhibiting protein synthesis indeed destabilized the place fields in the long-term much as does inhibiting PKA (*81*).

In the course of this work, Kentros and Agnihotri found, remarkably, that, as is the case with explict memories in humans, a key feature in the stabilization of PKA and protein synthesis-dependent phase of memory is attention (*82*). When a mouse does not attend to the space it walks through, the man forms but is unstable after 3 to 6 hours. When the mouse is forced to attend to the space, however, the map invariably is stable for days!

Inhibitory constraints on explicit memory. Recently we (74) and others (75) have found that the threshold for hippocampal synaptic plasticity and memory storage is determined by the balance between protein phosphorylation governed by PKA and dephosphorylation (74, 76). To determine whether the endogenous Ca^{2+}-sensitive phosphatase calcineurin acts as a constraint on this balance, we inhibited calcineurin and examined the effects on synaptic plasticity and memory storage. Isabelle Mansuy, Gael Malleret, Danny Winder, Tim Bliss, and I found that a transient reduction of calcineurin activity resulted in facilitation of LTP both in vitro and in vivo (74). This facilitation persisted for several days in the intact animal and was accompanied by enhanced learning and strengthening of short- and long-term memory on several spatial and non-spatial tasks requiring the hippocampus. These results, together with previous findings by Winder and Mansuy showing that overexpression of calcineurin impairs PKA-dependent components of LTP and memory (76, 77), demonstrate that endogenous calcineurin can act as a negative regulator of synaptic plasticity, learning, and memory (Fig. 6C).

An Overall View

Our studies of the storage component of memory, the molecular mechanism whereby information is stored, have led to two general conclusions.

First, our research suggests that the cellular and molecular strategies used in *Aplysia* for storing short- and long-term memory are conserved in mammals and that the same molecular strategies are employed in both implicit and explicit memory storage. With both implicit and explicit memory there are stages in memory that are encoded as changes in synaptic strength and that correlate with the behavioral phases of short- and long-term memory. The short-term synaptic changes involve covalent modification of preexisting proteins, leading to modification of pre-existing synaptic connections, whereas the long-term synaptic changes involve activation of gene expression, new protein synthesis, and the formation of new connections. Whereas short-term memory storage for implicit and explicit memory requires different signaling, long-term storage of both implicit and explicit memory uses as a core signaling pathway PKA, MAPK, and CREB-1. At least in the mouse, additional components are likely recruited. In both implicit and explicit memory the switch from short-term to long-term memory is regulated by inhibitory constraints.

Second, the study of learning has revealed new features of synaptic transmission and new cell-biological functions of synaptic signaling. For example, different forms of learning recruit different modulatory transmitters, which then act in one of three ways: (i) They activate second-messenger kinases that are transported to the nucleus where they initiate processes required for neuronal growth and long-term memory; (ii) they mark the specific synapses for capture of the long-term process and regulate local protein synthesis for stabilization; and (iii) they mediate, in ways we are just beginning to understand, attentional processes required for memory formation and recall.

Most important, the study of long-term memory has made us aware of the extensive dialog between the synapse and the nucleus, and the nucleus and the synapse (Fig. 5). In the long-term process the response of a synapse is not determined simply by its own

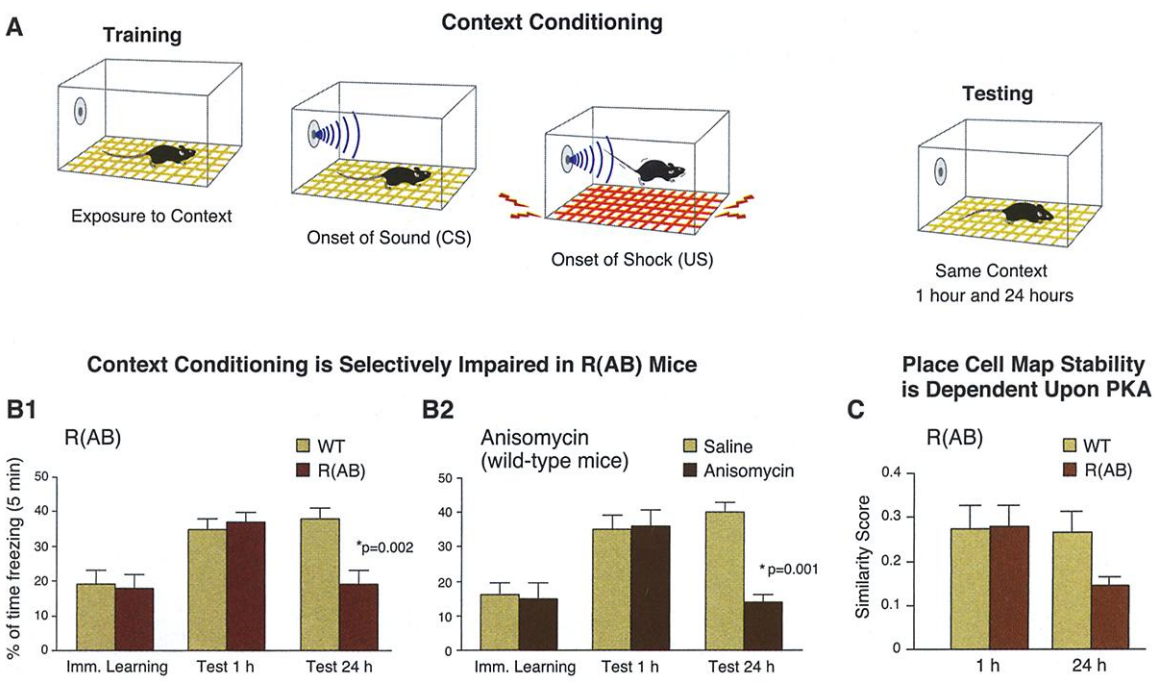

Fig. 7. (**A**) The protocol for context conditioning consists of exposure to the context followed by a tone and then a shock. The animals are then tested 1 hour and 24 hours after training. [From (*70*)] (**B1**) Mutant mice that express the R(AB) gene in the hippocampus, blocking the action of PKA, have a selective defect for long-term contextual memory. Mice that express R(AB) were conditioned to freeze to the context. After becoming familiar with the context, the mice heard a sound and received a shock through the electrified grid in the floor. As a result the animals learned to associate the context of the space with shock and to freeze when placed in the box at a future time. These mice had good short-term memory at 1 hour for freezing to context, but at 24 hours they no longer froze to context, indicating a defect in a form of long-term explicit (declarative) memory that requires the hippocampus. (**B2**) Wild-type mice exposed to anisomycin, an inhibitor of protein synthesis, during training show a similar defect for long-term memory when tested 24 hours after conditioning. [From (*70*)] (**C**) Place cell stability for R(AB) and wild-type mice. R(AB) mice with a defect in PKA and late LTP form place fields that are stable at 1 hour. These fields are not stable at 24 hours. [From (*73*, *80*)]

history of activity (as in short-term plasticity), but also by the history of transcriptional activation in the nucleus.

I started this essay by pointing out that 40 years ago, at the beginning of my career, I thought that a reductionist approach based on the use of a simple experimental system such as *Aplysia* might allow us to address fundamental questions in learning and memory. That was a leap of faith for which I have been rewarded beyond my fondest hopes. Still, the complexity of explicit memory is formidable, and we have only begun to explore it. We as yet know little about the molecular mechanisms that initiate or stabilize the synaptic growth associated with long-term memory. What signaling molecules lead to the cytoskeletal rearrangements during synaptic remodeling? How do they relate to the molecules that control synapse formation during development?

In addition, we have here only considered the molecular mechanisms of memory storage. The more difficult part of memory—especially explicit memory—is a systems problem. We still need to seek answers to a family of important questions. How do different regions of the hippocampus and the medial temporal lobe—the subiculum, the entorhinal, parahippocampal and perirhinal cortices—interact in the storage of explicit memory? How is information in any of these regions transferred for ultimate consolidation in the neocortex? We do not, for example, understand why the initial storage of long-term memory requires the hippocampus, whereas the hippocampus is not required once a memory has been stored for weeks or months (*21*, *78*). What critical information does the hippocampus convey to the neocortex? We also know very little about the nature of recall of explicit (declarative) memory, a recall that requires conscious effort. These systems problems will require more than the bottoms-up approach of molecular biology. They will also require the top-down approaches of cognitive psychology, neurology, and psychiatry. Ultimately we will need syntheses that bridge the two approaches.

Despite these complexities, these and other questions in the biology of learning no doubt will be vigorously addressed in the near future. For the biology of the mind has now captured the imagination of the scientific community of the 21st century, much as the biology of the gene fascinated the scientists of the 20th century. As the biological study of the mind assumes the central position within biology and medicine, we have every reason to expect that a succession of brain scientists will be called to Stockholm and honored for their own leaps of faith (*81*).

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