Calcium-Mediated Reduction of Ionic Currents: A Biophysical Memory Trace

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Summary. Learning behavior similar to vertebrate classical conditioning was demonstrated for the mollusc *Hermissenda crassicornis*. Postsynaptic membrane changes within well-defined neural systems that mediate the learning play a causal role in recording the learned association for later recall. Specific ionic currents in neural tissue undergo transformations lasting days after associative training with physiologic stimuli. During acquisition the intracellular calcium increases; this increase is accompanied by specific potassium current reduction that lasts for days after conditioning. The increase of calcium enhances calmodulin-dependent phosphorylation of proteins that either regulate or are part of ion channels. These currents and the conditions that precede their transformation occur in many types of vertebrate neurons, and hence this biophysical basis of *Hermissenda* learning could have relevance for species other than the gastropod studied.

Among the many cellular mechanisms for learning that have been proposed are growth and formation of new neuronal branches and synapses (*1*), reverberating circuits (*2*), use-dependent modification of existing circuits (*3, 4*), and swelling of post- and presynaptic processes (*5*).

The study of cellular learning mechanisms includes precise identification of sites on individual neurons where learned information is actually stored. Even for the simpler animals, such as molluscs, this has proved difficult, especially with respect to associative learning, which concerns the relationships of stimuli in time. An individual learns to associate one stimulus with another when the two stimuli occur together repeatedly with the same order and approximately the same intervening delay. It has now become possible to determine anatomic sites within identified neurons of the nudibranch *Hermissenda crassicornis* where associative learning is encoded by ionic current changes lasting for days.

Little is known of cellular mechanisms for human learning. What is known derives from the effects of lesion-induced deficits and reports by operating-room patients who have received intracranial electrical stimulation (*6*). Understanding how the function of neurons as they send signals to each other contribute to learning requires more direct and quantitative techniques and manipulations than possible with lesions alone. Thus there is a need for cellular analyses of animal learning which resembles or can serve as a model for human learning. With animal learning, however, obvious differences are immediately apparent. Compared to humans, animals are more limited in the range of stimuli to which they can respond, and they are also more limited as to which stimuli they can learn to associate.

The sensory and perceptual physiology involved in some animal learning such as the learning of a maze by a rat is not yet well understood, and therefore a search for cellular mechanisms of maze learning would be premature. Even cellular mechanisms of learned behaviors which are analyzable and which share common features of complex learning, such as temporal and stimulus specificity and long duration, have been sought but have not yet been found. Classical conditioning of an eye-blink response and related facial movements, for example, has been demonstrated in cats, rabbits, and humans (*7*). Some closely correlated changes of neuronal activity during and after this and similar conditioning have been observed in several vertebrate preparations (*8*). Correlation of extra- and intracellularly recorded electrical activity with acquisition of such learned behavior has been combined with lesion experiments in an effort to find areas of vertebrate brain which participated in learning the conditioned response (*8*).

Finding areas necessary for acquisition or expression of a learned behavior, however, does not reveal the rate-limiting steps in the process or where and how the learned information is actually stored. Elimination of a conditioned response (and preservation of the unconditioned response) by lesioning an area together with correlated neuronal activity does not reveal a storage site, since the lesioned area may only be important for the expression of a conditioned response rather than the learning of this response. It is necessary to demonstrate that membrane, biochemical, or structural changes are intrinsic to individual neurons, once the conditioning has occurred. Thus far with vertebrate preparations changes of neurons that are storage sites for associative learning have not been identified.

The technical limitations of the vertebrate preparations have led to the development of invertebrate models for making cellular analyses of learning. An example of invertebrate associative learning has been provided by studies on the bee. Bees learn to associate colors with food (*9*). This learning is specific and long-lasting. Because the bee brain has somewhat more than 800,000 neurons, a detailed map of the sensory pathways involved is not very accessible. Thus, although some change of impulse activity has been correlated with this learning (*10*), storage sites and basic mechanisms have not yet been discovered. Definition of sensory pathways may also prove difficult in the analysis of learning of another arthropod, the locust (*11*), for which changes of motor neuron impulse activity lasting some minutes have been correlated with learned changes of the leg position (*12*).

In the first studies of nonassociative gastropod learning on a cellular level, Kandel and his colleagues recorded decrement of excitatory postsynaptic potentials (EPSP's) in correlation with decrement (habituation) of the gill withdrawal reflex of *Aplysia californica* (*13*). They also measured enhancement of EPSP's during another form of nonassociative learning, sensitization, of the same reflex in response to neck stimulation and later tail shock (*13*). Subsequently, a number of investigators undertook to demonstrate associative learning with gastropod molluscs (*14, 15*). Substantial progress has been made in analyzing ele-

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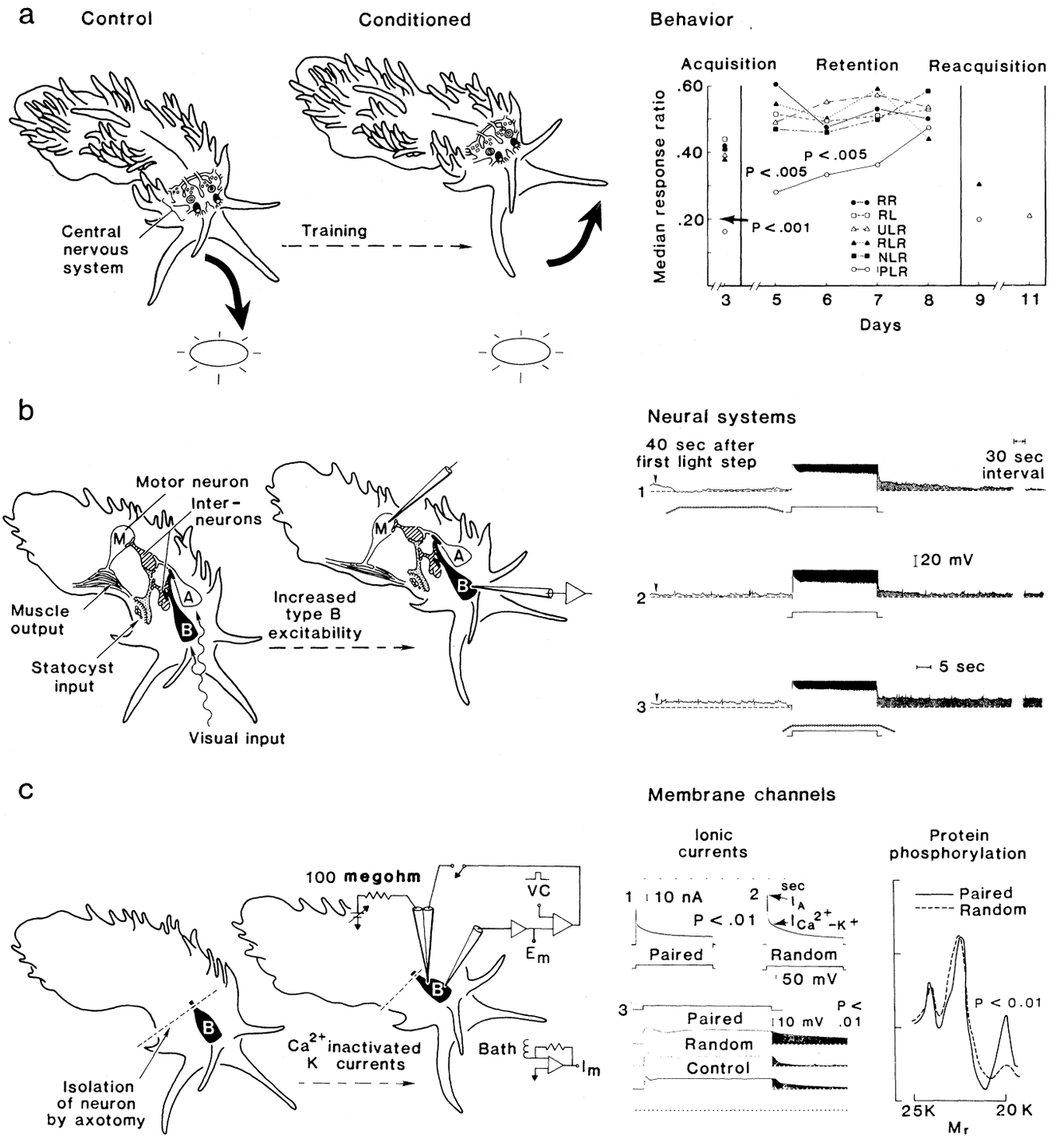

Fig. 1. Cellular analysis of *Hermissenda* associative learning. (a) Behavior. Reduction of positive phototaxic response in *Hermissenda* as a result of conditioning procedure. The plot on the right (*15*) represents data on the median response ratios for acquisition, retention, and reacquisition of a long-term behavioral change after a light stimulus [random rotation (●), random light (□), unpaired light and rotation (△), random light and rotation (▲), nothing (■), and paired light and rotation (○)]. (b) Neural systems. Critical input and output neurons, a few of many (*27*), that mediate learning the association of light and rotation. Type B and type A photoreceptors are indicated by B and A; M indicates the pedal ganglion motor neuron whose activity regulates turning movements. Intracellular recordings from intact type B cells during and after light and rotation stimuli. (Trace 1) Responses to the second of two succeeding 30-second light steps (with a 90-second interval intervening) each preceded by rotation (*30*). (Trace 2) Responses to the second of two light steps without rotation. (Trace 3) As in traces 1 and 2 with the onset of each light step followed by onset after 1 second of maximal rotation. (c) Membrane channels. Type B cells are isolated by axotomy subsequent to behavioral measurements made before and after training. The circuit for the voltage clamp experiment with two microelectrodes (*26, 32, 34, 35*) is shown in the center. The ionic currents were measured from holding potential of −60 mV. (Trace 1) Paired I_A (early peak outward K^+ current) and $I_{Ca^{2+}-K^+}$ (late current, maximum at 300 msec after onset of command) are smaller (*26, 28*) than random (trace 2) or control values (not shown). (Trace 3) Paired steady-state depolarization during and after (shaded areas) a light step (monitored by top trace) are larger than random and control (*24*). The data on protein phosphorylation are represented by densitometric scans of autoradiograms of samples of eye proteins in the molecular weight range of 20,000 to 25,000 obtained 1 to 2 hours after animals received the third training session of paired or random light and rotation (*37*).

ments of relevant neural circuitry in some of these preparations (*13, 16*). In addition, mechanisms have been proposed based on cellular analogues of *Aplysia* learning (*17*), although neural correlates of *Aplysia* associative learning are not yet available.

Hermissenda crassicornis

To identify neuronal sites which store a learned association, my colleagues and I undertook to define sensory pathways (*18*) of the nudibranch mollusc *Hermissenda crassicornis* from receptors to interneurons and motor neurons. It was necessary to find loci of convergence, neurons that received input from at least two distinct sensory pathways. If, as seemed possible, repeated activation of convergent sensory pathways with stimuli to which the animal could respond in its natural environment might produce associatively learned behavior, then convergence of sensory pathways might reveal what *Hermissenda*, which has several thousand neurons in its central nervous system, was capable of learning to associate.

In fact, it was well-defined intersensory convergence that led to the discovery of associative learning of *Hermissenda*. The animal's positive phototactic response, mediated by its two eyes, is reduced (Fig. 1a) after repeated pairings of light with rotation, an aversive stimulus, mediated by its two statocysts (*14, 15*). In an early study (*15*), the phototactic behavior was measured by a response ratio in the form of $1 - [A/(A + B)]$, which compared the latency during the test (A) (Fig. 1a) with baseline response latency (B) (Fig. 1a). Randomized light and rotation (that is, when the two stimuli have no fixed temporal relation) did not produce the learning, an indication of its truly associative or temporally specific nature (*15*). Repeated presentation of either the conditioned stimulus (light) alone or the unconditioned stimulus (rotation) alone caused no long-lasting behavioral change, a result that ruled out habituation and sensitization, respectively. Repetition of explicitly unpaired light and rotation (*15*), rotation preceding light, or rotation onset after light offset (*19*) does not produce a retained change of behavior. The learned behavior, which can be retained for many weeks, is extinguished by repeated presentations of light steps after training with paired stimuli (*20*). The learning is degraded by the interposition of additional light steps or rotation intervals between paired presentations of stimuli, showing a requirement for contingency (*21*). Responses to stimuli not presented during training are not modified, consistent with the stimulus specificity of the learned behavior. Even though animals "forget," they relearn the pairing-specific behavior with fewer training trials, indicating savings (*15*). Finally, recent experiments (*22*) show that, as a result of training, light (the conditioned stimulus) now elicits a contraction of the animal's caudal foot, a behavior that is reliably elicited by rotation (the unconditioned stimulus) but not by light prior to training.

Although the associate learning just described for *Hermissenda* is in many ways similar to vertebrate classical conditioning, there are dissimilarities. For example, vertebrate conditioning, unlike the *Hermissenda* conditioning, often requires some delay between the onset of the conditioned stimulus and that of the unconditioned stimulus for substantial conditioning to occur.

Neuronal Changes During *Hermissenda* Learning

On days after associative training of intact *Hermissenda*, changes of light-evoked neuronal activity (Fig. 1, b and c) have been recorded intracellularly from sensory receptors, interneurons, and putative motor neurons within the visual pathway (*23–26*). That these correlated neuronal changes survive dissection of the animal after training indicates lack of vulnerability to generalized responses of the nervous system during its isolation. Observations from a number of experimental procedures indicate that the learning-correlated neuronal changes in the visual pathway (mediating the response to the conditioned stimulus) arise from a learning-induced bias in the excitability (*27*) of photoreceptors relative to each other within the *Hermissenda* eye. These observations further indicate that memory of the light-rotation association is actually stored by long-term modification of specific membrane currents (*26, 28*) of individual identified photoreceptors, with the result that certain photoreceptors become more excitable and others less excitable. These observations imply that a means for storing the memory might be a sequence of biophysical and biochemical transformations (within the type B cell) (*27*) that result from the integrated response of the visual-statocyst network to repeated light-rotation pairing.

In each of the two *Hermissenda* eyes (*18*), there are three type B photoreceptors (located in the more caudal and dorsal portion of the eye) and two type A photoreceptors (located anteroventrally). The conditioning-induced increase of excitability of the medial type B cell was shown in two ways. (i) Injection of a small electric current through a microelectrode inserted inside the type B cell causes a change of the type B membrane potential. The potential change caused by the same microcurrent is greater for type B cells of conditioned animals as compared to the potential change of cells from animals exposed to randomized light and rotation and other control procedures (*23–26*). The type B cell membrane is more excitable because a greater potential change—which means a large electrical signal—is elicited for the same magnitude of current flowing across its membrane. (ii) Current also flows across the type B membrane in response to light. Larger potential changes (Fig. 1, b and c) are evoked by light for type B cells from conditioned animals (compared to cells from control animals). This larger light response is a second manifestation of increased type B excitability. Another index of increased excitability is the larger number of propagated impulses triggered by current or light-evoked potential changes of the type B membrane—also found only in conditioned animals (*23, 25*).

The medial type B cell inhibits the medial type A cell which excites via identified interneurons individual motor neurons (MNI cells) (Fig. 1b) which cause turning of the animal's foot in the direction of a light source (*29*). Increased light-evoked responses of the type B cell (Fig. 1, b and c), therefore, means decreased turning (via this pathway) and thus movement toward light, as was manifest in measures of phototactic responses of conditioned animals. Recent experiments also indicate that the medial type A cell itself undergoes a decrease of excitability in conditioned animals (*20*). The interneurons and MNI cells, however, are not the only neurons that receive input from the type A and type B photoreceptors.

A Causal Role for Type B Membrane Changes

The observations supporting a causal role for type B cell changes in the production of associative learning are summarized as follows.

1) The known synaptic interactions between photoreceptors, interneurons,

and motor neurons, as just mentioned, would be expected in conditioned *Hermissenda* to transmit the measured increase of type B photoreceptor excitability by light so as to cause a decreased excitation of motor neurons responsible for turning movements toward light. Learning-induced increases of type B excitability, measured with coded procedures, in fact, were found to predict learning-induced decreases of motor neurons excitability (*25*).

2) That the excitability increases were intrinsic to the type B membrane (and not simply a passive reflection of other changes of cells presynaptic to the type B cells) was demonstrated by isolating the type B somata from all other neurons. Type B somata, with all synaptic activity and impulse generating membrane eliminated by axotomy, showed enhanced responses to light (Fig. 1c) and to current injections in conditioned but not control animals during the learning-retention period (*24*). These learning-induced changes of excitability, which were intrinsic to the type B somata, were of sufficient magnitude to account for increases of impulse activity during and after a light step presented to intact nervous systems of the conditioned animals (*23, 24*). The increases of type B impulse activity were of sufficient magnitude to substantially account for the measured decreases of motor neuron responses to light in conditioned animals (*25*). Conditioning-induced changes at specific membrane currents (see below) were also shown to be intrinsic to the type B somata (*26, 28*).

3) For intact nervous systems, repeated pairing of light and rotation (but not unpaired stimuli) caused, as an integrated response of the visual-vestibular network (Fig. 2), cumulative membrane depolarization of the type B cell (Fig. 1b) (*30*), and prolonged elevation of mean intracellular Ca^{2+} (Fig. 3b) (*31*). In response to each presentation of the light stimulus, depolarization of the type B cell during and after the stimulus was greater for light ($\sim 10^4$ erg cm^{-2} sec^{-1}) paired with rotation (generating about $1.0g$, with the caudal portion of the nervous system oriented away from the center of rotation) than for light alone or light preceded by rotation (Fig. 1b). With repetitions of these stimuli at 90-second intervals, depolarization of the type B cell above the resting level of membrane potential (Fig. 1b) became progressively greater. Progressive steady depolarization of isolated type B cell bodies by injection of positive current (to simulate cumulative membrane depolarization in response to repetitive paired natural stimuli) together with repeated pairings of light and depolarizing current pulses (to simulate the synaptic effects of rotation) caused the same long-lasting changes of type B excitability observed for intact animals and intact nervous systems. Production of these prolonged changes of type B excitability depended on the concentration of intracellular calcium ion (*32*).

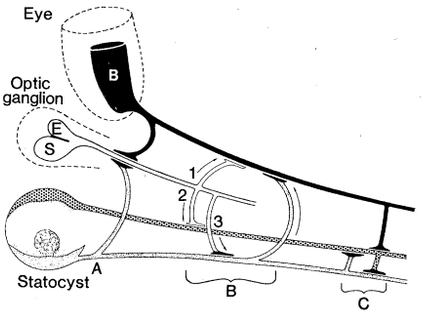

Fig. 2. Intersensory integration by the *Hermissenda* nervous system. A shows convergence of synaptic inhibition from type B and caudal hair cells on S-E cell. B indicates positive synaptic feedback onto type B photoreceptor. 1 indicates direct synaptic excitation; 2 indicates indirect excitation: E-S excites cephalic hair cell that inhibits caudal hair cell and thus disinhibits type B cell. 3 indicates indirect excitation: E-S inhibits caudal hair cell and thus disinhibits type B cell. Indirect excitation where B cell inhibits C cell, and thus disinhibits E cell, is at the bottom. C cell effects are not illustrated. C indicates intra- and intersensory inhibition. Cephalic and caudal hair cells are mutually inhibitory. Type B cell inhibits mainly the cephalic hair cell. All filled endings indicate inhibitory synapses; open endings indicate excitatory synapses (*41*).

4) When paired with light, current injections that simulated the effects of natural stimuli on specific hair cells or critically involved interneurons produced the same changes of excitability in photoreceptors. This was true for isolated nervous system preparations (*19*) as well as living animals (*33*). The experiment which provided the most conclusive evidence for causality required the impalement of type B cells in living *Hermissenda*. The measured excitability changes produced by light-current pairings (but not control procedures) predicted the observed long-lasting behavioral changes measured for the same animals on the days that followed impalement.

Although these data demonstrate that the type B soma membrane changes store learned information that contributes to the expression of the learned behavior, other neuronal changes may, nevertheless, also participate in the storage and recall processes.

Calcium-Mediated Changes of Ionic Currents in *Hermissenda* Neurons

Once intrinsic membrane changes have been found, they can be analyzed as to their involvement of individual ionic conductances (*26, 32, 34, 35*). These changes, largely responsible for *Hermissenda* conditioning, were within a cellular compartment (type B photoreceptor somata) which was accessible to measurement by voltage clamp techniques. This voltage clamp technique, with two microelectrodes, makes it possible to keep the voltage of a neuronal membrane constant while current carried by individual ions is measured. Four major ionic currents flow across this cell's membrane in the dark (that is, not induced by light). In that these currents were elicited in a nonlinear manner by causing the inside of the cell to become more positively charged, that is, causing it to depolarize, they could be classified as voltage-dependent. In the dark, voltage-dependent currents are (i) an early, rapidly inactivating outward K^+ current, with some of the properties of currents previously designated as I_A (and therefore referred to as I_A) blocked by 1 m*M* 4-aminopyridine; (ii) a delayed outward K^+ current, which shows much less and slower inactivation, and blocked by 100 m*M* tetraethylammonium ion; (iii) a rapidly activating, inward Ca^{2+} current sustained (showing almost no inactivation) and blocked by 10 m*M* Cd^{2+}; and (iv) a Ca^{2+}-dependent outward K^+ current, $I_{Ca^{2+}-K^+}$, which is blocked by intracellular iontophoresis of ethylene glycol tetraacetic acid (EGTA) (which binds and therefore lowers free calcium). The light-dependent currents are (i) an early, rapidly inactivating inward Na^+ current, which is not voltage-dependent and (ii) an outward Ca^{2+}-dependent K^+ current. Measurements of these currents (from coded animals) revealed that I_A and $I_{Ca^{2+}-K^+}$ were substantially reduced (Fig. 1c) across type B somata isolated from conditioned animals on days after the training experience (*26–28*). The magnitude of I_A and $I_{Ca^{2+}-K^+}$ reduction (30 to 40 percent compared to controls) was large enough to account for much of the measured enhancement of the isolated soma response to light as well as to current injection.

A sequence of biophysical and biochemical steps is believed to precede and ultimately result in reduction of I_A and $I_{Ca^{2+}-K^+}$ for days after training with paired stimuli. Steady depolarization of the type B cell becomes progressively greater (Fig. 1b) (*30*) during and after the

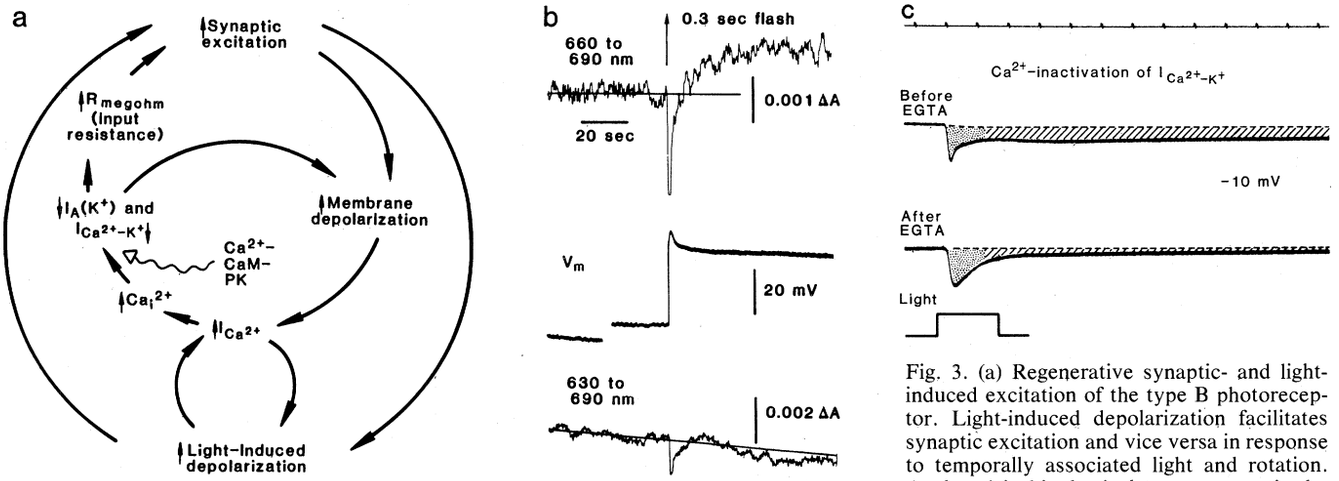

Fig. 3. (a) Regenerative synaptic- and light-induced excitation of the type B photoreceptor. Light-induced depolarization facilitates synaptic excitation and vice versa in response to temporally associated light and rotation. Analyzed in biophysical terms, synaptic depolarization causes transient activation and then prolonged inactivation of I_A and $I_{Ca^{2+}-K^+}$ and enhancement of a voltage-dependent Ca^{2+} current. Increased intracellular Ca^{2+} causes further inactivation of I_A and $I_{Ca^{2+}-K^+}$ and thus a further increase of effective input resistance. These in turn cause more membrane depolarization. Inactivation of I_A and $I_{Ca^{2+}-K^+}$ by elevated intracellular Ca^{2+} may occur via increased activity of Ca^{2+}-calmodulin–dependent protein kinase (Ca^{2+}-CaM-PK). (b) Prolonged elevation of intracellular Ca^{2+} accompanies depolarizing response of isolated type B cell to light. The cell was previously injected with arsenazo III. Absorbance changes at 660- to 690- and 630- to 690-nm wavelength pairs (top and bottom records) and membrane voltage response (middle) after a 0.3-second light flash. Differential absorbance changes at 660 to 690 measure changes of intracellular Ca^{2+} while those at 630 to 690 measure changes of *p*H (*31*). (c) Ca^{2+}-inactivation of $I_{Ca^{2+}-K^+}$. Isolated type B cell soma is placed under voltage clamp at −60 mV in ASW. After 10 minutes' dark adaptation, the onset of command depolarizing step to −10 mV (15 seconds) is followed by brief light step ($10^{3.5}$ erg cm^{-2} sec^{-1}). Long-lasting apparent inward current following light step results from intracellularly released calcium causing inactivation of steady-state $I_{Ca^{2+}-K^+}$. With the same state of dark adaptation, the light induced decrease of $I_{Ca^{2+}-K^+}$ is reduced after injection (under isopotential conditions) of EGTA (−2.0 nA, for 4 minutes). Calcium inactivation of $I_{Ca^{2+}-K^+}$ occurs in the absence of any inactivation of $I_{Ca^{2+}}$ (*36*).

responses of the visual-vestibular networks to repeated light and rotation pairings (but not unpaired and other control procedures). This stimulus and pairing-specific depolarization is accompanied by elevation of intracellular Ca^{2+} (Fig. 3, a and b) (*31*). Elevation of intracellular Ca^{2+} directly reduces I_A and $I_{Ca^{2+}-K^+}$ (*32, 36*) (Fig. 3c). Over the periods of days during which the learning is retained the type B cell is no longer depolarized (that is, its membrane potential has returned to its pretraining level) but I_A and $I_{Ca^{2+}-K^+}$ remain reduced. Available evidence indicates that Ca^{2+}-dependent phosphorylation is involved in the transition from short-term (minutes and hours) to long-term (days) biophysical neuronal changes. Biochemical analyses showed that Ca^{2+}-dependent phosphorylation of specific proteins was changed (Fig. 1c) only in neurons from the eyes of conditioned animals (*37*), and that these differences of Ca^{2+}-dependent phosphorylation could be related to the inactivation of I_A and perhaps $I_{Ca^{2+}-K^+}$ (*38*). In addition, intracellular iontophoresis of Ca^{2+}-dependent protein kinase (*39*) also produced I_A and $I_{Ca^{2+}-K^+}$ reduction which persisted for the duration of the recording period (up to 2 hours). Further analyses may reveal more specific roles of particular proteins (*37*) and their interaction with Ca^{2+} in the actual composition or regulation of I_A and $I_{Ca^{2+}-K^+}$ channels. However, the biochemical pathways that directly link the electrical responses of neurons to ionic channels are complex and at present still largely unknown.

Demonstration of primary membrane changes (reduction of I_A and $I_{Ca^{2+}-K^+}$) that serve as a memory trace in the cell body as just described, does not, of course, rule out other changes at other sites within the same cell, at other sites within other cells (*20*), or a role for other membrane current changes that could facilitate or help maintain the reduction of I_A and $I_{Ca^{2+}-K^+}$.

Modulation of Ca^{2+}-Mediated Inactivation of K^+ Channels

Our results thus far indicate that conditioning-induced neuronal changes do not result from the action of any particular synaptic transmitter substance or circulating neurohormone. Rather, the sequence of cellular steps for the production of long-lasting conditioning-induced reduction of I_A and $I_{Ca^{2+}-K^+}$ critically involves membrane depolarization accompanied by elevation of intracellular Ca^{2+}. These initial changes can occur in the absence of any neurohumoral influence, as was demonstrated with isolated type B cell somata (*32*). Repeated injections of a positive current, each paired with a light step (to simulate associative training), cause the same Ca^{2+}-mediated reduction of K^+ currents (as measured after conditioning) across type B somata that are devoid of all impulse activity, synaptic interactions, or physical proximity to any other neurons of the circumesophageal nervous system.

These results do not exclude possible contributions by substances released from presynaptic endings during the acquisition of the learning. For example, Farley has found that extracts of optic ganglia can cause increased type B input resistance and enhanced responses to light (*40*). It had been shown that the S-E optic ganglion cell (Fig. 2) exerts presynaptic control over EPSP's received by the type B cell (*41*). During acquisition the frequency of these EPSP's increased after paired light and rotation stimuli (as compared to control stimulus patterns). That optic ganglion presynaptic input to the type B cell included release of dopaminergic or adrenergic substances (or both) was suggested by the finding (*42*) that a single optic ganglion cell consistently showed green fluorescence by the Falk-Hillarp staining method.

Clonidine and norepinephrine, α_2-adrenergic receptor agonists (unlike dopamine), cause marked reduction of the same two currents reduced during learning, the I_A and $I_{Ca^{2+}-K^+}$ (*43*). This reduction of I_A and $I_{Ca^{2+}-K^+}$, which is largely

reversible at concentrations ≤500 μM, occurs in the absence of any effect on $I_{Ca^{2+}}$. Clonidine (like norepinephrine) also caused an increased type B input resistance and enhanced depolarizing response during and after a light stimulus for intact type B cells as well as after isolation by axotomy. Yohimbine, an α_2-adrenergic receptor antagonist, caused a reduction of type B input resistance and light-elicited depolarization for both intact and isolated cells (*43*). Yohimbine also caused a marked reduction of light-induced Ca^{2+}-mediated reduction of $I_{Ca^{2+}-K^+}$. Finally, yohimbine eliminated EPSP's received by the type B cell from the optic ganglion. These data suggest that, during acquisition of the learning, a presynaptic optic ganglion cell (marked by green fluorescence) may release an α_2-receptor agonist that amplifies that I_A and $I_{Ca^{2+}-K^+}$ reduction resulting from elevation of intracellular Ca^{2+} accompanying conditioning-induced depolarization of the type B cell. This amplification presumably occurs via electrical spread at depolarization from the region at synaptic interaction to the cell body ~60 micrometers away.

The relation of neurohumoral regulation of membrane currents to learning has been studied in *Aplysia* (*44*). Serotonin reduces K^+ currents (*45*), which may be independent of intracellular calcium (*46*), but whether these currents remain reduced in *Aplysia* after the serotonin treatment or after training for periods of hours or days is still unknown. Furthermore, those presynaptic neurons implicated in causing sensitization have been shown not to contain serotonin (*47*). Nevertheless, serotonin-like substances may play a role in *Aplysia* sensitization analogous to that of an α_2-receptor agonist in *Hermissenda* conditioning, that is, to amplify training-induced changes of membrane currents via elevation of intracellular calcium. Evidence for neurohumoral amplification of learning has been obtained for vertebrate preparations (*48*).

Calcium-Mediated Changes of Ionic Currents in Vertebrate Neurons

Membrane properties of many kinds of vertebrate neurons appear to resemble those of the type B soma. Evidence for voltage-dependent Ca^{2+} and calcium-dependent K^+ currents has been obtained for spinal cord motor neurons (*49*), dendrites of inferior olive neurons (*50*), and pyramidal cells of the hippocampus (*51*). The I_A occurs in hippocampal neurons (*51*) and inferior olive neuron somata (*50*). The necessary biophysical conditions, such as repeated and prolonged activation of voltage-dependent Ca^{2+} currents which precede conditioning-induced changes of ionic channels within type B soma membranes, could also precede similar conditioning-induced changes within the central nervous system of vertebrates. If vertebrate membranes at particular loci have the necessary densities of Ca^{2+} channels, the rise of intracellular Ca^{2+} may be great enough to cause persistent (days) reduction of voltage-dependent K^+ currents such as I_A and $I_{Ca^{2+}-K^+}$, but not great enough to affect the Ca^{2+} current itself. [Ca^{2+} current inactivation by elevated intracellular calcium in other neurons has been well studied (*52*).]

Recent experiments also suggest that calcium-mediated enhancement of membrane excitability could also explain long-term changes of hippocampal neurons. Long-term potentiation of postsynaptic responses to electrical afferent stimulation has been used for some time as a model for cellular modification during vertebrate learning (*53*). Lowering of external calcium or injection of EGTA, which lowers internal calcium, reduces or eliminates the potentiation effect (*54*). The expression of long-term potentiation, therefore, may require prolonged elevation of intracellular calcium during high frequency stimulation of afferent input to the hippocampal pyramidal cells. Prolonged and marked elevation of intracellular calcium inside pyramidal cells has in fact now been directly measured (*55*).

The data summarized above as well as that from other *Hermissenda* studies (*27*) represent an example of how a causal basis for associative learning can be established in a relatively simple nervous system, that is, one with several thousand neurons. That gastropod associative learning will never be identical to conditioning as defined, for example, by the phenomenology of the eye-blink preparations, is consistent with many known differences between gastropod and vertebrate nervous systems. Vertebrate species almost never have intersensory convergence at the visual receptor level and usually not in the retina at all. Gastropod action potentials last tens of milliseconds while those of vertebrates may last only several milliseconds. The delay between a presynaptic action potential and the onset of a postsynaptic potential can be many milliseconds for a gastropod but a fraction of a millisecond for a vertebrate (*56*). Therefore, a *Hermissenda* or an *Aplysia* probably will not have the necessary neuronal machinery to allow optimal associative learning for the same precise interval of time between the conditioned stimulus and the unconditioned stimulus as was demonstrated by Gormezano for the conditioned eye-blink reflex of rabbits (*57*). It is also not probable that gastropods will have the necessary neural systems to allow extensive generalization of the association to other related stimuli or situations while, at the same time, preserving appropriate specificity for the stimuli which were associated.

Establishing a causal basis for the learning of higher vertebrates will not be the same as for *Hermissenda*, where conditioning-induced changes of specific neurons within a small network cause a learned associative behavioral change. It is more probable that learning-induced changes will be diffusively represented throughout vertebrate neuronal aggregates and that only the collective effect of changes in distributed sets of neurons will actually store a learned association. Vertebrates could resemble *Hermissenda* in that similar conditioning-induced membrane changes, resulting from modulation of specific calcium-mediated biochemical control mechanisms, might be responsible for storing the learned information. It may not be possible, however, to verify the causal role of these changes, as it was for *Hermissenda*, by producing these membrane changes in individual neurons or neuronal aggregates and thereby the learned behavior. It may be possible, nevertheless, to establish, using slices from vertebrate brain, for example, that changes of excitability due to conditioning are intrinsic to identifiable neuron types. Such a demonstration may eventually become possible by applying the brain-slice technique to these vertebrate learning preparations—that is, finding changes in cells of recognizable types in slices made from critically involved brain areas of conditioned animals. Intracellular recordings from hippocampal slices, for example, have recently revealed conditioning-induced electrophysiologic changes in CA1 cells (*58*). On days after the rabbit was conditioned, the afterhyperpolarization which follows a positive current pulse is substantially lower for conditioned as compared to pseudoconditioned and unconditioned (naïve) animals (*58*). This difference probably reflects a reduction on one of the same ionic currents (that is, $I_{Ca^{2+}-K^+}$) which encode *Hermissenda* associative learning. These changes in slices specific to

conditioned animals might then be related to the electrophysiologic and biochemical changes that result from direct electrical stimulation of an area such as the hippocampus (*59*) but which have not been shown to occur with learning, or to the extracellular correlates of learning obtained by Thompson *et al.* (*59a*).

Postsynaptic Model for Vertebrate Learning

The anatomic means of heterosynaptic interaction (interaction between two different synaptic inputs) most frequently observed so far in the vertebrate central nervous system is the sharing of a common postsynaptic process between two distinct presynaptic terminal branches (*60*). Often this is a shared dendritic branch rather than a shared neuronal cell body. By contrast, axo-axonic synapses and synapses on presynaptic endings have been observed much less frequently. Thus, dendritic branches provide likely sites for convergence, already existing within vertebrate neural systems, which could mediate interaction of associated stimuli. Dendritic branches would also allow separation of hosts of pairs of presynaptic terminals that have the potential of interacting. With conditioning, a dendritic membrane could undergo an increased excitability change in response to input from a conditioned stimulus, which previously had some excitatory effect, without significant changes of excitability in response to input from other stimuli located on other dendritic branches of the same neuron. For stimulus pairing of classical conditioning, dendritic membrane postsynaptic to the presynaptic terminal activated by the conditioned stimulus could become more excitable with conditioning while the dendritic membrane postsynaptic to the terminal activated by the unconditioned stimulus would not change. This might be accomplished if the postsynaptic membrane receptive to the conditioned stimulus had a sustained Ca^{2+} current which, when sufficiently activated, resulted in a substantial rise of intracellular Ca^{2+}, while the unconditioned stimulus membrane had a much smaller capacity for voltage-dependent Ca^{2+} flux.

The potential change caused by the unconditioned stimulus is presumed to add to that of the conditioned stimulus and thus significantly enhance voltage-dependent currents similar to the voltage-dependent Ca^{2+} current of the type B photoreceptor or the voltage-dependent Ca^{2+} current responsible for action potentials of olivary dendrites. Biophysical characteristics of the conditioned stimulus effect on its postsynaptic membrane and of the unconditioned stimulus effect on its postsynaptic membrane can (as illustrated by the *Hermissenda* visual-vestibular system) provide necessary constraints to encode only temporally associated stimuli. Increasing the intensity of the unconditioned stimulus, rotation, for example, does not produce ever-increasing synaptic depolarization, which follows an initial inhibition. Rotation-induced synaptic depolarization quickly approaches a maximum. Similarly, increasing the intensity of the conditioned stimulus, light, will not duplicate the cumulative depolarization produced by stimulus pairing. With repetition, very bright lights ($> \sim 10^4$ erg cm^{-2} sec^{-1}) will cause more rapid light adaptation and ultimately less depolarization of the type B cell. Light (conditioned stimulus)-induced depolarization then must interact with extrinsic synaptic-induced depolarization (unconditioned stimulus), and vice versa, to cause sufficient cumulative depolarization to result in the prolonged (days) Ca^{2+}-mediated inactivation of the K^+ currents.

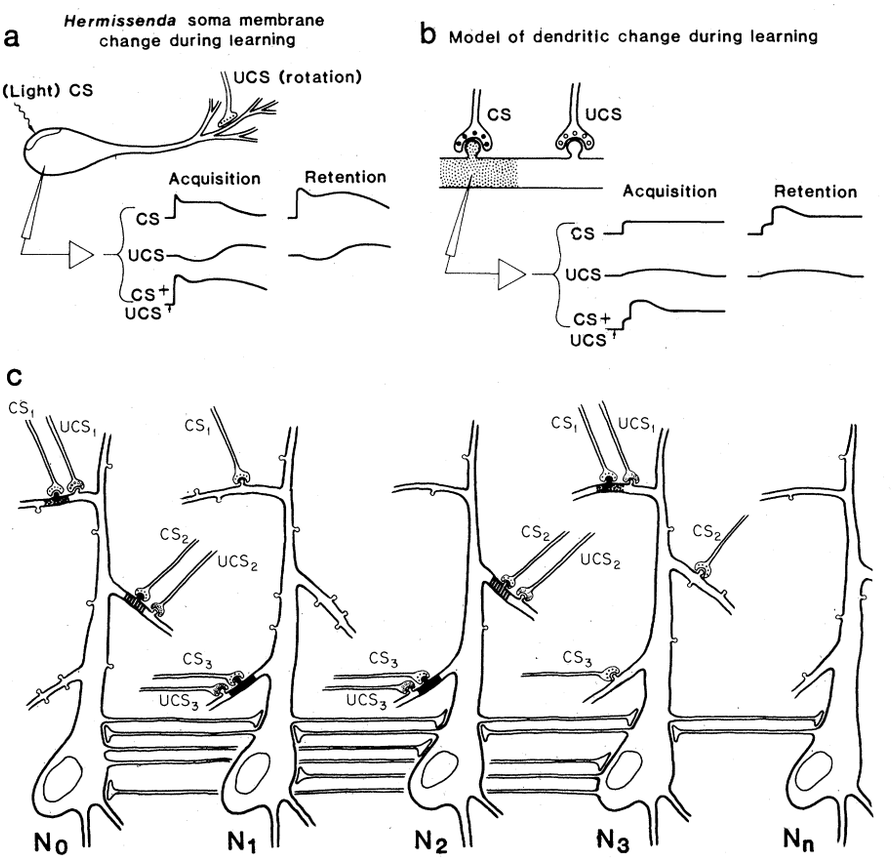

Fig. 4. (a) Schematic summary of conditioning-induced soma membrane changes in *Hermissenda*. Paired light and rotation are followed in the type B cell soma by an enhanced long-lasting depolarizing response (LLD) to light. Repeated pairings (lower left records) lead to cumulative membrane depolarization, enhanced response during and after the light, decreased voltage-dependent K^+ currents, increased input resistance, and thus increased excitability. On the days after training, that is, during retention of the learning, the cumulative depolarization is no longer present, whereas the other changes remain (*24, 27, 30*). (b) Model of conditioning-induced membrane changes in a dendritic branch. The genesis of these membrane changes could arise in a manner analogous to that observed for *Hermissenda* type B cells. (c) Hypothetical neural system changes during associative learning. Potential conditioned stimulus pathways synapse on postsynaptic dendritic membrane in proximity to postsynaptic sites for unconditioned stimulus pathways. Repetition of temporally associated stimulation of preexisting conditioned and unconditioned stimuli inputs which share a common postsynaptic dendritic branch cause persistent increases of postsynaptic excitability at the conditioned stimulus site (shaded areas). For a given set of neurons (N_o . . . N_n) different combinations of neurons will show increased excitability in response to a conditioned stimulus, depending on which stimuli were paired during training and depending on the genetically constrained features of the already formed neural systems. Horizontal processes ending on cell bodies represent inhibitory presynaptic endings.

If the basic neural circuits, and the Ca^{2+}-activated changes of membrane excitability, mediating the visual-vestibular association by *Hermissenda* were multiplied many times and if instead of soma membrane (Fig. 4a) postsynaptic dendritic membrane (Fig. 4b) included loci of convergence with potential for stimulus-pairing-induced modification, the same neurons could participate in generating hosts of different memories. One neuron could have a great number of convergences distributed throughout its dendritic tree. A particular association might activate single convergence sites on dendritic membranes of neurons distributed throughout a neuronal aggregate or a group of neuronal aggregates. Mutually inhibitory interactions could serve to amplify the activation of a particular set of neurons during recall of an association by reducing the excitability of those neurons not included in the activated set. Just as occurs in the five-cell network of the *Hermissenda* eye, the inhibitory interactions could exaggerate the learning-induced bias of a vertebrate system's response to a conditioned stimulus. Which memory recalled would depend on which combination of dendritic convergences was activated and which combination of neurons was activated (Fig. 4c).

Conclusion

The above describes how learned information could be encoded and stored with associatively induced changes of dendritic membranes. The scheme depends on the specificity of each set of neurons that are activated by temporally related input from converging stimuli. This model is the one most consistent with our observations on *Hermissenda*. Modifications of or alternatives to the model can of course be guided by data when they become available. The underlying principles of the model are summarized as follows.

1) Stimulus associations begin with pairing-specific responses of neural systems whose genetically determined synaptic organization includes sites of convergence which, as shown with *Hermissenda*, precede and determine the potential for learning.

2) Growth and development of neuronal processes constrain the potential for learning by determining the state of synaptic organization and thus which convergences exist at various life stages of an organism. Developmental processes are not directly involved in learning lasting for days. However, since morphological differences have been produced by prolonged exposure of an organism to controlled environmental stimulation (*61*), it would not be unreasonable to suppose that some structural expression eventually results from biophysical and biochemical changes during acquisition and retention of long-term learning, that is, lasting weeks or longer.

3) Postsynaptic dendritic and (to a lesser extent) soma membrane are more likely than the membrane of presynaptic terminals (although these may also provide sites) to serve as sites where stimulus patterns initiate sequential changes responsible for storing and recalling an organism's past experience.

4) The initiation of these sequential changes, as shown by the *Hermissenda* results, does not require that there be direct action of neurotransmitters released by stimulus inputs converging on critical membrane sites. However, neurochemical agents might facilitate the effects of summated voltage changes on ionic currents produced by distinct synaptic effects which are not electrically remote from each other.

5) It is not absolute magnitude of membrane current changes which critically encodes a learned association. Rather, it is the change of relative excitability (due to complementary current changes) within a neural system which determines a new network bias.

6) The summated voltage changes result in shifts of ionic and biochemical regulation of membrane channels which are ubiquitous within animal nervous systems. These membrane channels are present in organisms ranging from *Paramecium* (*62*) to *Hermissenda* to mammals. Because they are not tied to particular neurotransmitters and their metabolic pathways, the means for learning can be available to many and widely distributed neural systems, providing the necessary capability for the multiple and widely distributed representation characteristic of vertebrate learning.

The hypothetical and unfinished form of this model and its underlying principles recommend that it not be mistaken for an answer to the question of how our own learning is accomplished at the level of cellular physiology. The model derives from membrane changes demonstrated for identified neurons within neural pathways with tens or at most hundreds of *Hermissenda* neurons. Learning of higher vertebrates may depend not only on modified relationships within small sets of neurons (for example, increased type B excitability and decreased type A excitability) but on new integrative properties that emerge from the function of vast arrays or "ensembles" (*63*) of neurons. Nevertheless, as a summary of what we know thus far, the model suggests how future experimental questions might be phrased.

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RESEARCH ARTICLE

Role of the Conserved AAUAAA Sequence: Four AAUAAA Point Mutants Prevent Messenger RNA 3′ End Formation

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In bacteria, messenger RNA's (mRNA's) generally are primary transcripts and are exact replicas of DNA sequences in the genome. In eukaryotes, however, a primary transcript becomes an mRNA only after a collection of physical modifications: capping, cleavage to form a new 3′ terminus, polyadenylation, splicing, base methylation, and the transport of mRNA from nucleus to cytoplasm (*1–3*). In principle, each maturation step provides a means of regulating mRNA formation. This article focuses on defining the sequences within a primary transcript that are necessary for three of these steps: cleavage, polyadenylation, and transport.

Interest in these three steps stems first from their universality (*1–3*). Almost all cellular and viral mRNA precursors that have been examined are cleaved to generate a new 3′ terminus to which polyadenylic acid [poly(A)] is added (*1–4*) [for an exception, see (*5, 6*)]. Only mRNA's with a mature 3′ terminus are transported to the cytoplasm. Cleavage involves at least one endonucleolytic scission (*7*). The site of this scission is unknown; it may coincide with the polyadenylation site, or may lie downstream. Similarly, the protein or nucleic acid factors that catalyze the reaction have not yet been characterized.

Interest in these maturation steps also stems from their potential to determine how much mRNA, and what type of mRNA, a cell contains. For example, the same gene can produce two or more mRNA's with different 3′ termini in different cell types (*8–10*) or at different stages of viral infection (*2*).

Formation of SV40 (simian virus 40) mRNA's, like that of most mRNA's, involves posttranscriptional processing. This small double-stranded DNA virus genome is transcribed into two families of mRNA's in monkey cells (Fig. 1A). All mRNA's that accumulate late in viral infection (late mRNA's) are cleaved (*11*) and polyadenylated at the same site (*12*).

Sequences involved in cleavage, polyadenylation, and transport of mRNA in animal cells have not yet been completely defined, but must include the highly conserved AAUAAA sequence located 6 to 26 bases before the polyadenylation site of nearly all animal cell mRNA's (*13*). Fitzgerald and Shenk (*10*) demonstrated that deletion of this sequence

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