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Molecular Biology of Learning: Modulation of Transmitter Release

Eric R. Kandel and James H. Schwartz

Behavior is modified by experience through two processes—learning, the acquisition of new knowledge about the environment, and memory, its retention. Depending on how long the change in behavior persists, the memory is termed short term (minutes to hours) or long term (days to years). The mechanisms by which learning is acquired and memory retained are central to both neurobiology and psychology. Despite its importance for neurobiology, learning has not been approachable with the techniques of cell biology until recently.

The ability to study learning on the cellular level results from the realization that it is a universal feature of nervous systems (1). Not only are all animals capable of elementary forms of learning, but several learning processes first described in mammals—for example, habituation, sensitization, and classical conditioning—occur in similar form in mollusks and other invertebrates (1–6). This suggests that the cellular mechanisms for these forms of learning are the same throughout phylogeny and therefore can be studied effectively in higher invertebrates. Some of these animals have nervous systems made up of larger (as large as 1000 micrometers) and fewer (10^5 to 10^6) neurons than are found in vertebrates (10 to 100 μm; 10^{12}).

The body of neurophysiological work on vertebrates and invertebrates carried out during the past half century has suggested that both learning and memory are somehow expressed through changes in nerve cells. Recent studies in the marine snail Aplysia and in several other higher invertebrates indicate that simple forms of associative and nonassociative learning occur at identified cellular loci, the synaptic connections between specific neurons (7–9). Memory persisting for weeks can result from changes in the strength of already existing contacts (7, 10, 11). Prolonged changes in synaptic strength can be achieved by changing the amount of chemical transmitter released by the presynaptic terminals of specific neurons (9, 12).

With the recognition that certain forms of learning can alter the strength of specific synaptic connections, comes the possibility of investigating the molecular basis of the learning by defining the biochemical mechanisms that underlie the persistent changes in transmitter release. Do these mechanisms require expression of specific genes that lead to the synthesis of entirely new proteins? Are there posttranslational modifications of already existing molecules that alter their activities? Or do learning and memory make use of both processes? The large size of the nerve cells of higher invertebrates and the ability to identify individual neurons have already permitted electrophysiological analysis of the synapses at which learning takes place. These experimental advantages now also promise to be useful for exploring the molecular mechanisms underlying learning because biochemical processes can be causally related to functional (plastic) changes in specific nerve cells, and these plastic changes in turn can be causally related to the behavioral modification.

The purpose of this article is to describe how a behavioral system in Aplysia can be used to examine the mechanisms of several forms of learning at different levels of analysis: behavioral, cell-physiological, ultrastructural, and

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molecular. At the behavioral level we can rigorously characterize various forms of learning and obtain the time course for the short- and long-term memory of each form. At the physiological, ultrastructural, and molecular levels it is possible to specify, for each form, the locus and mechanism in individual identified neurons. Most important, the information from any one level of analysis can be related to information obtained at the others.

We shall focus our discussion primarily on one type of learning, short-term

tioning. We shall first consider sensitization and habituation and then describe classical conditioning.

Sensitization is an elementary form of nonassociative learning in which an animal learns to strengthen its defensive reflexes and to respond vigorously to a variety of previously neutral or indifferent stimuli after it has been exposed to a potentially threatening or noxious stimulus. Habituation, an even simpler form of learning, is a process functionally reciprocal to sensitization in which an animal learns through repeated presentation to

Habituation and Sensitization Have a Specific Neuronal Locus

Most of the nerve cells mediating the gill- and siphon-withdrawal reflex have now been identified (Fig. 3) (15–18). There are 13 identified central motor cells (5 for the gill, 7 for the siphon, and 1 for both gill and siphon) and 30 peripheral motor cells for the siphon. These motor cells are activated by two populations of sensory neurons, each containing about 24 cells. One population (the left E cluster) innervates the siphon skin; the other (the right E cluster) innervates the mantle shelf (17). Finally, there are several interneurons, at least one of which inhibits and others that excite. The sensory neurons synapse onto interneurons and motor neurons; the motor neurons synapse directly onto the muscles that effect the behavior (Fig. 3).

By surveying the various cells of the neural circuit that controls the behavior during habituation and sensitization in the intact animal, Castellucci *et al.* and Byrne *et al.* (7, 9, 12, 19) found that the critical changes underlying both habituation and sensitization occur at the synapses made by the sensory neurons onto motor neurons and interneurons. Thus both forms of learning take place at a common locus, the same set of sensory synapses.

Changes in the size of the synaptic potential—in the strength of the direct (monosynaptic) excitatory connections between sensory neurons and their follower cells—account for both habituation and sensitization (Fig. 4) (9, 12, 19, 20). With repeated sensory stimulation at rates that produce habituation in the intact animal, the monosynaptic excitatory connections between the sensory and motor neurons are functionally depressed (7, 21) because less transmitter is released by each impulse in the sensory neurons. Transmitter at chemical synapses is released in multimolecular packets called quanta (22). An analysis of habituation in terms of the quantal components of synaptic transmission indicates that, in this form of learning, fewer transmitter quanta are released from the terminals by each action potential. [The sensitivity of the receptor molecules in the postsynaptic cells is not altered (9).] Similarly, sensitization produces presynaptic (heterosynaptic) facilitation at the terminals of the sensory neurons by increasing the number of transmitter quanta released per impulse (12). Thus the amount of transmitter released by the sensory cells is modulated in opposite ways by habituation and sensitization.

Summary. Until recently, it has been impossible to approach learning with the techniques of cell biology. During the past several years, elementary forms of learning have been analyzed in higher invertebrates. Their nervous systems allow the experimental study of behavioral, neurophysiological, morphological, biochemical, and genetic components of the functional (plastic) changes underlying learning. In this review, we focus primarily on short-term sensitization of the gill and siphon reflex in the marine mollusk, *Aplysia californica.* Analyses of this form of learning provide direct evidence that protein phosphorylation dependent on cyclic adenosine monophosphate can modulate synaptic action. These studies also suggest how the molecular mechanisms for this short-term form of synaptic plasticity can be extended to explain both long-term memory and classical conditioning.

sensitization, because it has been analyzed most completely on the molecular level. The studies of sensitization provide the first direct evidence that protein phosphorylation mediated by cyclic adenosine monophosphate (cyclic AMP) can serve as a mechanism for modulating synaptic strength (13). We shall also suggest how the molecular mechanisms for the short-term form of synaptic plasticity might be extended to explain long-term memory for sensitization and classical conditioning.

A Simple Reflex Shows Both Associative and Nonassociative Learning

To study the learning capabilities of *Aplysia*, we have used the defensive withdrawal reflexes of the external organs of the mantle cavity (Fig. 1A). In mollusks, this cavity, a respiratory chamber housing the gill, is covered by a protective sheet, the mantle shelf, which terminates in a fleshy spout, the siphon. When the siphon or mantle shelf is stimulated by light touch, the siphon, mantle shelf, and gill all contract vigorously and withdraw into the mantle cavity. This reflex is analogous to vertebrate defensive escape and withdrawal responses, which can be modified by experience. In *Aplysia* this simple reflex can be modified by two forms of nonassociative learning, sensitization and habituation, as well as by associative classical condi-

ignore a weak stimulus, the consequences of which are neither noxious nor rewarding. Thus, an animal will initially respond to a weak tactile stimulus to the siphon by briskly withdrawing its gill and siphon (Fig. 1B). When repeatedly touched, the animal will learn to ignore the stimulus and, after 10 to 15 stimuli, will exhibit reflex responses that are reduced to one third their initial value (Fig. 1B). If a noxious sensitizing stimulus is presented to the tail, the response to the next stimulus will be enhanced; this enhancement persists for minutes to hours depending on the intensity of the sensitizing stimulus (Figs. 1B and 2). Repeated stimulation prolongs the enhancement of the reflex for weeks.

In *Aplysia*, as in vertebrates, sensitization is not the removal of preexisting habituation (4, 14) but, rather, a generalized enhancement of a wide range of responses akin to behavioral arousal. Two independent sensory pathways originate in the skin of the siphon and the mantle shelf. The distinction between habituation and sensitization can be demonstrated by stimulating only one of these pathways. Although repeated stimulation of either pathway habituates withdrawal of the gill, habituation is restricted to the activated pathway and does not generalize to the other, unstimulated one. In contrast, a sensitizing stimulus delivered subsequently enhances the response of both the habituated and the nonhabituated pathway.

Synapses of the Modulatory Neurons Identified by Electron Microscopy

Sensitization is mediated by an individual modulator neuron, L28, and by a small group of electrically coupled cells, the L29 cells. The cell bodies of L28 and the L29 group are located in the abdominal ganglion and are activated by stimuli that produce sensitization (Fig. 3). L28 is activated by shocks to the head, and L29 by shocks to the tail (20). The properties of the L29 cells have been studied in detail. Stimulating one of the L29 cells causes presynaptic facilitation of the connections between the sensory and motor neurons. The axons of the L29 cells run out of the ganglion in the left and right pleuroabdominal connective. Terminals of their axon collaterals have been identified and characterized morphologically in thin sections of the neuropil region of the abdominal ganglion. Electron microscopy reveals that they contact terminals of the sensory neuron near the sites at which the sensory neuron releases its transmitter (23). Combined electron microscopic, radioautographic, and pharmacological studies suggest that L28 and the L29 cells are mediated by serotonin (23, 24) (Fig. 5A). Applying serotonin, but not other transmitters, produces presynaptic facilitation.

Sensitization Depends on Serotonin and Cyclic AMP

That the formation of new protein is not required for short-term sensitization (25) drew our attention to the possibility that the behavioral change might result from a change in concentration of a small molecule—perhaps a second messenger, like cyclic AMP. To test this idea, Cedar et al. (26) examined the effects on cyclic AMP content of stimulating axons in the connective, the pathway that mediates sensitization. Strong stimulation doubled cyclic AMP in the abdominal ganglion, and the effect was simulated by applying serotonin in the bath. This analysis has recently been extended to the cellular level (27): individual sensory neurons undergo a three- to fourfold increase in the intracellular concentration of cyclic AMP after the isolated ganglion is exposed to serotonin for 5 minutes.

Brunelli et al. (24) next exposed the abdominal ganglion to dibutyryl cyclic AMP and found that it increased synaptic facilitation at the sensory-to-motor neuron synapse. Moreover, facilitation also occurred when cyclic AMP was injected into sensory neurons (Fig. 5B).
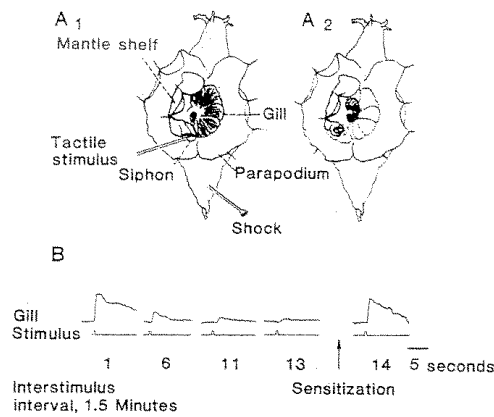
Fig. 1. Short-term sensitization of the gill-withdrawal reflex in *Aplysia*. (A₁) Experimental arrangement for behavioral studies in the intact animal showing the gill in a relaxed position. A gill-withdrawal reflex is elicited by a water jet (tactile) stimulus to the siphon. The sensitizing stimulus is a noxious mechanical or electrical stimulus to the neck or tail. (A₂) Gill after withdrawal. The relaxed position is indicated by the dotted lines. (B) Photocell recordings showing sensitization and habituation of the gill-withdrawal reflex. After 13 stimuli, the reflex response was reduced to less than 30 percent of its initial value. Arrow: noxious sensitizing (electrical) stimulus applied to the tail (arrow).
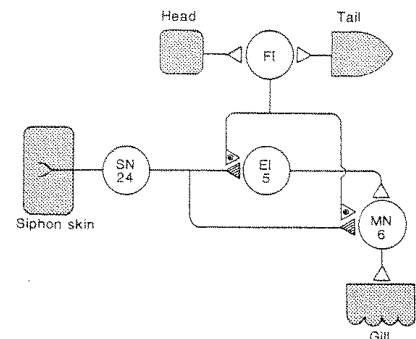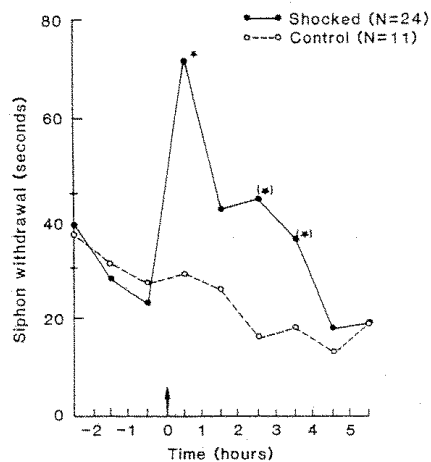
Fig. 2 (left). Time course of sensitization after a single strong electrical shock to the tail. The siphon-withdrawal reflex was tested once every 0.5 hour, and the mean of each two consecutive responses is shown. Even this low rate produced some habituation. After the third siphon stimulus, the experimental group received a single shock to the tail (arrow). After this sensitizing stimulus the experimental animals had significantly longer withdrawals than controls for up to 4 hours (59). Fig. 3 (right). Neural circuit of the gill component of the defensive withdrawal reflex to siphon stimulation. The interneurons and the motor cells are all unique, identified cells (15, 17, 20). Abbreviations: SN, sensory neuron; MN, motor neuron; EI, excitatory interneuron; and FI, facilitating interneuron.
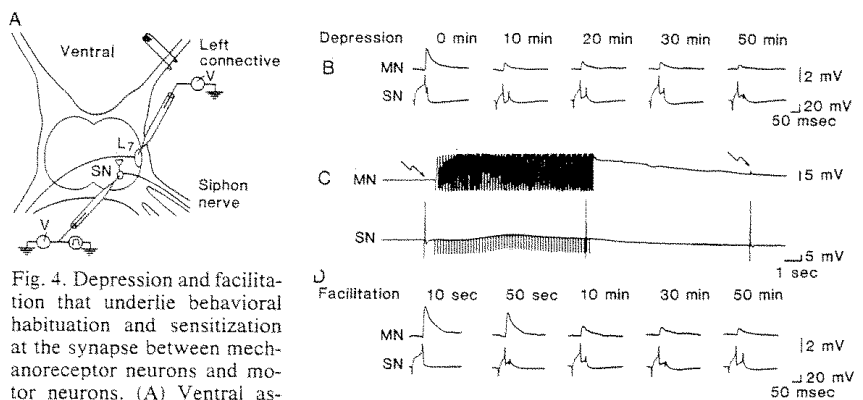
Fig. 4. Depression and facilitation that underlie behavioral habituation and sensitization at the synapse between mechanoreceptor neurons and motor neurons. (A) Ventral aspect of the abdominal ganglion of *Aplysia*, illustrating simultaneous recording from gill motor neuron L7 and a mechanoreceptor sensory neuron. Stimulation of the left pleuroabdominal connective is used as the facilitating stimulus. The connective carries information from the head and the tail to the abdominal ganglion. (B–D) Depression and subsequent facilitation of the monosynaptic excitatory postsynaptic potential (EPSP) after a strong stimulus. (B) Progressive depression of EPSP in the motor neuron that occurs when the sensory cell fires once every 10 seconds for 50 minutes. (C) Effects of the facilitating stimulus (a train of shocks to the left connective at 6 hertz for 10 seconds). Arrows, last EPSP before the facilitating stimulus and the first EPSP after the stimulus. (D) Gradual decline of facilitation over the next 50 minutes, during which the sensory neuron was again stimulated once every 10 seconds.

This effect is pharmacologically specific: neither cyclic guanosine 5'-phosphate nor cyclic AMP produces the facilitation. These observations suggested that the facilitating neurons mediate sensitization by releasing serotonin, which then acts on the terminals of the sensory neurons to increase intracellular cyclic AMP. Elevation of cyclic AMP, in turn, enhances the release of the sensory transmitter.

Serotonin and Cyclic AMP Modulate the Ca²⁺ Current of Presynaptic Terminals

We suggest that serotonin and cyclic AMP enhance the influx of Ca^{2+}, which is critical for transmitter release. This ion is required for the binding of vesicles to discharge sites—a necessary step in exocytotic release of transmitter (28). Influx of Ca^{2+} into the terminal is triggered by the depolarizing effect of the action potential. Klein and Kandel (29) found that the presynaptic facilitation underlying sensitization enhances a voltage-dependent influx of Ca^{2+} into the sensory neuron. This influx is initiated by the depolarizing action of the spike that invades the terminals. According to this view, cyclic AMP enhances the influx of Ca^{2+} into the terminals, as it does in the vertebrate heart in response to norepinephrine, another aminergic transmitter (30).

Because the sensory terminals are small, it has not yet been possible to examine the action of cyclic AMP on them directly. Nevertheless, they are sufficiently close to the cell body that somatic changes reflect changes in the terminals (29, 31, 32). In addition, the properties of somatic Ca^{2+} channels seem to resemble those of the terminals (33, 34). Klein and Kandel (29, 31) therefore examined changes in membrane properties of the cell body of the sensory neurons during presynaptic facilitation and found that synaptic actions produced by serotonin and cyclic AMP altered the currents underlying the action potential, thereby changing its duration.

The inward Ca^{2+} current in both cell body and terminals is normally masked by much larger outward K^+ currents (Fig. 6A). To uncover the Ca^{2+} current, the ganglion was exposed to tetraethylammonium (TEA), an agent that blocks the delayed K^+ channels (Fig. 6B) (35). This treatment prolongs the action potential and gives rise to a characteristic plateau, the duration of which is sensitive to slight changes in Ca^{2+} or K^+ current because the two currents are balanced during the plateau that precedes the descending limb of the action potential (34). The duration of the spike in the TEA-treated sensory neuron, even though a good assay for changes in Ca^{2+} influx, does not show whether the Ca^{2+} current increases because of a direct

action on the Ca^{2+} channel, or indirectly because an opposing K^+ conductance is reduced.

Klein and Kandel (29) found that in TEA the Ca^{2+} current was prolonged by (i) stimulating the connective that carries sensitizing input to the abdominal ganglion from the head and the tail; (ii) exposing the sensory cell to serotonin, the putative mediating transmitter; (iii) injecting cyclic AMP intracellularly; and (iv) incubating the ganglion with isobutyl-methlxanthine (IBMX), a phosphodiesterase inhibitor that increases the amount of endogenous cyclic AMP (Fig. 7A). Of 24 transmitter candidates tested, only serotonin was found to mediate presynaptic facilitation with this assay system, and the spike broadening induced was blocked by cinanserin (24). Firing a single L29 facilitator neuron also produced broadening of the action potential in the sensory neuron treated with TEA (36).

Prolonged modulation of a voltage-sensitive Ca^{2+} current is a powerful and efficient mechanism for controlling synaptic effectiveness that can both enhance and depress synaptic transmission (37). Thus, habituation progressively decreases Ca^{2+} current simply as a result of the repeated invasion of the terminals by action potentials. During repeated stimulation of the TEA-treated sensory neuron at rates that produce habituation, the spike narrows progressively, reflecting a corresponding decrease in Ca^{2+} current. This decrease in duration of the action potential parallels the decrease in amplitude of the excitatory postsynaptic potential (EPSP).

Enhancement of Ca²⁺ Influx Results from Depressing a K⁺ Current

The increase in Ca^{2+} influx during presynaptic facilitation could occur because the facilitatory transmitter has a direct effect on the Ca^{2+} channels of the presynaptic terminals or because the action potential in the sensory neuron is prolonged by a synaptically mediated decrease in K^+ current. To distinguish between these alternatives, Klein and Kandel (31) voltage-clamped the cell bodies of the sensory neurons and examined the response of specific Ca^{2+} and K^+ currents to serotonin and to stimulation of the facilitatory pathway. These currents could be examined in isolation by blocking the other channels pharmacologically.

With Na^+ and K^+ channels blocked, current through the Ca^{2+} channel decreased with repeated depolarizing commands (designed to simulate the repeat-
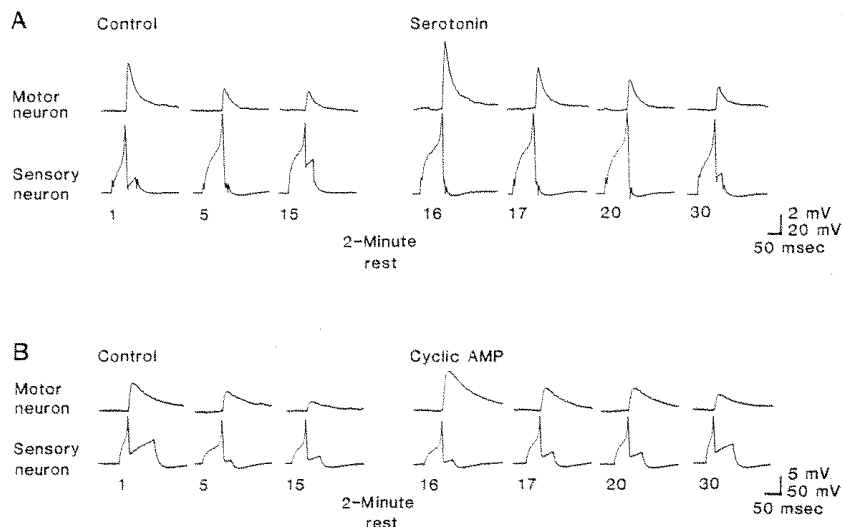

Fig. 5. Serotonin (A) and cyclic AMP (B) simulate presynaptic facilitation. (A) A sensory neuron stimulated once every 10 seconds produced a monosynaptic EPSP in a gill or siphon motor neuron. Between the 15th and 16th action potential was a 2-minute rest during which the ganglion was exposed to $10^{-4}M$ serotonin. (B) A sensory neuron was first stimulated once every 10 seconds for 15 stimuli (1 to 15). During a rest, cyclic AMP was introduced into the cell body of the sensory neuron by iontophoresis. Hyperpolarizing current pulses (lasting 1 second) were applied every 2 seconds for 2 minutes from one barrel of a double micropipette filled with $1.5M$ cyclic AMP; the recording barrel was filled with $2M$ potassium citrate. Thirty seconds after the end of the injection a second series of 15 stimuli was given (16 to 30). The amplitudes of the evoked EPSP's of the second series are higher than the amplitudes of comparable EPSP's evoked in control experiments (24).

ed activation of the neuron by habituation), indicating that the depression of transmitter release underlying habituation results from inactivation of Ca^{2+} channels. When a pulse of serotonin was then added briefly to the bath, a step depolarization failed to elicit changes in the inward currents. In contrast, when the Ca^{2+} channels were blocked and the K^+ channels examined in isolation, serotonin effectively decreased the outward current (38). These experiments indicate that the facilitatory transmitter decreases a K^+ current rather than influencing the Ca^{2+} channel directly (39).

Serotonin and Cyclic AMP Modulate a Novel Species of K^+ Channel

Only one of several possible K^+ channels is affected by serotonin. At least four types of K^+ channels have been found in various vertebrate and invertebrate neurons: (i) an early K^+ channel; (ii) a delayed K^+ channel; (iii) a Ca^{2+}-dependent K^+ channel (40); and (iv) the M channel, a K^+ channel modulated by the interaction of acetylcholine with muscarinic receptors (41). Using voltage-clamp analysis in combination with pharmacological blocking agents, Camardo et al. (42) found that serotonin did not affect any of these known channels but seemed to modulate a new one. Unlike the four K^+ currents described, the current through this channel is activated at the resting level, is large at the peak of the action potential, does not inactivate readily, does not depend upon Ca^{2+} influx, and is not blocked by Ba^{2+}. By analogy to the M current (I_{K_M}) described by Brown and Adams (41), Camardo et al. (42) call the current through this novel channel the S current (I_{K_S}) because it is modulated by serotonin. Siegelbaum et al. (43) defined the properties of this channel with patch-clamp techniques and have found that serotonin acts by decreasing the probability of its opening, not by altering its conductance or selectivity.

This novel K^+ channel decreased by serotonin normally contributes to the repolarization of the action potential. By decreasing this K^+ current, serotonin and cyclic AMP cause the action potential to broaden by 10 to 20 percent, allowing greater inflow of Ca^{2+} and increased release of transmitter (31). To determine if this small increase in duration accounts for the large increase in synaptic potential produced by the sensory neurons as a result of presynaptic facilitation, Klein et al. (37, 44) examined the duration of presynaptic depolarization and the amplitude of the EPSP's

under voltage clamp and found the relationship to be quite steep. For short pulses of the duration of the action potential, increasing the duration of the command step from 3.0 to 3.5 milliseconds quadrupled the size of the EPSP's. This large effect supports the idea that the small increases in spike duration that occur normally with presynaptic facilitation could cause significant synaptic facilitation.

Cyclic AMP, Transmitter Regulation, and the Multiple Control of Ionic Channels

In 1957 Grundfest (45) suggested that gated channels can be activated either by voltage or by chemical transmitter but not by both. This suggestion gave rise to what seemed to be a fundamental distinction in channels of excitable membranes: voltage-gated channels are electrically excitable but chemically inexcitable, and chemically gated channels are chemically excitable but electrically inexcitable. Grundfest's theory, which guided research on excitable membranes for 20 years, has been supported by many examples, with only minor exceptions such as the chemically gated channels of the neuromuscular junction, whose opening time depended slightly on

voltage (46). It has become clear, however, that there is an important class of channels whose gating is multiply regulated (30–32, 39, 47).

The experiments reviewed above suggest that the I_{K_S} channel belongs to this new class of channels and that it is regulated both by voltage and by a chemical transmitter. The channel has two other novel features (29, 31, 37). (i) The channels are present in synaptic terminals, where they participate in controlling release of transmitter. (ii) Dual regulation of the K^+ channel endows terminals with unusual plastic capabilities because it makes the release of transmitter sensitive to both electrical (membrane potential) and chemical (cyclic AMP) modulatory signals.

In addition to specifying the ionic locus of the action of serotonin, these studies illustrate the usefulness of voltage-clamp analysis for membrane biochemistry (46). The various ionic currents flow through distinct channels, each of which is composed of specific intrinsic membrane proteins (48). Identifying and chemically characterizing the presynaptic channel that is modified in sensitization and relating it to the biochemical mechanisms of the short-term memory are tasks that will be made easier by the knowledge that only one of
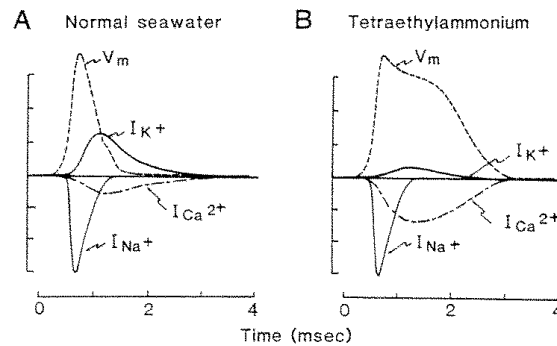

Fig. 6. Modulation of the Ca^{2+} component of the action potential in sensory neurons. Action potential (V_m) and the Na^+, K^+, and Ca^{2+} currents I_{Na}, I_K, and I_{Ca}. These currents give rise to the action potential. Application of TEA reduces I_K, which results in a slowed repolarization that unmasks and enhances the Ca^{2+} current.
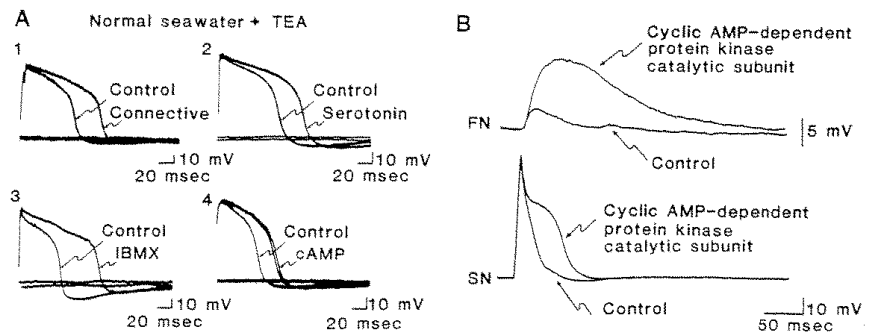

Fig. 7. Spike broadening in a sensory neuron obtained by intervention at several steps in the proposed mechanism for presynaptic facilitation of the sensory neuron-to-motor neuron synapse. (A_1) Stimulation of the connective, the facilitating pathway. (A_2) Application of $10^{-4}M$ serotonin. (A_3) Application of the phosphodiesterase inhibitor IBMX. (A_4) Intracellular injection of cyclic AMP (29). (B) Intracellular injection of the catalytic subunit of cyclic AMP–dependent protein kinase (51).

several types of K$^+$ channels is affected by serotonin. For example, these studies suggest that the protein of the K$_S$ channel resembles certain metabolic enzymes whose activities can be modified both by allosteric ligand interactions and by covalent modifications.

A Molecular Model of Sensitization

Klein and Kandel (*31*) have proposed a specific sequence of molecular steps initiated by the facilitating transmitter (Fig. 8). Serotonin, the likely transmitter for presynaptic facilitation, stimulates synthesis of cyclic AMP. Following Walsh *et al.* (*49*), who showed that cyclic AMP stimulates glycolysis in muscle by means of a cyclic AMP–dependent protein kinase, Kuo and Greengard suggested that all actions of cyclic AMP in eukaryotes are mediated by protein phosphorylation (*50*). Klein and Kandel (*31*) therefore proposed that the serotonin-stimulated increase in cyclic AMP causes the dissociation of regulatory subunits from a cyclic AMP–dependent protein kinase in the terminals of the sensory neurons. The catalytic subunit of the protein kinase would then phosphorylate a novel species of the K$^+$ channel protein or a regulatory protein that is associated with it. This phosphorylation inactivates the channel and thereby slows repolarization of the action potential (*33*), which allows more Ca^{2+} to flow into the terminals. This allows more synaptic vesicles to bind to release sites and consequently more transmitter to be released (*31*).

Sensitization Activates Cyclic AMP–Dependent Protein Kinase

The specific biochemical cascade proposed leads to several predictions: (i) that serotonin brings about the phosphorylation of a membrane protein by cyclic AMP–dependent protein kinase, (ii) that this protein is associated with a particular class of K$^+$ channels, and (iii) that phosphorylation of this K$^+$ channel leads to its closing.

To examine the relationship between cyclic AMP–dependent protein phosphorylation and the closing of the K$^+$ channel, Castellucci *et al.* (*51*) injected (under pressure) the purified catalytic subunit of the cyclic AMP–dependent protein kinase obtained from bovine heart (*52*) into cell bodies of individual sensory neurons in isolated abdominal ganglia. They assayed the Ca^{2+} current and the K$^+$ current while also measuring transmitter release postsynaptically by
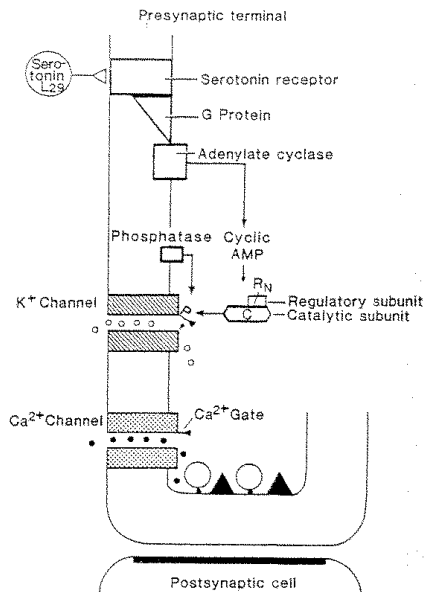
Fig. 8. Molecular model of presynaptic facilitation underlying sensitization.

recording the synaptic potential either in an interneuron or in a motor neuron of the gill-withdrawal reflex. If the actions produced by behavioral sensitization, serotonin, and cyclic AMP involve phosphorylation of the K$^+$ channel leading to enhanced transmitter release, we might expect that intracellular injection of the kinase would (i) increase influx of Ca^{2+}, (ii) decrease the K$^+$ current, and (iii) increase the amount of transmitter released by the sensory cell. Castellucci *et al.* (*51*) obtained direct support for each of these three predictions (Fig. 7B).

If the molecular basis of enhanced transmitter release involves cyclic AMP–dependent protein kinase, we also might expect that serotonin, cyclic AMP, and the catalytic subunit of the kinase would stimulate phosphorylation of specific proteins. We find that the phosphorylation of several proteins is stimulated by a 5-minute exposure to serotonin of both the abdominal and the pleural ganglia, both of which contain clusters of sensory cells (*53, 54*). Some of these proteins are contained in membrane fractions, some are soluble, and others are associated with cytoskeleton preparations. This is consistent with the physiological and ultrastructural evidence that the cyclic AMP–dependent protein kinase acts on several protein substrates. Stimulation of some of these proteins is seen when only the sensory cells from these ganglia are used. The identification, distribution, and compartmentalization of these phosphoproteins currently are being investigated (*54*). In cell-free homogenates prepared from these sensory cells, the phosphorylation of each of the membrane-associated pro-

teins is stimulated by cyclic AMP, by Ca^{2+}, or by both second messengers. As a result, it might be possible to isolate these proteins in amounts sufficient to obtain antibodies for immunohistochemical localization. In addition, antibodies may be pharmacologically useful for blocking the enhancement of transmitter release and thereby expression of the learning.

The Rate-Limiting Step in the Short-Term Memory for Sensitization

That sensitization is simulated when the catalytic subunit of protein kinase is injected intracellularly indicates that phosphorylation of a protein is an obligatory step in the mechanism of short-term memory. To determine what step in the sequence of biochemical reactions (Fig. 8) contains the memory for short-term sensitization, Castellucci *et al.* (*55*) used a specific protein inhibitor of the kinase (*56*). If the presumed channel protein remains phosphorylated in the absence of a sustained elevation in the concentration of cyclic AMP, then after serotonin has brought about the release of the endogenous catalytic subunit, injection of kinase inhibitor should be ineffective (Fig. 9A). If the time course depends on continuous activation of the kinase resulting from a sustained elevation of cyclic AMP, injecting the inhibitor should restore the spike duration and the Ca^{2+} current to normal at any time, even after stimulation with serotonin (Fig. 9B). Castellucci *et al.* (*55*) found that injection of the kinase inhibitor immediately narrows the action potential previously broadened by serotonin (10^{-6}M) (Fig. 9C), indicating that the time course of the memory is not determined by a prolonged state of protein phosphorylation but rather by sustained enhancement of kinase activity. This is consistent with the observation that cyclic AMP remains elevated more than an hour after a brief pulse of serotonin; its time course actually parallels that of the presynaptic facilitation (*27, 57, 58*). Thus the available evidence suggests that the memory resides either in the continued synthesis of cyclic AMP by adenylate cyclase or in diminished degradation of the cyclic nucleotide by phosphodiesterase.

A Possible Mechanism for Long-Term Memory

Fundamental to the study of memory is the relationship between the short-term and the long-term forms. Behavioral (*59*) and electrophysiological (*11*) ex-

periments suggest that short-term sensitization grades into long-term sensitization. A single noxious sensitizing stimulus produces a memory that lasts several hours. With four consecutive noxious stimuli, the memory lasts 1 day. Sixteen consecutive stimuli prolong the memory to several days, and with 16 spaced stimuli (four per day for 4 days) it lasts several weeks (59). Two independent experiments suggest that short- and long-term sensitization also have a common cellular locus. (i) Physiological experiments indicate that the strength of synaptic connections made by sensory neurons is enhanced in long-term sensitization (11, 60). (ii) Bailey and Chen (61) have found that terminals of the sensory neurons undergo striking morphological changes. Transmitter vesicles are released from varicosities of the axon terminals at specialized regions called active zones. In naïve animals, only 40 percent of the varicosities of the sensory neurons have active zones. In long-term sensitized animals, 70 percent of varicosities have these release sites. In addition to the increase in number, sensitized animals also show an increase in the area of each active zone.

What are the molecular mechanisms by which these functional and morphological changes are produced? Experiments on short-term sensitization suggest several molecular schemes in which long-term memory shares with short-term memory both synaptic locus and ionic mechanisms. The final step in the long-term process could also be enhanced transmitter release caused by modification of Ca^{2+} influx, brought about when a cyclic AMP–dependent protein phosphorylation alters the configuration of a K^+ channel protein (Fig. 10A). But to convert the short-term to the long-term process, some distinctive molecular events must be added, and these might also explain the morphological changes. Whereas the short-term process does not require synthesis of new macromolecules (25), its conversion to a long-term process might require expression of new genes in response to learned experiences. This notion would be consistent with earlier proposals, based on studies in vertebrates, that the synthesis of new proteins is essential for long-term memory (62).

As an example, we shall consider one possible molecular explanation for long-term memory that involves a specific change in gene expression (Fig. 10B). We suggest a new regulatory subunit of the cyclic AMP–dependent protein kinase as a likely candidate for the long-term change. We favor the regulatory subunit because this species of protein

exhibits molecular variation in different cells and tissues (63). Serotonin, acting repeatedly on the terminals of the sensory neuron (training), could induce a new class of regulatory subunit for the protein kinase. The inducer might be cyclic AMP (64). Thus, the prolonged elevation of cyclic AMP that occurs in short-term sensitization may induce the new regulatory subunit by phosphorylation of nuclear protein. Synthesis of the new class of subunit could be permanent or it could slowly decay if not reinforced by subsequent training.

We posit the induction of a new regulatory subunit with two novel features: (i) it would have greater sensitivity to cyclic AMP, thereby allowing the catalytic subunit to dissociate readily; and (ii) it would be site-specific, allowing the kinase to be bound to the presynaptic

membrane near the K^+ channels that are to be modulated (Fig. 10B). The synthesis of a regulatory subunit with greater affinity for cyclic AMP would allow the cyclic AMP–dependent protein kinase to work at relatively normal concentrations of the cyclic nucleotide (65). As a result, slight elevations above the normal concentrations of cyclic AMP of the sort that accompany the sensitizing or arousing stimuli of everyday life (inadequate to evoke the short-term process in the untrained terminal) would now be sufficient to provide (by modification of ion channels) the enhanced influx of Ca^{2+} required to increase release of transmitter (Fig. 10, B_2 and B_3).

It is unlikely that a regulatory subunit with greater affinity for cyclic AMP would occur without site specificity, since this could disturb other pathways
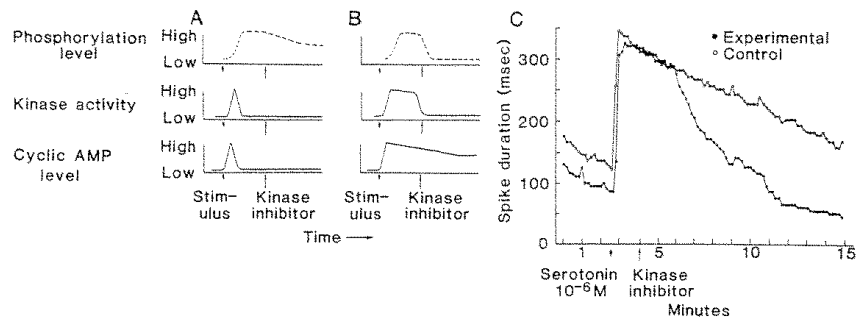

Fig. 9. (A and B) Two alternative models of the time course for the memory for short-term sensitization. (C) Results obtained after injection of the protein kinase inhibitor. In each graph, the first arrow indicates serotonin and the second indicates the inhibitor.
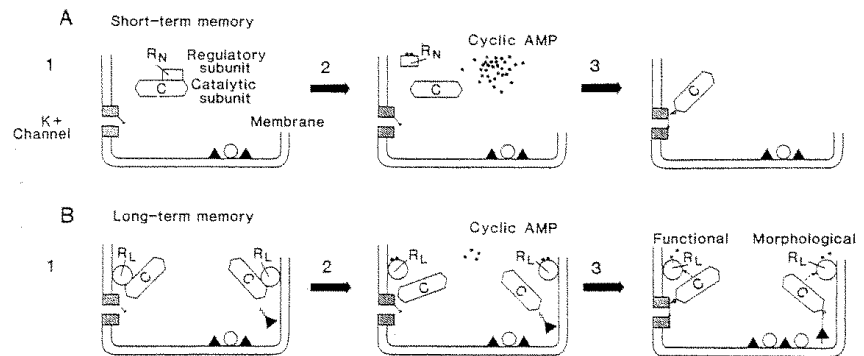

Fig. 10. A model for the biochemical basis of long-term memory. (A_1) In short-term sensitization the cyclic AMP–dependent protein kinase is proposed to have a normal regulatory subunit (R_N) and no particular orientation with respect to a substrate membrane protein associated with the K^+ channel. In naïve terminals, relatively high concentrations of cyclic AMP are needed to activate (C) the catalytic subunit (A_2) to phosphorylate (P) the membrane protein (A_3) which brings about enhanced release of transmitter, the neurophysiological event underlying sensitization. The memory is brief because the concentration of cyclic AMP diminishes soon after stimulation with serotonin. (B_1) In trained neurons, a new class of regulatory subunit (R_L) has been induced. As a result, the protein kinase differs from the naïve enzyme in being site-specific, and thus being advantageously oriented both to the channel and to the mechanism that governs the organization of dense projections at the active zone where synaptic vesicles line up to release transmitter. In addition, this new kinase has higher affinity for cyclic AMP. (B_2) Consequently, lower concentrations of cyclic AMP are required to phosphorylate these target proteins. (B_3) Functionally, as in (A_3), the K^+ channel is inhibited as long as the channel protein remains phosphorylated. Morphologically, protein phosphorylation leads to the stable enlargement of the synapse. In this form of sensitization, the memory persists for longer periods of time because it is embodied in R_L, a protein molecule.

regulated by cyclic AMP within the neuron. As in general metabolism, regulation would affect the first branch-point enzyme in the pathway to be controlled. Induction of a site-specific regulatory subunit would, for example, avoid unnecessary changes in carbohydrate metabolism that might occur if other elements of cyclic AMP metabolism were altered. Although we predict that indiscriminate phosphorylation of proteins would not occur in long-term sensitization, a specific protein kinase could trigger a family of parallel cyclic AMP–dependent changes in the sensory neuron. Thus the same cyclic AMP–dependent kinase which produces the change in functioning of the K^+ channel protein could also be optimally situated to alter the assembly of the protein components that constitute the active zone (Fig. 10B) and thereby initiate the striking change in morphology of sensory terminals that have recently been observed by Bailey and Chen (61). These two molecular changes, both caused by the same cyclic

AMP–dependent kinase, would operate together to bring about enhanced transmitter release from the long-term sensitized neuron.

Although obviously premature because of the lack of supporting experimental data, this speculative explanation for long-term memory can be tested. An important general prediction is that both the short-term and the long-term forms of sensitization share the cascade of events that result in increased release of transmitter. This prediction can now be tested electrophysiologically. Another prediction is that, unlike the short-term form, long-term sensitization will be blocked by inhibitors of protein synthesis. Moreover, characterization of regulatory subunits in vertebrates is an active field of biochemical research (63, 66). In *Aplysia*, Eppler *et al.* (67) have characterized these proteins and have found that nerve endings contain at least nine different forms. Of these, four are associated with membrane and are possible candidates for regulatory subunits that

influence ion channels. Finally, even the prediction that cyclic AMP affects nuclear proteins is testable in identified *Aplysia* neurons. Indeed, these large cells offer special experimental advantages for molecular genetic studies of the nervous system since nuclei of individual cells can be isolated by hand dissection (25, 68). In addition, recombinant DNA techniques have recently allowed genes of known function to be isolated in *Aplysia* (69).

The Relationship Between Sensitization and Associative Learning

Elementary forms of learning are divided into two general categories: nonassociative (habituation and sensitization) and associative (classical and operant conditioning). For associative learning to take place, two stimuli or a stimulus and a response must be temporally associated. For example, in classical conditioning an initially weak or ineffective conditioned stimulus (CS) acquires novel behavioral significance only after it has been paired with a strong unconditioned stimulus (US). After an animal has been conditioned it behaves as if the CS predicts the US. Thus, through classical conditioning, we learn about causal relationships in the environment. In contrast, nonassociative learning does not require temporal pairing of stimuli and does not teach the animal to expect any relationship between stimuli. Frequently a reflex shows both sensitization and classical conditioning to the same CS and US. In those instances, the enhancement produced by paired presentations of CS and US will be greater or last for a longer time than that produced by unpaired presentations.

Recently, Carew *et al.* (70) found that, in addition to being enhanced by sensitization, the siphon- and gill-withdrawal reflexes of *Aplysia* can also be classically conditioned. To produce this associative form of learning, Carew *et al.* (70) paired a mild tactile stimulus to the siphon (the CS), which elicits feeble withdrawal of the siphon and gill, with a strong electrical stimulus to the tail (the US) that produces powerful withdrawal (Fig. 11A). After 15 pairing trials, conditioned animals showed greater withdrawal in response to the weak CS, both immediately after training and as long as 4 days later, than do sensitized animals that received the US alone or control animals that received the CS and US in a specifically unpaired or random manner. Thus, classical conditioning of this reflex was acquired rapidly and persisted for days.
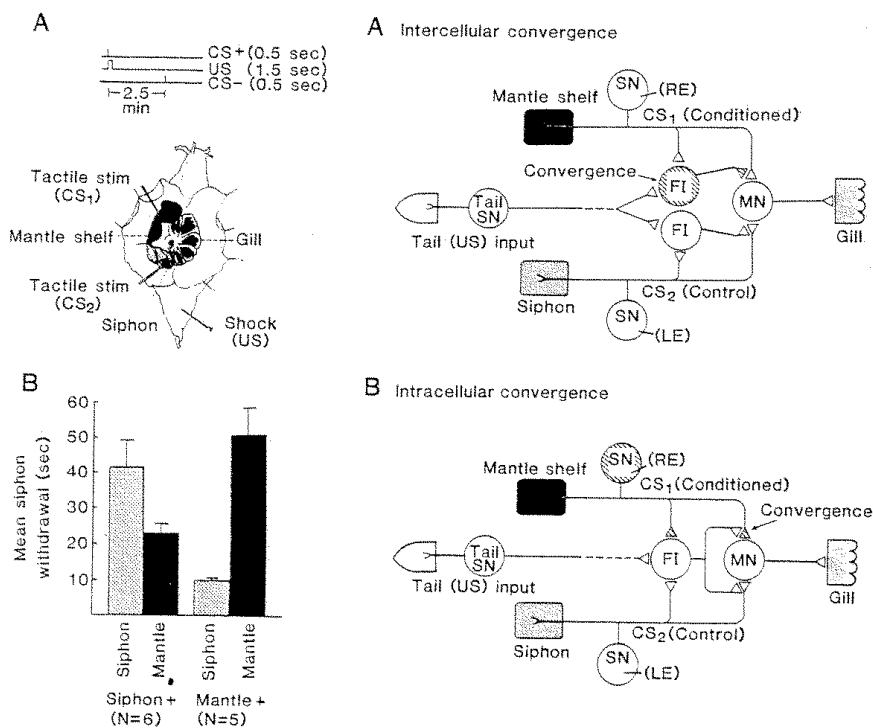

Fig. 11 (left). Mechanisms for differential classical conditioning of siphon- and gill-withdrawal reflexes. (A) Dorsal view of *Aplysia* illustrating the two CS pathways (siphon and mantle shelf) and the US pathway used for differential conditioning. The conditioning protocol is indicated above. CS+ and CS−, paired and unpaired inputs. Before conditioning, weak, 10-second withdrawals of the siphon resulted from either of the two CS stimuli. (B) After differential conditioning animals receiving siphon + training show significantly greater responses to the siphon CS+ than to the mantle CS−; the reverse is true for animals receiving mantle plus training. Fig. 12 (right). Two models for classical conditioning. In each, conditioning consists of a memory component, responsible for the time course of the learning, and a temporal, discriminative component responsible for associative specificity. In each model, presynaptic facilitation provides the mechanism for the memory component, but temporal specificity is achieved in different ways. (A) Temporal specificity is achieved by convergence of the CS and US on a common facilitator neuron, which is shaded to indicate spike activity. (B) Temporal specificity results from convergence of inputs on the sensory neuron where the spike activity occurs.

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Carew *et al.* (*71*) have now found that this reflex system can also be differentially conditioned by pairing a weak CS to either the siphon or the mantle shelf with a strong shock US to the tail (Fig. 11). As we have discussed, the siphon and mantle shelf is innervated by its own population of sensory neurons (the LE cluster innervates the siphon, the RE cluster the mantle shelf), and each pathway can be activated independently to serve as a conditioned stimulus. Animals in which a CS to the siphon is paired with a tail shock and in which a CS to the mantle is specifically not paired show, after training, a greater response to the siphon than to the mantle. Conversely, when a stimulus to the mantle is paired, and one to the siphon is not paired, the response to the mantle stimulus is greater (Fig. 11B). Differential learning allows each animal to serve as its own control and facilitates the analysis of classical conditioning on the cellular level. In addition, since two sensory inputs to a common motor output can be differentially conditioned, at least some aspects of the learning appear to be localized in the sensory part of the reflex.

Because classical conditioning and sensitization share similar components, it has often been thought that they are related in mechanism (*72*). It is therefore attractive to think that classical conditioning consists of the components that account for sensitization, and an additional mechanism which gives associative learning temporal specificity (*73*). According to this line of thought, cyclic AMP–mediated enhancement of transmitter release could serve as the basic mechanism for strengthening synaptic connections in associative as well as in nonassociative learning. Optimal enhancement might require that the CS and US be temporally paired. Temporal specificity, the additional step involved in associative learning, could be explained by a convergence of the CS and US at one of two loci in the neural circuit (Fig. 12): (i) the CS and US pathways might converge on a facilitating interneuron that is distinctive for each CS pathway (siphon and mantle shelf) but common to that pathway and the US pathways (Fig. 12A), and (ii) the CS and US might converge within the terminals of the CS sensory neurons because of impulse activity in the sensory neurons that is coincident with synaptic input from the US neurons (Fig. 12B).

Hawkins *et al.* (*74*) have tested both models and provided direct support for the second. They found that, after a series of pairing trials in which action potentials in a sensory neuron immedi-

ately precede activity in the US pathway, the sensory neuron releases more transmitter than when action potentials in the sensory neuron are not paired with the US. Thus, at least some of the mechanism for the temporal specificity of classical conditioning occurs within the sensory neuron itself. Similar results have been obtained independently by Walters and Byrne (*75*), who have found temporally specific activity-dependent synaptic facilitation in indentified sensory neurons that innervate the tail of *Aplysia*. The discovery of temporally specific and activity-dependent facilitation in another population of sensory cells suggests that this mechanism is general.

Temporal specificity utilizing the basic mechanisms of sensitization could be achieved in several ways by modulating one or more steps in the cyclic AMP cascade. For example, activation of cyclic AMP synthesis by serotonin might be enhanced in a terminal which has just experienced one or more of the four events that occur during the action potential: depolarization, Na^+ influx, Ca^{2+} influx, or K^+ efflux. One attractive possibility proposed both by Abrams *et al.* (*76*) and by Walters and Byrne (*75*) is that Ca^{2+} might activate adenylate cyclase. According to this idea, the action of the serotonergic facilitating neuron would be more effective in classical conditioning than in sensitization because activation of the cyclase by serotonin is preceded by an influx of Ca^{2+} within the sensory neuron. Thus, a basic presynaptic regulatory mechanism involving enhancement of transmitter release by cyclic AMP–dependent phosphorylation could be used in different ways (conventional and amplified) to achieve both nonassociative and associative learning.

Reductionist Strategies for Studying Learning

Because its nervous system is advantageous for biophysical and biochemical analysis, *Aplysia* has provided molecular insights into a simple form of learning. We suggest that the mechanisms for sensitization that we have considered here may not be unique to *Aplysia* or even peculiar to sensitization and classical conditioning but operate in other animals and, with variations, in more complex forms of learning. Indeed, there are single-gene mutants of *Drosophila* (dunce) that cannot master associative learning tasks (*77*) and that lack cyclic AMP phosphodiesterase (*78*). Dunce has abnormally high concentrations of cyclic

AMP (*78*) and is deficient not only in learning associative conditioning tasks but also in learning sensitization (*79*). It has as yet been difficult to analyze the mechanisms involved in detail because the locus of sensitization has not been identified electrophysiologically in the nervous system of the fruit fly, which contains neurons that are quite small. Nevertheless, genetic, cell biological, and biochemical approaches support the idea that cyclic AMP modulates the strength of behavior in sensitization and in certain forms of classical conditioning.

Reductionist strategies have been successfully applied to the analysis of various biological mechanisms including muscle contraction, the genetic code, protein synthesis, secretion, active transport, membrane excitability, and electrical and chemical transmission between nerve cells. Still, the reductionist approach to learning and memory has not yet been fully accepted. Some psychologists and biologists still hesitate: they believe that only higher animals exhibit complex forms of learning and that analysis of learning in any simpler animal may not be relevant to the mammalian brain and particularly to humans (*80*). Within the last year, this view has been challenged in two studies that have compared the details of two forms of classical conditioning in invertebrates and in mammals. Walters *et al.* (*81*) found that *Aplysia* shows a conditioned association between a neutral stimulus and a central defensive motivational state that resembles the conditioning of fear in mammals. Even more striking, Sahley *et al.* (*5*) found that the land snail *Limax* can learn higher order forms of associative learning (including blocking, second-order conditioning, and preconditioning) previously thought to be restricted to mammalian intelligence. These studies show that invertebrates, like mammals, can form a central representation of the CS and learn about its predictive properties.

We emphasize that the term "learning" is likely to cover a repertoire of mechanisms and not be a single process. As we have indicated, learning varies in time course, specificity to pairing, and complexity. Fortunately, in addition to *Limax* (*82*) and *Aplysia* (*74, 81*), other invertebrates capable of being studied on the cellular level—*Pleurobranchaea* (*83*), *Hermissenda* (*84*), locusts (*85*), and leech (*86*)—are also capable of associative learning. We can therefore expect a broad attack on the problem of associative learning to provide answers to questions central to the study of learning: What kinds of cellular changes account

for the long-term storage of information in the various forms of learning? Do these processes involve expression of specific genes? If new gene products are induced, what sort of environmental signals trigger the induction? Is the biochemical mechanism underlying long-term memory specific to the synaptic process or are there generalized effects on other aspects of neuronal metabolism? What molecular mechanism underlies the morphological changes that occur during synaptic plasticity? How do molecular mechanisms of the long-term forms of plasticity relate to those of short-term plasticity? How do the various molecular mechanisms of nonassociative learning relate to those activated by associative learning? We believe that it will soon be possible to answer all of these questions explicitly, in terms that are understandable both to the psychologist and to the cell biologist.

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