WHAT WE CAN LEARN FROM INVERTEBRATE LEARNING

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INTRODUCTION

Interest in the learning abilities of lower animals dates back to Romanes (1895), who argued that ability to learn provides an operational definition of mind. However, about 30 years ago a special interest in invertebrate learning was kindled in a search for learning phenomena that might yield to physiological analysis. Progress was rapid and striking, and instances of habituation, sensitization, and classical conditioning began to be analyzed in terms of cellular mechanism. However, soon after the first important invertebrate studies appeared, synapses of the mammalian hippocampus that were subject to one kind of long-term potentiation (LTP; recent reviews in Hawkins et al 1993, Bliss & Collingridge 1993) were found to have the features that Hebb (1949) had speculated might underlie associative learning. Attempts to circumscribe parts of the mammalian brain involved in learning were also increasingly successful, and some of the regions identified were found to contain synapses subject to LTP (see Carlson 1994). An explosion of interest in mammalian LTP has resulted.

Nevertheless, the exact relationship between findings at the cellular level and the behavioral phenomenon of learning remain complex and obscure in mammals. With invertebrate preparations, however, relationships between cellular and behavioral phenomena are much more readily clarified. Also, as rapid as progress has been in understanding mammalian LTP, the cellular understanding of certain forms of invertebrate plasticity is significantly more advanced.

WHAT INVERTEBRATES LEARN

Simple decreases of the innate response to a stimulus with repetition (habituation) or increases as a result of repetition or strong stimulation (types of sensitization) occur in all invertebrates, even the protozoa (e.g. Jennings 1906), and associative learning, at least in particular contexts and situations, is well developed in cephalopod molluscs and in many arthropods (see Corning et al 1973). Such learning can be robust, play significant roles in the lives of the animals, and last a very long time. Thus, a bee’s recollection of the color or location of a flower from which it received nectar can last a lifetime (Menzel 1990); and from a single inspection flight lasting only a few minutes, a digger wasp can memorize the locations and amounts of food needed to provision some 15 burrows containing developing young (Baersends 1941 as cited in Gould 1982).

Though the nervous systems of invertebrates that can learn have many fewer neurons than do typical vertebrate species in which learning is studied, their nervous systems are far from simple and, like ours, are ill understood.
The physiologist requires specific behavior patterns that are produced by neural circuitry simple enough to understand, but that are also subject to modification by experience. The relative ease of elucidating neural circuits of behavior in invertebrates follows perhaps less from their simplicity than from their nonredundant use of neurons. Processing tasks that in vertebrates appear to involve massive numbers of neurons of rather similar function operating in parallel are carried out in invertebrates by circuitry in which each individual neuron has a relatively unique role. Once these unique neurons are found and characterized, the same identified neuron can be studied from one animal to another, greatly facilitating the task of functional analysis. It is unknown whether this difference between vertebrate and invertebrate nervous systems leads to different processing strategies.

**Physiologically Analyzed Learning Paradigms**

**Modification of Aplysia Defensive Withdrawal Reflexes** If a sea hare (Aplysia) is stimulated at appropriate bodily locations, it protectively withdraws its gill, respiratory siphon, and/or tail. A siphon-gill withdrawal reflex of the abdominal ganglion mediates withdrawal of the siphon and gill in response to stimulation of mechanoreceptors on the siphon and part of the mantle shelf (see Kandel 1976), and a tail-siphon reflex of the pleural-pedal and abdominal ganglia mediates withdrawal of the tail and siphon in response to stimulation of tail mechanoreceptors (see Walters et al 1983). These two reflexes appear to have sufficiently similar properties that here we do not usually distinguish them.

Defensive withdrawal to gentle stimulation habituates (i.e. gets weaker and less prolonged) when stimuli are repeated (e.g. 5–10 stimuli at 1 per 3 min) and becomes sensitized following one or a few strong stimulations of various body parts including the head and tail (Pinsker et al 1970). Habituation and sensitization produced by limited training lasts under an hour, but with sufficiently spaced training they persist for weeks (Carew et al 1972, Carew & Kandel 1973, Pinsker et al 1973).

Although strong stimulation sensitizes reflex reactions somewhat to all stimuli, much more profound sensitization occurs for stimuli that are applied just before (ideally 1/2 s) each sensitization-producing stimulus (Carew et al 1981, Carew et al 1983, Hawkins et al 1983, Walters & Byrne 1983). Thus, the reflex can be classically conditioned to particular stimuli. Conditioning also shows some response specificity. The form of reflexive siphon withdrawals is adapted to help direct a defensive ink secretion toward the source of disturbance, and when a rostral conditioned stimulus (CS) is paired with a caudal unconditioned stimulus (UCS), the conditioned response adopts a caudal form (Walters & Erickson 1986, Walters 1989, Hawkins et al 1989). Classical
conditioning can last for more than three days (Carew et al 1972), but its duration has not been evaluated fully.

MODIFICATIONS OF LATERAL GIANT ESCAPE IN CRAYFISH When threatened, crayfish produce all-or-none flexions of their abdomens that thrust them through the water away from the source of disturbance (see Krasne & Wine 1987 for a review). Most studied has been a lateral giant (LG) neuron–mediated response caused by mechanical stimulation of the abdomen. The probability of LG escape diminishes with repeated stimulation. This habituation recovers partially over a period of several hours but accumulates over days with repeated experience (Wine et al 1975, Krasne & Teshiba 1994). Traumatic stimulation also increases the probability of LG escape to its normal stimuli for up to a few hours (sensitization; Krasne & Glanzman 1986), but associative conditioning of the response has not been reported.

CONDITIONED SUPPRESSION OF HERMISSENGA PHOTOTAXIS The marine gastropod *Hermisenda crassicornis* is normally positively phototactic but innately adheres to available surfaces under turbulent conditions. Following repeated simultaneous pairing of vestibular stimulation (produced by rotation but intended to simulate turbulence) and light (e.g. 50 pairings/day for 3 days), but not after asynchronous light and rotation, phototaxis is suppressed for as long as 18 days (Alkon 1974, Harrigan & Alkon 1985). Training reduces responses to contrast differences, slows onset of movement and rate of locomotion, and causes shortening of the foot, which also occurs as part of the unconditioned response to vestibular stimulation (Lederhendler et al 1986, Lederhendler & Alkon 1987); these changes are presumed to be responsible for reduced phototaxis.

CLASSICALLY CONDITIONED RESPONSES TO ODOR IN INSECTS AND SLUGS Classical conditioning persisting for days is reliably produced in bees, fruit flies (*Drosophila*), and the slug, *Limax maximus*, by one or a few pairings of an odorant (the CS) with either positive or negative stimuli. In bees, the odorant is presented just before sugar water, which elicits innate proboscis extension; after conditioning the odorant elicits a similar response (Takeda 1961, Bitterman et al 1983; see Menzel 1990). In *Drosophila* (Tully & Quinn 1985) and *Limax* (Sahley et al 1981a) subjects are exposed simultaneously to the odorant and either shock (*Drosophila*) or a bitter substance (*Limax*). When subsequently given a choice between the CS and a different odor, subjects avoid the CS.

**Choice of Paradigms**

Most physiological studies have used simple classical conditioning paradigms because it was suspected that the associative bond presumed to form during
such conditioning might be due to the strengthening of synaptic connections between neurons whose activity represents the events associated. However, over the past 20 years the discovery of phenomena such as blocking (Kamin 1969) and attenuation of learning by unpredicted UCSs (Rescorla 1967) has made it clear that vertebrate classical conditioning is governed by statistical features of the flow of events more sophisticated than the mere co-occurrence of CSs and UCSs.

Rescorla & Wagner (1972) have argued that most observations can be understood if it is assumed that co-occurrence of a CS and UCS leads to the formation of an association between them only if other CSs present at the same time are not already strongly associated to the UCS. However, Gallistel (1990) has argued forcefully that the full range of phenomena of classical conditioning, as now understood, shows that what an animal learns is not a connection between representations of events but rather the times, locations, and descriptions of significant events, which are then utilized to guide performance at the time of testing. Learning, he argues, is a matter of storing values of variables, not forming connections. Therefore, he reasons, physiologists should utilize learning situations, such as those involving navigation, in which it is apparent that values of variables are being learned.

To what degree do invertebrates in fact show the features of classical conditioning that raise these questions? *Limax* trained to avoid a mixture of carrot and potato odors subsequently avoid potato; but such learning is attenuated if the animals were previously trained to avoid carrot, a clear blocking result (Sahley et al. 1981b). Similarly, classical conditioning of *Aplysia* defense responses is attenuated if animals are trained in a context where they were previously shocked (Colwill et al. 1988). But conditioning of proboscis extension responses in bees does not seem to be subject to blocking (Menzel 1990, Couvillon et al. 1983).

Unpaired UCS presentations appear to attenuate conditioning of defensive withdrawal in *Aplysia* (Hawkins et al. 1986), phototactic suppression in *Hermissenda* (Farley et al. 1987a,b), and, to a slight extent, proboscis extension in bees (Menzel 1990). However, these effects may have been at least partly due to habituation to additional UCS presentations. Furthermore, the unpaired UCS presentations in *Hermissenda* were only effective in attenuating conditioning if they came immediately after the offset of light and rotation. This specificity, which can be explained by vestibular inhibition of the long-lasting depolarization (a depolarization that follows pairing and is suspected of inducing neural changes; see below) suggests that contingency degradation in *Hermissenda* is not comparable to that in vertebrates.

Thus, although blocking and contingency degradation effects can occur in invertebrates, they are not general and are unlikely to be a consequence of fundamental cellular mechanisms. Hawkins & Kandel (1984) have suggested...
plausible circuit-based explanations for some invertebrate blocking and contingency degradation results, and several authors have suggested that even in mammals, blocking may depend on specialized circuitry (Fanselow 1986, Thompson 1990).

We know of nothing in the invertebrate literature that would force abandonment, as Gallistel proposes, of the view that conditioning is the result of strengthening of synapses in circuits mediating conditioned responses. Furthermore, it is widely believed that nervous systems code the values of variables by activating populations of neurons from within multidimensional topographic maps having the variable values as axes. Given this, the formation of connections between neurons representing a qualitative event and the neurons representing a value such as the time or location of the event seems a plausible information storage strategy, even for the kinds of learning that Gallistel considers basic.

Phenomena such as associative conditioning of *Aplysia* defensive withdrawal or *Hermissenda* phototactic suppression may work because investigators have capitalized fortuitously on neural attributes that are not necessarily used for learning in the life of the animals and that therefore may not tell us how animals really learn. For example, Walters and colleagues have suggested that apparent classical conditioning in *Aplysia* may be the product of a mechanism that evolved to increase the local sensitivity of skin regions that have been traumatically stimulated (Walters 1987, Woolf & Walters 1991). Thus, it may be only an accident of the way those mechanisms work that allows pairing of a traumatic stimulus in one bodily location with a neutral stimulus in another to increase responses to the neutral stimulus and so meet the formal requirements for classical conditioning. Such arguments are not easy to answer. However, they do not seem to apply to the kinds of learning studied in insects and in *Limax*, which is one reason for pursuing with more vigor physiological analyses in those animals.

**LOCI OF FUNCTIONAL CHANGES RESPONSIBLE FOR LEARNING**

In invertebrates, where one is commonly dealing with relatively well-defined neural circuits, one seeks specific circuit elements whose functions are altered rather than anatomically defined locations. One can envisage a variety of aspects of neuron function that might change. However, characteristics such as critical firing levels and resting potentials that would be expected to simultaneously affect a neuron's responses to input over many of its many input lines would not allow nearly as large a storage capacity as changes at individual synapses. Therefore, synapses are commonly expected to be the site of functional changes responsible for learning.
Aplysia

As first characterized by Kupfermann & Kandel (1969; see also Kandel 1976) the circuit for defensive withdrawal is monosynaptic; sensory neurons innervating various parts of the body make direct synapses with the motor neurons that produce the response (Figure 1). Early studies of short-term habituation found pronounced depression of transmission at the sensory-motor synapses and detected no significant contribution of either sensory adaptation or neuromuscular fatigue to the decline of the behavioral response (Castellucci et al 1970, Kupfermann et al 1970, Byrne et al 1978). These transmission changes were due to reductions of excitatory postsynaptic potential (EPSP) amplitude, and analysis of the statistical properties of variations in EPSP amplitude due to probabilistic release of transmitter from single synaptic vesicles (quantal analysis) indicated that decreased release from sensory neuron terminals was responsible (Castellucci & Kandel 1974). EPSP depression occurred normally when firing of interneurons was prevented by the use of high divalent cation bathing media to reduce excitability; thus, depression was apparently intrinsic to the sensory neurons rather than being the result of presynaptic inhibitory modulation from outside the basic circuit (Kandel 1976). This conclusion is supported by the observation that short-term depres-

![Figure 1](image_url)

**Figure 1** Circuit for *Aplysia* defensive withdrawal. Most fully studied circuitry is in bold; * indicates the most studied site of plasticity. The relevant central nervous system (CNS) circuitry is primarily within abdominal and pedal-pleural ganglia; s = sensory neurons; m = muscles; p = peripheral motor neurons; box = interneuron circuitry.
sion of EPSPs occurs when sensory neurons that have formed synapses on motor neurons in cultures of isolated sensory and motor neurons are stimulated at frequencies similar to those used in behavioral experiments (Montarolo et al 1988, Rayport & Schacher 1986).

Long-term habituation was also found to be associated with decreased monosynaptic transmission (Castellucci et al 1978), but it apparently cannot be established by repetitive activity at cultured sensory-motor synapses and might involve presynaptic inhibition at least during its induction (Montarolo et al 1988; see Initiation of Change section below). Both short- (Kupfermann et al 1970, Castellucci & Kandel 1976) and long-term (Frost et al 1985) sensitization were similarly shown to be due to facilitated transmission at sensory-motor neuron synapses. Quantal analysis showed short-term facilitation to be due to increased transmitter release (Castellucci & Kandel 1976), and broadening of the sensory neuron action potential (see below) provided further evidence of presynaptic change.

Sensitization appears to be induced by facilitatory neurons that release transmitters to the presynaptic terminals of sensory neurons (see below). One of several transmitters used is 5-HT; exogenous 5-HT produces effects similar to those caused by sensitizing stimuli (Brunelli et al 1976) and has been used in many experiments to produce neural changes thought to underlie sensitization. Application of 5-HT to cultured sensory-motor synapses causes facilitation lasting for minutes when applied briefly and lasting at least 24 hr when applied repeatedly or for a longer period, showing that the facilitation of transmission is intrinsic to the sensory-motor synapses (Montarolo et al 1986, Rayport & Schacher 1986). Quantal analysis at cultured synapses has also established that long-term facilitation is due to increased transmitter release, without changes in postsynaptic sensitivity to transmitter (Dale et al 1988).

Classical conditioning procedures produce EPSP and sensory neuron changes similar to those produced during simple facilitation (see below), but they are exaggerated in extent in those sensory neurons that were active just before traumatic stimulation or serotonin application (Hawkins et al 1983, Walters & Byrne 1983, Abrams 1985). Hence, classical conditioning is said to be due to an activity-dependent amplification of facilitation (or activity-dependent facilitation) and is believed to result from the same kinds of changes that occur during sensitization.

Thus, habituation, sensitization, and classical conditioning of Aplysia defensive withdrawal are all associated with alterations of transmitter release at the monosynaptic connection between sensory and motor neurons, and the biophysical and molecular biological bases of these alterations, which are discussed below, have been studied intensively. However, these changes appear to be merely the tip of the iceberg.
Sensitizing events also can cause threshold decreases to mechanical stimulation (Walters 1987, Billy & Walters 1989a,b), other sensory neuron changes (Klein et al 1986), motor neuron input impedance increases (Pieroni & Byrne 1992), and persisting increases of spontaneous firing rates, with resulting development of neuromuscular facilitation (Frost et al 1988). More importantly, evidence is accumulating that defensive withdrawal responses are by no means primarily monosynaptic. Eberly & Pinsker (1984) have shown that interneurons that normally produce spontaneous "respiratory pumping" movements of gill and siphon (Byrne 1983) are also responsible for a large fraction of motor output in reflexive responses of freely behaving animals though not of acute preparations. And in acute preparations, suppression of almost all interneuron firing by elevating divalent cation concentration reduces the area under motor neuron EPSPs by more than 75% (Trudeau & Castellucci 1992). Thus, understanding learned changes in defensive withdrawal will require analyzing changes in the polysynaptic as well as monosynaptic pathways (see Figure 1).

Excitation of interneurons that contribute to defensive withdrawal is normally truncated by an immediately following inhibition (Trudeau & Castellucci 1993a, Fischer & Carew 1993), and reduction of this inhibition is substantially responsible for response increases due to sensitizing stimulation (Trudeau & Castellucci 1993a,b). Augmentations of the inhibition during repetitive stimulation may also contribute to habituation (Fischer & Carew 1993), and it has been reported that habituation of the tail-siphon reflex is entirely due to the polysynaptic pathway (Stopfer & Carew 1994).

Some complexities are also introduced by the existence outside the CNS of a neural plexus containing additional motor neurons that under some circumstances contribute to defensive withdrawal (see Pearlman 1979, Lukowiak 1979). In addition, Colwill et al (1988) found that, in freely behaving Aplysia, defensive reactions to constant test stimuli are heightened in animals placed in a situation where they were previously shocked. This means that the circuitry that mediates defensive withdrawal is subject to learning-induced external modulation, and it opens the possibility that in freely behaving animals, learning-altered defensive behavior with training is due to altered modulation originating in circuitry outside the defensive reflex pathway itself.

**Crayfish**

The lateral giant (LG) escape response of the crayfish is named for the bilateral pair of LG command neurons of the circuit that mediates the behavior (Figure 2; see Krasne & Wine 1987). The LGs fire, usually only once, when they receive sufficient summed input from primary afferents and sensory interneurons (monosynaptic input alone is usually subthreshold); the single firing produces a vigorous tail flip. Presumably as an adaptation favoring rapid-
Figure 2  Circuit for crayfish LG escape. Most fully studied circuitry is in bold; * indicates the most studied site of plasticity; s = sensory neurons; m = muscles; TI = tonic inhibitory system.

ity, most of the synapses of the circuit are electrical; only those between sensory neurons and first-order interneurons are purely chemical (cholinergic) (Zucker 1972a, Edwards et al 1991, Miller et al 1992).

Most evidence has pointed to these chemical synapses as the locus of change responsible for habituation because they are depressed, at least partly due to lowered release, by repetitive activation, whereas transmission of other circuit synapses is fairly stable (Krasne 1969, Zucker 1972b, Krasne 1976). However, recent findings (Krasne & Teshiba 1994), though confirming that intrinsic depression makes some contribution to behavioral habituation, appear to have established that in the freely behaving crayfish, cessation of response is mostly due to the onset of activity in a descending tonic inhibitory pathway (Figure 2; Vu et al 1993) that directly innervates the LGs.

Sensitization also has been associated with altered transmission at the chemical sensory neuron synapses (Krasne & Glanzman 1986). Octopamine in crayfish, like serotonin in *Aplysia*, facilitates transmission at these synapses (Glanzman & Krasne 1983), though the relation of this effect to sensitization by traumatic stimulation is speculative. Individual primary afferents appear to make multiple synapses, some strong and some with little effect, on their interneuronal targets; octopamine augments transmission mainly at those synapses that initially were not effective (Bustamante & Krasne 1991). Thus, plasticity could reside in a subset of synaptic contacts specialized for the purpose.

As with the excitatory interneurons for defensive withdrawal in *Aplysia,* the excitation of the LGs is normally truncated by activity of an inhibitory pathway that parallels the excitatory one (Vu et al 1987). However, in crayfish there is no evidence that excitability of escape is altered by changes in this inhibition.
**Hermissenda**

Whereas the circuits for *Aplysia* defensive withdrawal and crayfish LG escape span the entire reflex arcs, only the afferent end of the circuit involved in *Hermissenda's* conditioned suppression response is worked out in detail (Figure 3). The eye possesses two types of photoreceptors, denoted A and B (Alkon 1973). Statocysts, which provide information about bodily displacements and are the sensors of the rotational UCS used in conditioned suppression experiments, also contain two cell types, cephalic and caudal, which are differentiated on the basis of their responses in a centrifugal force field (Farley & Alkon 1980). As explained below, the B photoreceptors and the caudal hair cells (but not the A photoreceptors or cephalic hair cells), along with a class of optic ganglion neurons called the S/E cells, are part of a network of mutually interacting neurons that appear to play a role in detecting the coincidence of visual CSs and vestibular UCSs and thus in determining whether a conditioned response will develop (see below).

Although the charting of the circuitry that stands between the photoreceptors and motor apparatus is incomplete, this preparation has been able to provide a great deal of interesting information, because training produces changes in the B photoreceptors themselves, which show an augmented re-

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**Figure 3** Circuit for *Hermissenda* phototaxis. Most fully studied circuitry is in bold; * indicates the most studied site of plasticity; A = type A and B = type B photoreceptors; HC = vestibular hair cells; S/E = S/E optic ganglion cells (see text).
response to light after CS-UCS pairing but not after various appropriate control procedures (Crow & Alkon 1980, West et al 1982). It seems paradoxical that an increased response to light should decrease phototaxis. A definitive resolution of this conundrum is not available, but a proposed explanation is based on the fact that A photoreceptors, which are suspected of mediating turning toward the light, are inhibited by B photoreceptors; thus they may drive turning less strongly in conditioned animals (Goh & Alkon 1984, Goh et al 1985, Lederhendler & Alkon 1987). In contrast, B photoreceptors are suspected of promoting antagonistic responses that disrupt locomotion (e.g. clinging to the substrate); these responses would increase in conditioned animals (Akaike & Alkon 1980, Lederhendler et al 1986). The inhibitory synapses of type B onto type A photoreceptors may also become strengthened by training (Schuman & Clark 1994, Frysztak & Crow 1994).

Increased responses of photoreceptors of trained animals to light after surgical removal of the synaptic portion of the receptors indicates that the photoreceptors themselves are altered (West et al 1982), but since testing was done soon after removal, it is not certain to what degree these clearly intrinsic changes are self sustaining. Pairing of light and intracellular depolarization of a single type B photoreceptor, which is meant to mimic the effects of paired light and rotation on the cell (see below), causes behavioral phototactic suppression 24 hr later (Farley et al 1983). This observation indicates that the changes in the B photoreceptor contribute significantly to altered behavior, although this treatment may also have caused central nervous system activity leading to changes elsewhere in the system.

Insects

Some of the brain structures thought to be important for olfactory learning in bees are shown in Figure 4. With a few exceptions, analysis in terms of identified neurons has not yet been possible. Olfactory information projects to antennal lobes and thence to the calyces of the so-called mushroom bodies (MBs) (see Menzel 1990, Hammer 1993, Mauelshagen 1993, Mobbs 1984). A neuron that appears to mediate reinforcement by the UCS (Hammer 1993; see below) also distributes profusely to the antennal lobes, lateral protocerebrum, and calyces of the MBs, making all three sites points of CS-UCS convergence. Studies of the effects on learning of local cooling (Erber et al 1980, Menzel 1990), selective destruction of MB neuroblasts (de Belle & Heisenberg 1994), and MB structural mutations (Heisenberg et al 1985) as well as localization of abnormal gene products in learning mutants (Han et al 1992, Nighorn 1991) and findings of anatomical changes produced by various forms of experience (see Belle & Heisenberg 1994) all point to the MBs as having a special role in learning.
Intrinsic Change or Extrinsic Modulation?

In most of the work reviewed above the focus has been on changes intrinsic to the neurons of the circuits that actually mediate the learned behaviors. It may seem somewhat surprising that animals with some degree of encephalization should abdicate to what is rather low-level circuitry, analogous to our spinal reflexes, control over the excitability of behavior patterns that may be crucial.

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**Figure 4** Neural structures involved in insect olfactory conditioning. * indicates the most studied site of plasticity; MB = mushroom body; AL = antennal lob; SOG = sub-esophageal ganglion; VUMmxl = VUMmx1 neuron; VM = visual medulla; VL = visual lobula.
for survival. That training produces changes paralleling behavioral learning in such circuitry seems clear. But the role of higher-level control circuitry may be underestimated because it is liable to be dysfunctional in more or less dissected and restrained animals. There is now evidence both from crayfish and *Aplysia* that the role of extrinsic modulation by higher-level circuitry in freely behaving animals cannot be ignored. The detailed analysis of changes in the low-level circuitry has progressed remarkably, but this analysis may tell only a small part of the story.

**Parallel Distributed Processing?**

Parallel distributed processing theories of learning propose that learning may be the result of a multiplicity of changes in neural circuits each of which is too small to affect behavior significantly on its own but that in the aggregate can produce large effects on behavior when the circuits are used in certain ways. Thus, the changes responsible for learning (engrams) are said to be “distributed.” Lockery & Sejnowski (1993) have used a back-propagation algorithm to generate distributed engrams that would produce the sorts of behavioral changes seen during leech habituation and sensitization. In the circuits produced by the algorithm, changes at individual synapses were all near or below practical limits of detectability, and the behavioral consequences of changes at any one synapse would have been negligible.

Sensitization and habituation of defensive behavior in *Aplysia* and crayfish clearly are due to changes in synaptic efficacy (resulting from intrinsic change or extrinsic modulation) distributed over a number of sites within the circuits that mediate the behaviors. However, in contrast to the leech modeling results, changes at individual synapses are detectable and are thought to have significant behavioral consequences. Indeed, Falk et al (1993), using voltage-sensitive dyes, have visualized altered responses of hundreds of abdominal ganglion neurons that are engaged by test stimuli during habituation training in *Aplysia*, and Frost et al (1988) have speculatively proposed specific, qualitatively different functions such as determination of response specificity or response duration for various changes they found during sensitization.

Nevertheless, some of the fundamental properties of parallel distributed processing might be operative in these invertebrate circuits. This would be the case if it could be shown, for example, that enhanced transmitter output of sensory neurons in sensitized *Aplysia* contributed substantially to augmented defensive responses when combined with changes at interneuronal synapses but not when responses not involving interneurons were tested. The same principle might be demonstrable in the crayfish LG circuit if first-synapse depression operating in conjunction with tonic inhibition of the LGs were an essential ingredient in LG habituation but had relatively little influence on slow flexion reflexes innervated by the same afferent pathways.
Altered Ion Channels

Ion channel alterations appear to be an important cause of learned changes of behavior. In *Aplysia*, strong stimulation or 5-HT application causes statistically increased closures of a serotonin sensitive K⁺ channel (here called KS; Klein & Kandel 1978, 1980; Klein et al 1982; Siegelbaum et al 1982) and also decreases the delayed voltage-dependent K⁺ current that promotes repolarization following action potentials (Baxter & Byrne 1989). Both effects increase the duration of sensory neuron spikes, thus extending the period during which Ca²⁺ enters terminals and hence the amount of transmitter released to excite defense reflex motor neurons (Klein & Kandel 1978, 1980; but see Klein 1994). These effects also cause a slight depolarization of the sensory neuron and contribute to a decreased tendency toward accommodation. Long-term sensitization has also been associated with reductions of an outward current thought to be mediated by closure of the KS channels (Scholz & Byrne 1987).

Activity-dependent increases in transmission are associated with exaggerated broadening of presynaptic spikes, and decreases of a presumptive KS current, suggesting that activity merely amplifies the effects produced during ordinary sensitization (Hawkins et al 1983, Abrams 1985, Hawkins & Abrams 1984). Conversely, the synaptic depression responsible for habituation is associated with a slight shortening of presynaptic spikes and has been attributed to inactivation of Ca²⁺ channels (Klein & Kandel 1980, Eliot et al 1994b). Much of this picture emerges from voltage-clamp and patch-clamp studies, which must be done on sensory neuron somata rather than on the presynaptic terminals themselves. Although the soma and terminal cannot be counted on to have the same channel types, findings from terminals using Ca²⁺-sensitive dyes (Eliot et al 1993) and growth cones (Belardetti et al 1986) are consistent with those from somata.

Closure of K⁺ channels is also thought to be responsible for the increased responsiveness of type B photoreceptors associated with suppression of phototaxis in *Hermissenda*. Input resistance of the photoreceptors increases, resulting in stronger (less shunted) responses to light both acutely (Crow & Alkon 1980) and a day or more following more protracted training (West et al 1982). Voltage-clamp studies indicate that the resistance increases result from decreases in a rapidly activating and inactivating depolarization-dependent K⁺ current (Iₐ) and a Ca²⁺-dependent K⁺ current (Iₖ-Ca) (Alkon et al 1982, 1985). Using patch-clamp recording, a 64 pS K⁺ channel, possibly the mediator of Iₐ, has been found in which the probability of opening is greatly reduced at 24 hr after classical conditioning (Etcheberrigaray et al 1991, 1992).
Undefined Physiological Changes

The discovery of ion channel changes as the cause of altered function in learning is appealing because of the well-defined nature of the change, which can then be studied in still more detail (see below). However, modeling studies first suggested that the quantitative details of neither the synaptic facilitation thought to be responsible for sensitization nor the depression responsible for habituation in *Aplysia* can be accounted for by the K$^+$ and Ca$^{++}$ channel changes initially thought responsible for them, and physiologically undefined processes labeled “transmitter mobilization” and “depletion” were postulated to account for the discrepancies (Gingrich & Byrne 1985). Experimental evidence obtained subsequently showed that 5-HT enhances transmission via mechanisms independent of the presynaptic spike broadening produced by altered K$^+$ currents (Hochner et al 1986a,b; Pieroni & Byrne 1992; Klein 1994). It has been suggested that alterations in Ca$^{++}$ handling (Boyle et al 1984) or a modulation of the release process itself (Pieroni & Byrne 1992) might be responsible. Increases in spontaneous miniature EPSPs appear to be a correlate of this spike broadening–independent process (Dale & Kandel 1990).

Anatomical Change

In *Aplysia*, both repeated traumatic stimulations leading to long-term sensitization in vivo (Bailey & Chen 1988a,b) and 5-HT application causing synaptic facilitation that lasts more than a day in sensory and motor neuron co-cultures (Glanzman et al 1990) cause increases in presynaptic varicosities, synaptic active zones, length of active zones, and numbers of synaptic vesicles immediately adjacent to these zones. Conversely, repeated stimulation leading to behavioral habituation that lasts weeks, as well as applications of FMRFamide, which causes long-term decreases of synaptic efficacy in culture (see below), result in effects largely opposite to those produced by sensitizing procedures (Bailey & Chen 1988a, Schacher & Montarolo 1991). In the case of 5-HT–produced facilitation, some changes have been detected within 30 min of the start of 5-HT application (Bailey et al 1993). Elevated numbers of varicosities and active zones in behaviorally trained animals persist about as long as does augmented transmission (>3 weeks), but increases in active zone length and numbers of adjacent vesicles outlast training by only a few days (Bailey & Chen 1989).

Pairing of light and rotation that causes suppression of phototaxis in *Hermissenda* decreases the range of the neuronal arbor of B photoreceptors (Alkon et al 1990). It has been suggested that this could reflect a selection process in which a few “useful” branches increase while others decrease.
THE INDUCTION OF NEURONAL CHANGES RESPONSIBLE FOR LEARNING

Phosphorylation is the predominant means used to regulate the activity of proteins such as enzymes and receptors in animal cells. Thus, it is not surprising that this mechanism is intimately involved in the changes responsible for learning (see Byrne et al 1993). Indeed, understanding the induction of changes largely reduces to understanding what activates the second messenger cascades responsible for triggering phosphorylation (i.e. how change is initiated) and understanding what the cascades do (i.e. how changes are implemented).

Initiation of Change

SENSITIZATION  Induction of sensitized defensive reactions must be triggered by evidence of danger. Because significant danger generally causes defensive reactions, vigorous activity of the motor circuitry that produces these reactions might provide a signal that could trigger the responsible changes. But in Aplysia much of this motor circuitry is also engaged during spontaneous respiratory pumping (see above) and therefore cannot provide a reliable signal of danger; thus, independent circuitry is used to recognize danger. Such circuitry is ill-charted but some of the output neurons involved have been identified. These output neurons, which are believed to be relatively few in number, fire in response to strong stimuli and distribute widely, innervating the presynaptic terminals of defense reflex sensory neurons at their contacts with both motor and interneurons (Mackey et al 1989, Hawkins et al 1981, Hawkins & Schacher 1989). The release of transmitters to their presynaptic targets induces the facilitated state. Facilitators differ with respect to receptive field and output properties, as would be expected from differences in response topography for traumatic stimulation of various bodily regions (see above; Walters & Erickson 1986, Erickson & Walters 1988). Facilitators also differ with respect to transmitter released, duration of firing to a transient stimulus, and post-activity persistence of the facilitation they produce (both on the order of seconds to tens of minutes) (Mackey et al 1989).

The transmitters released by facilitators have not been fully characterized. Serotonin, as well as two endogenous peptides, the small cardioactive peptides (SCPs), mimic most of the effects of trauma (Brunelli et al 1976, Abrams et al 1984), although 5-HT appears to be the more faithful mimic. Serotonergic processes are found near terminals of defense reflex primary afferents (Kistler et al 1985, Longley & Longley 1986), and SCPs are present in abdominal ganglion neuropile (Lloyd et al 1985). The properties of the facilitation produced by activity of some facilitators are somewhat different from those of any of the known mimetic agents (Hawkins & Schacher 1989). But some facilita-
tors have been identified as serotonergic (Mackey et al 1989), and pharma-
cological disruption of serotonergic transmission has been shown to reduce
behavioral sensitization and its neural correlates (Brunelli et al 1976, Glanz-
man et al 1989b, Mercer et al 1991). Thus, 5-HT is accepted as one known
mediator of sensitization. Its effects, both on intact Aplysia ganglia and sen-
sory-motor synapses grown in culture, have been studied extensively. Brief
applications cause presynaptic facilitation lasting several minutes (Rayport &
Schacher 1986, Eliot et al 1994a), whereas multiple spaced or a single longer
application produce sensitization lasting days (Montarolo et al 1986).

Both 5-HT and strong stimulation, acting via a membrane G-protein (Cast-
tellucci et al 1983, Schwartz et al 1983), stimulate adenylyl cyclase to catalyze
production of cAMP (Bernier et al 1982). As in other cells, cAMP exerts its
physiological effects by activating a cAMP-dependent protein kinase (PKA).
Thus, direct elevation of cAMP produces enhanced sensory-motor transmis-
sion, sensory neuron spike broadening, and Kv channel closure (Brunelli et al
prevent PKA from phosphorylating its target proteins prevent these effects
(Castellucci et al 1982). PKA is formed by the association of two catalytic
subunits with two regulatory subunits that inhibit catalytic activity, and cAMP
activates the enzyme by binding to sites on the regulatory subunits, which
causes them to dissociate from the catalytic units, freeing them to act. Direct
injection of the catalytic subunit produces the same effects as exposure to
cAMP (Castellucci et al 1980). There may also be a modest contribution of the
Ca++- and phospholipid-dependent protein kinase (PKC) system to spike
broadening and enhanced transmission (Sugita et al 1992).

As discussed below, procedures causing changes that persist on the order of
days involve new protein synthesis. Daily repetition of treatments in which
individual measured effects do not last as long as the intertreatment intervals
can trigger long-term change. Where and how the requisite integration is being
made is unknown, but during extended periods of 5-HT treatment, the pattern
of protein phophorylation undergoes sequential changes (Homayouni et al
1991) that could be involved in the requisite processing (see further below).

In crayfish, sensitization-like effects on the lateral giant escape circuit are
produced by the naturally occurring neuromodulator, octopamine (Glanzman
& Krasne 1983), which also facilitates insect evasion responses (Casagrand &
Ritzman 1992, Sombati & Hoyle 1984). Octopamine appears to be a general
arthropod analog of adrenalin (e.g. Evans 1985, Orchard 1982, Battelle &
Kravitz 1978), but its actual role in sensitization is speculative.

HABITUATION Habituation of the protective responses studied in physiological
work becomes appropriate when evidence accumulates that a specific distur-
bance can be safely ignored. To be adaptive, habituation must be somewhat
stimulus specific. In principle its induction could be triggered either by activity per se or by receipt of a special safety signal analogous to the danger signal provided by the facilitators that trigger sensitization. Insofar as habituation is the result of tonic inhibitory modulation, as in crayfish LG escape, its analysis must await charting of the circuitry that generates the inhibition. However, remarkably little is known even about mechanisms of intrinsic depression.

Depression has been ascribed to processes such as inactivation of Ca\(^{++}\) currents, depletion of transmitter available for release, and reduced invasion of spikes in terminal arbors (see above and Zucker 1989). At least in the short-run such changes might be expected to be the result of intrinsically slow rates of recovery of the relevant processes (e.g. transmitter mobilization, recovery from Ca\(^{++}\) channel inactivation; see Zucker 1989 for a discussion of proposed mechanisms); however, active down-regulation mediated via phosphorylation or dephosphorylation cannot be discounted, although it has been little discussed.

One possible scenario involves presynaptic transmitter autoreceptors. Many presynaptic terminals have autoreceptors whose stimulation down-regulates release (e.g. Trimmer & Weeks 1989, Chesselet 1984), and in guinea pig ileum, synaptic depression that develops at low stimulation frequencies is prevented by blocking presynaptic muscarinic autoreceptors pharmacologically (Morita et al 1982). The depression-prone first-order synapses of the crayfish LG reflex are also known to have muscarinic autoreceptors whose transient activation by cholinergic agents appears to produce a depression of release lasting about 90 min (Miller et al 1992); however, there is presently no evidence that these autoreceptors play a role in habituation.

Although short-term depression occurs normally at cultured *Aplysia* sensory-motor synapses, repetitive activation does not lead to long-term depression of transmission, suggesting the need for a factor that is not present in these simple co-cultures (Montarolo et al 1988). In fact, several spaced 5-min applications of FMRFamide, a peptide that causes presynaptic inhibition of release from *Aplysia* sensory neurons via an arachadonic acid second messenger cascade, causes cultured synapses to undergo a long-term, protein-synthesis–dependent reduction of transmission accompanied by the same kinds of morphological changes seen following long-term habituation training (Piomelli et al 1987, Schacher & Montarolo 1991, Schacher et al 1993, Bailey et al 1992). Thus, the intrinsic changes responsible for long-term habituation in *Aplysia* may require receipt of a special FMRFamide-like safety signal. To achieve the stimulus specificity required of habituation, this signal would have to be directed selectively to those sensory neurons activated by the repeating stimulus, or the long-term effects of the FMRFamide-like agent would have to be activity-dependent, which FMRFamide’s short-term inhibitory effects in fact are (Small et al 1989).
**Dishabituation**  Habituation resulting from intrinsic depression, whatever the means of its induction, can only be adaptive if it can be prevented from developing and reversed under conditions of danger, and there is considerable evidence that traumatic stimulation breaks up both short-term and long-term habituation (Krasne & Glanzman 1986, Pinsker et al 1970, Carew et al 1971, Carew et al 1979, Fitzgerald et al 1990). The same mechanisms used for sensitization might mediate dishabituation. But in *Aplysia*, dishabituation (i.e. trauma-produced recovery of habituated reflexes) appears before sensitization (i.e. enhancement of unhabituated reflexes) during development (reviewed in Carew 1989; but see Glanzman et al 1989b), and recovery from synaptic depression is heavily dependent on transmitter mobilization not involving presynaptic spike broadening and Ks channel closure (Hochner et al 1986a,b). This mobilization, which like sensitization is well mimicked by 5-HT (Hochner et al 1986b, Pieroni & Byrne 1992), appears to be dependent on PKC-catalyzed phosphorylation, although PKA-mediated processes amplify it somewhat (Braha et al 1990, but see Ghiradi et al 1992). A separate mechanism for dishabituation could provide a means of restoring excitability to prehabituation levels in a way that does not result in heightened sensitivity to novel stimuli, but this would require an unknown external means of recruiting the PKC- and not the PKA-mediated process.

**ASSOCIATIVE LEARNING**  Conditioned responses become appropriate when the environment provides evidence that one stimulus predicts another in a biologically significant context.

**Activity-dependent facilitation in Aplysia**  The condition for development of activity-dependent facilitation in *Aplysia* is pairing of noxious stimulation with the activity of defense reflex sensory neurons. Thus, the facilitation is believed to be simply an exaggerated form of simple facilitation (see above). Consistent with this theory, pairing of 5-HT exposure with sensory neuron activity (or depolarization by K+) causes a more than additive increase in (a) cAMP production (Abrams 1985, Ocorr et al 1985), (b) degree of presynaptic spike broadening (Eliot et al 1994a, Abrams 1985), and (c) transmitter release per spike (assessed by the evoked EPSP; Eliot et al 1994a) in intact ganglia and/or co-cultured sensory and motor neurons.

Sensitivity to the conjunction of presynaptic activity and trauma or 5-HT input depends on the presence of extracellular Ca++ (Abrams 1985), suggesting that Ca++ entering presynaptic terminals during activity potentiates the cAMP-producing effects of 5-HT. Ca++, acting via calmodulin, does in fact enhance the effectiveness of the enzyme, adenylyl cyclase, that catalyzes the formation of cAMP (Abrams et al 1991). In addition, PKA and Ca++/calmodulin-dependent protein kinase (CaM kinase) both can phosphorylate pro-
tein [cAMP-responsive-element (CRE)–binding protein; see below] that triggers induction of long-term facilitation when its phosphorylated form binds to a specific DNA recognition sequence. Moreover, PKA plus CaM kinase causes more transcription than does either kinase alone in a cell-free test system (Dash et al 1991). Therefore, transcription-triggering protein could provide another site sensitive to the conjunction of trauma and sensory neuron activity.

Classical conditioning in intact *Aplysia*, and activity-dependent facilitation of EPSP amplitude in isolated ganglia, depend on close forward pairing (0.5 s ISI) of sensory neuron activity and traumatic stimulation (Hawkins et al 1986, Clark 1984). It is not obvious how the biochemical conjunction–detecting mechanisms just described would produce this order sensitivity. Biochemical experiments have provided some evidence that rate of 5-HT–stimulated adenylyl cyclase catalysis of cAMP production is enhanced by Ca\(^{++}\)/calmodulin pulses that precede onset of 5-HT exposure (Yovell & Abrams 1992, Abrams et al 1991), but the time scale of these experiments is in minutes rather than seconds. It remains to be determined whether the precise temporal requirement for classical conditioning is an intrinsic property of the sensory neuron. In sensory-motor neuron co-cultures, activity and 5-HT exposure produce more facilitation when they overlap than when they are separated by a full minute (Eliot et al 1994a), but measurements of precise timing and order dependence have not been done. It has been suggested that perhaps prevention of backward conditioning is due to network properties; e.g., presynaptic inhibition that follows traumatic stimulation might prevent such conditioning by preventing amplification of facilitation by presynaptic activity (Mackey et al 1987).

**Classical conditioning in insects** Analysis of classical conditioning of the proboscis extension reflex of bees and of odor avoidance in *Drosophila* (see above) has provided evidence for the generality of the mechanisms seen in *Aplysia* as well as promising preparations for more detailed analysis.

A variety of *Drosophila* mutants (amnesiac, turnip, rutabaga, dunce, and others) with learning disorders but few other behavioral abnormalities have been established (Dudai et al 1976, Quinn et al 1974, Tully & Quinn 1985). Continuing analysis of the primary biochemical deficits of these mutants (see Tully 1988, Dudai 1989) indicates that dunce has low cAMP phosphodiesterase (see Qiu et al 1991), that rutabaga adenylyl cyclase is not activated by Ca\(^{++}\)/calmodulin (see Levin et al 1992), and that turnip has somewhat less active than normal adenylyl cyclase as well as drastic reductions in PKC activity (Smith et al 1986). These findings are striking, given the importance attributed to the PKA system in *Aplysia* and the postulated role of adenylyl cyclase Ca\(^{++}\)/calmodulin sensitivity in associative phenomena, as well as evi-
dence for major involvement of the PKC system in a variety of other learning phenomena (see above and below).

Induction of classically conditioned responses to odor in insects may also depend on the activity of special neurons analogous to the facilitators of *Aplysia* defensive reflexes. In bee brain a neuron has been identified that fires in response to sugar water UCSs and that when driven by direct intracellular depolarization produces no behavior but serves quite effectively in lieu of sugar water as a reinforcement in conditioning experiments (Hammer 1993). This neuron, the VUMmx1 cell (Figure 4), which is thought to be one of a small population of similar cells, has dendrites in the subesophageal ganglion and projects a profoundly branching axonal arbor into several structures including the calyces of the mushroom bodies, which, as reviewed above, are candidate loci for engrams. It might be expected from the *Aplysia* analogy that the cAMP pathway products of *dunce* and *rutabaga* genes would be localized preferentially in the calyces where the bee VUMmx1 cells terminate. This is true for the *dunce* phosphodiesterase, but *rutabaga* Ca\(^{++}/\)calmodulin-sensitive adenyl cyclase seems to be concentrated more along axons or at terminals of the mushroom body neurons.

*Long-term potentiation in Aplysia* Activity-dependent facilitation provides a means of selectively enhancing transmission at synapses whose presynaptic activity coincides with a widely broadcast facilitatory message. It does not, however, allow such enhancement to be limited to synapses between particular co-active pre- and postsynaptic neurons. Enhancement of transmission at co-active synapses was postulated by Hebb and subsequently established as the mechanism for one type of long-term potentiation (associative LTP) in mammalian brain (see Hawkins et al 1993, Bliss & Collingridge 1993). Initial tests for a role of postsynaptic activity in the activity-dependent facilitation of *Aplysia* found that depolarizing the postsynaptic neuron during presynaptic activity did not cause facilitation, and hyperpolarizing the neuron during pairing of traumatic stimulation and presynaptic activity did not reduce facilitation (Hawkins et al 1983, Carew et al 1984).

However, in these experiments the somatically injected current may not have adequately affected dendrites, and recent experiments on co-cultured sensory and motor neurons of *Aplysia* show that a phenomenon extremely similar to mammalian associative LTP occurs at *Aplysia* sensory-motor synapses (Lin & Glanzman 1994a,b). Repeated, brief sensory neuron tetani cause a persistent enhancement of transmission that is prevented by postsynaptic hyperpolarization. Pairing a weak presynaptic tetanus with direct depolarization of the motor neuron also causes enhancements, and these are blocked by the NMDA antagonist, APV, or postsynaptic injection of the Ca\(^{++}\) chelator, BAPTA. This *Aplysia* LTP-like mechanism (apLTP) is obviously quite inde-
dependent of activity-dependent facilitation, since no facilitators are present in the culture. Similarly, leech protective responses, which are also sensitized by a 5-HT-dependent mechanism, can still be classically conditioned, but not sensitized, after 5,7-DHT poisoning of their serotonergic systems (Sahley 1993).

If operative in vivo (Cui & Walters 1994), apLTP could be the basis for response-selective associative conditioning such as that reported by Walters (1989) and Hawkins et al (1989), which were attributed to other mechanisms. However, because gill and siphon motor neurons are activated during spontaneous respiratory pumping, fortuitous sensory activity during pumping could result in adventitious conditioned responses due to apLTP. Suitable three-way interaction effects between sensory, facilitator, and motor neuron activity (i.e. interactions between activity-dependent facilitation and apLTP) could resolve this problem. Indeed, this system could provide a useful model for physiological investigations of interactions between contiguity and reinforcement.

Classically conditioned phototactic suppression in *Hermissenda* Light applied to *Hermissenda*'s type B photoreceptors, whose increased responses and synaptic outputs are believed to be responsible for learned suppression of phototaxis, causes a strong depolarization, which at light-offset does not entirely abate for several minutes (Alkon & Grossman 1978). This persistent after-depolarization, which occurs even when the distal synapse-containing portion of a photoreceptor is cut away, is referred to as the LLD (long-lasting depolarization). Recordings during training show that pairing regimens that lead to successful conditioning are associated with enhanced LLDs, which tend to summate from one light-vestibular stimulation pairing to the next to produce a cumulative depolarization that may last for hours (Alkon 1979, Tabata & Alkon 1982, Farley & Alkon 1987, Alkon 1980). These augmented post-stimulation depolarizations might be secondary to decreased shunting associated with developing $K^+$ current reductions, but the possibility that they play a causative role in establishing longer term changes has received considerable attention.

How pairing might enhance the LLD, so that it could in turn cause long-term change, has been explained with reference to the network connecting B photoreceptors and hair cells (Figure 3; Tabata & Alkon 1982, Farley & Alkon 1987, Alkon 1979). Type B photoreceptors (here called B cells) and caudal (but not rostral) hair cells provide convergent hyperpolarizing inhibitory input to a group of S/E optic ganglion cells that in turn send excitatory input back to the B cells and inhibitory input to the hair cells. The S/E cells are prone to post-inhibitory rebound. Thus, when they are inhibited particularly strongly because of pairing of light and caudal hair cell stimulation, they produce substantial rebound excitation of the B cells at offset of light and vestibular stimulation. This excitation is further enhanced because the otherwise toni-
cally firing caudal hair cells are uninhibited and thereby prevented from inhibiting the B cells. The result is a barrage of EPSPs to the B cells that sum with the intrinsic light-produced LLD. If this explanation of the LLD enhancement following pairing is correct, and if it really does cause learning, then rotations designed to selectively stimulate the rostral hair cells, or light-vestibular stimulation pairings that do not co-terminate, should not cause conditioning. Both predictions have been confirmed (Farley & Alkon 1980, Grover & Farley 1987).

Voltage-clamp and use of the Ca\(^{++}\)-sensitive dye, Arsenazo III, show that the nonsynaptic portion of the LLD is due to a light- and depolarization-dependent Ca\(^{++}\) current (Alkon 1979) that, presumably because of its voltage dependence, is somewhat self-sustaining. Arsenazo measurements also show that light flashes paired with injections of depolarizing current, which start with the flash and extend for about a minute beyond it, simulating the effects of the S/E cell input that occurs during pairing, cause augmented Ca\(^{++}\) responses (Connor & Alkon 1984). A similar regimen of B-cell stimulation results in behavioral learning measured a day later (Farley et al 1983). Thus, B photoreceptor Ca\(^{++}\) elevations, acting via Ca\(^{++}\)-dependent protein kinases such as PKC or CaM kinase, have been suggested as the event that triggers learning-induced changes.

Both CaM kinase and PKC, under conditions intended to elevate intracellular Ca\(^{++}\), as well as phorbol ester, which directly activates PKC, cause reductions of I\(_A\) and I\(_{K\text{-}Ca}\) (Sakakibara et al 1986a,b; Alkon et al 1986, 1988; Farley & Auerbach 1986); however, the PKC-mediated effect is thought to mimic that of training more closely. Conversely, PKC inhibitors prevent induction of both early (Matzel et al 1990) and more persistent (>24 hr) learning-produced changes (Farley & Schuman 1991). As would be expected, changes do not develop in B photoreceptors injected with the Ca\(^{++}\) chelator, EGTA (Matzel et al 1992).

In the picture that thus emerges, sensitivity to pairing is partly a consequence of the convergence of photoreceptors and hair cells on the S/E cells, resulting in post-inhibitory depolarizing input to the photoreceptors, and partly to the joint dependence of a photoreceptor Ca\(^{++}\) current on light and depolarization. The assumptions of this schema have been incorporated in an equivalent circuit model that rather faithfully reproduces many experimental results (Werness et al 1992, 1993), but this is difficult to evaluate because the values of the many parameters used in the simulation have not been given.

In the above scenario, an LLD-associated Ca\(^{++}\) elevation is supposed to foster B photoreceptor change. But Matzel et al (1992) have reported that abolishing the LLD by clamping the photoreceptor membrane potential to its resting level right after paired light and vestibular input does not prevent development of conditioned changes.
Several other findings also complicate the picture. For example, exposure to exogenous 5-HT has been found to produce changes apparently similar, though not identical, to those produced by behavioral conditioning procedures (Crow & Bridge 1985, Schuman & Clark 1994, Farley & Wu 1989, Acosta-Urquidi & Crow 1993), and pairing 5-HT with light amplifies this 5-HT effect (Crow & Forrester 1986, 1991; Frysztak & Crow 1994). Kinase inhibition using the partially selective PKC inhibitors H7 and sphingosine prevents induction of this effect in the short term, but long-term changes still develop (Crow & Forrester 1993a, b). Serotonergic neurons that respond to both visual and vestibular stimulation and innervate the B photoreceptors have been found (Cheyette & Farley 1989), but a role for them in learning has not been established. Finally, procedures that interfere with 5-HT release or action reduce short-term conditioning effects of paired light and caudal hair cell stimulation (Grover et al 1989). Thus, as with the original scheme, sensitivity to CS and UCS pairing may be dependent on both convergence on a common target (the serotonergic cells) and on an intrinsic B cell interaction between synaptic input and light.

A quite different possibility is suggested by the recent discovery that when GABA, which may be the transmitter the caudal hair cells release to the B photoreceptors during paired light and vestibular stimulation, is applied to depolarized B cells, an increase in input resistance occurs similar to that produced by training (Matzel & Alkon 1991). This effect requires external Ca$$^{++}$$ and is H7-sensitive. Pairing of GABA and depolarization also seems to transform the hyperpolarizing inhibition produced by hair cell synapses on B photoreceptors into a depolarizing response that is apparently caused by release of Ca$$^{++}$$ from internal stores (Alkon et al 1992). This hair cell–engendered Ca$$^{++}$$ response could also contribute to the induction of further B cell changes.

THE LOCUS OF DECISIONS TO CHANGE—GENERALITIES  The initiation of change depends on an evaluation that can be made by the potentially changing cell itself (i.e. proximally) or by other neurons (i.e. distally). Of the cases we have examined, Aplysia sensitization, which simply depends on whether facilitators fire, falls into the latter category whereas Aplysia short-term habituation seems to fall into the former. Sensitivity to pairing in classical conditioning of Aplysia appears to be dependent on a proximal integration at the level of adenylyl cyclase and perhaps also at the CRE-binding protein, whereas in Herrnissenda it depends, at least in one theory, on both the distal network illustrated in Figure 3 and a synergistic interaction between synaptically induced depolarization and light stimulation of the B photoreceptor.

Two general points need to be made about these differing scenarios. First, distal strategies have the advantage that change can be made contingent on
potentially sophisticated computations made anywhere in the nervous system. Thus, it seems likely that at least in higher animals the distal approach will be the one commonly taken. Second, the information processing capabilities of single cells should not be underrated. The “and” gate formed by adenylyl cyclase’s joint Ca\textsuperscript{++} and 5-HT dependence is an example of a kind of biochemical information processing that could be elaborated to an extraordinary degree as the various second messenger and protein kinase systems of a cell interact. Sophisticated logical analysis based on various types of input to a cell as well as timing and counting operations are all at least in principle single-cell capabilities (Bray 1990).

**Implementation of Change**

Although there is significant information regarding the cytoplasmic signals activated by various training regimens in invertebrate learning paradigms, considerably less is known about the cellular changes that result from activation of these signals. One important common theme is that development and expression of short-term learning may involve covalent modifications of pre-existing proteins, whereas induction of long-term change usually requires protein synthesis (Goelet et al 1986).

Thus, although short-term sensitization in *Aplysia*, and generator potential enhancements produced by pairing light and 5-HT exposure in *Hermissenda* photoreceptors, develop in the presence of protein synthesis inhibitors, inhibition of protein synthesis during periods of training, or during exposure to facilitatory or inhibitory neuromodulators, prevents the development of the corresponding long-term changes (Castellucci et al 1989, Crow & Forrester 1990, Montarolo et al 1986). It also prevents the structural changes described above (Bailey et al 1992), long-lasting changes in protein phosphorylation (see below; Sweatt & Kandel 1989), and decreases in PKA regulatory subunits (see below) (Bergold et al 1990). However, inhibition of protein synthesis does not prevent long-term learning in bees (Wittstock et al 1993), and its effects have not been tested on long-term habituation in *Aplysia* or crayfish.

**PATTERNS AND ROLES OF PHOSPHORYLATION** During short-term sensitization, and at 24 hr after induction of long-term sensitization in *Aplysia*, a common set of 17 sensory neuron proteins show increased phosphorylation (Sweatt & Kandel 1989). The commonality of these patterns suggests a role for phosphorylation in expression rather than in induction of the sensitized state. However, because structural changes are probably developing at 24 hr after induction of long-term sensitization, it is surprising that the biochemical activity involved is not associated with some alterations in phosphorylation. Four *Hermissenda* eye proteins also show increased phosphorylation 24 hr after induction of phototactic suppression (Nelson et al 1990).
One of the phosphorylated *Aplysia* proteins could be the Ks channel itself or a closely associated membrane protein, because application of the catalytic subunit of PKA directly to inside-out patches of sensory neuron membrane causes closure of its channels (Shuster et al 1985). Also, one of the proteins that becomes phosphorylated by *Hermisenda* training reduces the two conditioning sensitive K$^+$ currents when injected into B photoreceptors (Nelson et al 1990).

In *Aplysia* the presynaptic inhibitory neuromodulator FMRFamide suppresses resting phosphorylation levels of 10 proteins, all of them within the group of 17 whose phosphorylation increases during sensitization. When applied in conjunction with 5-HT or cAMP, FMRFamide prevents all 17 increases of phosphorylation (Sweatt & Kandel 1988) as well as preventing any functional sensitization (Sweatt et al 1989, Belardetti et al 1987), possibly by activating phosphatases that break down phosphate-protein bonds (Ichinose & Byrne 1991).

Although short- and long-term sensitization are said to ultimately produce similar patterns of phosphorylation, these patterns change dynamically during extended periods of serotonin treatment (Homayouni et al 1991). This could reflect protein kinase system involvement in the evaluation of whether the history of facilitator input warrants initiation of long-term change (see Generalities section above). And some later developing phosphorylation could be involved in the recruitment of protein synthesis.

Cyclic AMP can induce transcription of a variety of so-called immediate early genes. This induction is thought to occur when cAMP-activated PKA phosphorylates certain proteins, the cAMP-responsive-element binding proteins, that in turn bind to a particular DNA nucleotide sequence called the cAMP responsive element (CRE) (Brindle & Montimony 1992). There is now considerable evidence that this sequence of events is involved in 5-HT-induced long-term facilitation in *Aplysia*: 1. Serotonin exposures that cause long-term sensitization, but not more limited exposures, cause fluorescently labeled catalytic subunits of PKA to break away from cAMP-bound regulatory units and enter the nucleus (Bacskai et al 1993). 2. *Aplysia* sensory neuron DNA contains the CRE (Schacher et al 1990a). 3. Foreign CREs fused to *Escherichia coli lacZ* genes injected into sensory neurons express LacZ gene product when the neuron is stimulated by a 5-HT protocol that produces long-term facilitation (Kaang et al 1993). 4. Injection of short nucleotide chains containing the CRE sequence in abundance, which should tend to capture limited amounts of natural phosphorylated CRE-binding proteins and thereby prevent transcription, prevented expression of the lacZ gene in the above experiment and also prevented induction of long-term but not short-term facilitation (Dash et al 1990). Alberini et al (1994) have identified a
protein (ApC/EBp) that is produced by a cAMP-inducible gene and appears to be essential for induction of long-term sensitization.

These data suggest intriguing possible roles for phosphorylation in producing long-term change. However, the lack of effect of kinase inhibitors on long-term *Hermissenda* photoreceptor conditioning produced by pairing 5-HT with light (see above) should not be forgotten.

**PATTERNS AND ROLES OF PROTEIN SYNTHESIS** Repeated 5-HT applications induce a complex, temporally orchestrated, pattern of increased and decreased synthesis of specific proteins in *Aplysia* sensory neurons (Barzilai et al 1989). Some of this synthesis must be associated with regulatory gene actions that establish altered patterns of continuing synthesis (see Castellucci et al 1988). Also, of 15 proteins whose rate of transcription alters within 15–30 min of the start of 5-HT exposure and continues for 1–3 hr (so-called early proteins), 5 appear to be related to structural changes. Four of these proteins, whose synthesis decreases, are *Aplysia* homologues of the vertebrate neural cell adhesion molecules (Schacher et al 1990a, Mayford et al 1992). These presumed *Aplysia* cell adhesion molecules (apCAMs) appear to play a role in neurite outgrowth and synapse formation. Thus, monoclonal antibodies to apCAM cause sensory neuron processes in culture to defasciculate (Mayford et al 1992), a process associated with the formation of synapses in sensory-motor neuron co-cultures (Glanzman et al 1989a, 1990). The application of 5-HT to sensory neurons, in addition to down-regulating apCAM synthesis, also causes protein synthesis–dependent endocytosis of existing cell surface apCAM molecules (Mayford et al 1992, Bailey et al 1992). One of the early proteins whose synthesis is increased is in fact critical for apCAM endocytosis and has been identified as the light chain of *Aplysia* clathrin, a protein homologous to one known to regulate receptor-mediated endocytosis in vertebrate cells (Hu et al 1993). Synthesis of several other known proteins such as actin, intermediate filament protein, and several endoplasmic reticulum proteins is also altered (Noel et al 1993, Kennedy et al 1992, Kuhl et al 1992).

Another consequence of altered protein synthesis is suggested by the discovery that 5-HT treatments that induce long-term facilitation cause a long-lasting reduction in concentration of regulatory (but not catalytic) subunits of PKA (Greenberg et al 1987, Bergold et al 1990). Thus, the phosphorylations normally mediated by cAMP can occur at lower concentrations of cAMP and possibly become endogenous, potentially explaining the persistence of the short-term pattern of Ks channel closure and protein phosphorylation after induction of long-term sensitization. Extended 5-HT treatment reduces regulatory subunits at 24 hr even in isolated synaptic terminals (Greenberg et al 1987), but integrity of protein synthesis during induction is necessary for regulatory subunits to remain low in intact cells, presumably because replace-
ment subunits eventually get synthesized unless their synthesis is down-regulated or synthesis of a protease is up-regulated as a protein synthesis–dependent result of the induction process.

In *Hermissenda*, long-term (>24 hr) phototactic suppression is associated with altered synthesis rates of a number of mRNA species (Nelson & Alkon 1990). However, the functions of the associated proteins are unknown.

**MEMORY**

Sensitization of *Aplysia* defensive withdrawal has provided a useful model for investigating the mechanism of retention over time. Since sensitization is mediated by protein phosphorylation, it might be expected that sensitization would be relatively long lasting because phosphates link to proteins via inherently stable covalent bonds. However, cells are rich in protein phosphatases that limit the longevity of these bonds; in *Aplysia* the activity of phosphatases is indicated by prolongation of spike broadening effects in the presence of phosphatase inhibitors (Ichinose & Byrne 1991). Indeed, injection of a protein kinase inhibitor into a sensory neuron abolishes spike broadening previously established by exposure to 5-HT within a few minutes, indicating that the phosphorylation responsible for broadening is not inherently stable (Castellucci et al 1982a). In fact, the short-term effects of 5-HT last only about 5 min (Bernier et al 1982, Eliot et al 1994a, Schacher et al 1990b, or perhaps less (Yovell et al 1987), after 5-HT exposure ends. Thus, the duration of short-term sensitization may be mostly dependent on sustained exposure to a facilitatory transmitter. This could result from persistent activity of facilitators (Mackey et al 1989), the mechanism for which is still unknown. It is also unknown how the effects of multiple 5-HT exposures separated by 15 min can sum to induce protein synthesis. Perhaps even very low levels of cAMP, when prolonged, can trigger protein synthesis or there may be some slowly degraded phosphoproteins that span the interstimulus interval.

Long-term sensitization involves growth, which is associated with altered levels of several proteins, and decreased levels of PKA regulatory subunit, presumably resulting from lowered synthesis or from increased production of a protease. Altered behavior due to structural changes or presence (or absence) of particular proteins would be expected to be more stable than that due to protein phosphorylation, but because cytoplasmic and cell membrane molecules turn over, persistence on the order of weeks and longer must still be explained. Induction of sensitization may establish a new self-maintaining pattern of gene expression analogous to the transcriptional and perhaps post-transcriptional controls that are put in place as cells differentiate during development. Long-term sensitization would then be the result of continuing export of particular proteins at regulated concentrations to sensory terminals. Emp-
tage & Carew (1993) have shown that at 24 hr after a 1.5 hr exposure of a sensory neuron soma to 5-HT, the terminals are sensitized even though they were not exposed to the facilitator and never displayed short-term sensitization (see also Clark & Kandel 1993).

Such a mechanism might well mediate *Aplysia* sensitization, in which all the terminals of a given sensory neuron may change in the same way. But it is believed that associative learning in vertebrates demands a capability for independent regulation of each of thousands of synapses of a given neuron, and it is implausible to suppose that the status of each of those synapses could be coded by self-sustaining patterns of gene expression. Thus, this sort of cell memory is not a suitable storage medium for large-scale associative learning. Rather, it must be supposed that changes established locally at each synapse, perhaps involving cooperative pre- and postsynaptic changes (Schuman & Madison 1994), are individually self renewing, (see Lisman 1985, Crick 1984, Hanson & Schulman 1992), albeit with presumed logistical support from the cell nucleus. It will be interesting to exploit *Aplysia* neuron co-cultures of the kind used to study LTP (Lin & Glanzman 1994a, b) or sensory neurons that have spatially well-separated output synapses (Emptage & Carew 1993, Clark & Kandel 1984, 1993) to study the development and maintenance of long-term changes that are specific to particular synapses.

**CONCLUSIONS**

The findings reviewed here provide an extent and depth of insight into mechanisms of simple learning that go far beyond anything that could have been envisaged a few decades ago. We find particularly impressive the analysis of sensitization in *Aplysia*, where one can trace an almost unbroken causal chain from experiential events responsible for the learning to anatomical and physiological changes responsible for the altered behavior. The relative simplicity of the neural circuits that mediate the learned responses demonstrates clearly the relationship between the cellular responses studied and the resulting behavior; this is the tremendous strength of the approach. We can feel quite confident that what is being studied are really mechanisms of learning and not laboratory curiosities or phenomena unrelated to learning. However, we can also see that the surface has only been scratched; there is extensive evidence that the changes studied so far are only a small part of the totality. Thus, behavioral learning has not yet really been explained, even in invertebrates. But the way seems clear to continuing the analysis with the same approaches that have brought us this far.
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