Archives Italiennes de Biologie, 135: 15-36, 1997.

NEUROBIOLOGICAL PRINCIPLES OF LEARNING AND MEMORY

M. BRUNELLI, M. GARCIA-GIL, R. MOZZACHIODI, R. SCURI AND M.L. ZACCARDI

Dipartimento di Fisiologia e Biochimica "G. Moruzzi", Università degli Studi di Pisa, Via S. Zeno 31, 56127 Pisa, Italia

INTRODUCTION

Memory allows single individuals to build up the consciousness of themselves and to store all those information that are utilized to change the behavior following experience throughout the life.

Learning is considered a process through which it is possible to collect information and to modify the behavior to environmental changes. Memory storage is the retention of that modification over time.

Studies on memory have shown that there are different types of memory processes. *Explicit memory* includes learning about people, places, and things that can be reported verbally and requires conscious awareness. *Implicit memory* includes forms of perceptual and motor learning that do not require conscious awareness. The formation and the recall of implicit memory are absolutely independent on awareness or cognitive processes. This type of memory, built up slowly through repetition over many trials, is expressed primarily by improved performance and cannot ordinarily be expressed in words. Such learning can be recalled automatically without deliberate effort. Implicit memory can be studied in a variety of reflex systems either in vertebrates or in invertebrates.

Two major classes of implicit learning and memory have been identified: *non associative* and *associative learning*.

Two forms of non associative learning are very common in everyday life: *habituation* and *sensitization*. Habituation is a decrease in response to a repeated light stimulus. Sensitization (or pseudoconditioning) is a strengthening of responses to a wide variety of stimuli following an intense or noxious stimulus. For example, a sensitized animal responds more vigorously to a mild tactile stimulus after it has received a painful pinch. Moreover, a sensitizing stimulus can override the effects of habituation. For example, after the startle response to a noise has been reduced by habituation, the response can be restored by delivering a strong pinch. This process is called *dishabituation*. Sensitization and dishabituation are not dependent on the precise timing of the intense stimulus relative to the weaker stimulus; no association between the two stimuli is needed.

Not all examples of non associative learning are as simple as habituation or sensitization. Many types of more complex learning have no obvious associational element (although hidden forms of association may be present). These types of

learning include imitative learning, a major component of the acquisition of language.

Many types of associative learning have been distinguished on the basis of the experimental procedures used to establish the learning. Two experimental paradigms have been extensively studied: 1) *classical conditioning*, 2) *operant conditioning*.

1. The essence of classical conditioning is the pairing of two stimuli. The conditioned stimulus (CS), such as light or tone, is chosen because it produces either no overt response or a weak response usually unrelated to the response that eventually will be learned. The unconditioned stimulus (US), such as food or shock to the leg, is chosen because it normally produces a strong, consistent, overt response (the unconditioned response), such as salivation or withdrawal of the leg. Unconditioned responses are innate; they are produced without learning. When a CS is followed by an US, the CS begins to elicit a new or different response called the conditioned response. Sometimes the conditioned response resembles the unconditioned response, but they can also differ. If the US is rewarding (food or water), the conditioning is termed *appetitive*; if the US is noxious (an electrical shock), the conditioning is termed *defensive* or *aversive*.

The intensity or probability of occurrence of a conditioned response decreases if the conditioned stimulus is repeatedly presented without the unconditioned stimulus. This process is known as extinction.

All forms of associative learning have evolved to enable animals to distinguish events that reliably and predictably occur from those that are only randomly associated. The brain seems to have evolved to detect causal relationships in the environment.

2. The second paradigm of associative learning, discovered by Edward Thorndike and studied by B. F. Skinner is the *operant conditioning* (also called trial-and-error learning). If an animal promptly receives food when it presses the lever in a test chamber, subsequent rate of pressing the lever will increase above the spontaneous rate. The animal can be described as having learned that, among its many behaviors (e.g. grooming, rearing and walking) the lever pressing is the behavior followed by food. Operant conditioning can be considered as the formation of a predictive relationship between a response and a stimulus. Thus, operant behaviors are said to be emitted rather than elicited, and when the behaviors produce favorable changes in the environment (when they are rewarded or led to the removal of noxious stimuli), the animal tends to repeat them.

As we have seen, animals generally learn to associate stimuli that are relevant for their survival; they do not learn to associate stimuli that are biologically meaningless.

I. *Stages of memory.*

It has long been known that a person after a trauma can have a selective memory loss for events that occurred before the blow (retrograde amnesia), as well as for events that occurred after the recovery of consciousness (anterograde amnesia).

Clinical studies provide evidence that brain trauma can produce particularly deep retrograde amnesia for events acquired within a few hours or at most days before the trauma. Thus the recently acquired memories are readily disrupted, whereas the oldest memories remain quite undisturbed.

Studies of retention and disruption of memory have supported a commonly used model of memory storage by stages. Input to the brain is processed into a short-term memory (S.T.). This has very limited capacity and, in the absence of rehearsals, persists only for a period of minutes. The information is later transformed by some processes into a more permanent long-term store (L.T.).

According to this model, the retention of memory can suffer because of destruction of the contents of a memory store. Alternatively, memories may be disrupted by interfering with the search-and-retrieval mechanism. This latter conclusion is supported by the observation that trauma sometimes only temporarily disrupts memory for recent events, while memory for past events gradually returns.

Retrieval of recent memories is easily disrupted until the memories have been converted into a L.T. memory form. Once converted, they are relatively stable. With time, however, both the stored information and the capacity to retrieve it gradually diminish, even in the absence of external trauma.

Concerning the neurobiology of learning, a major task is to assess how alterations in the brain are related to behavioral changes. A second task is to determine the mechanisms underlying synaptic changes associated with memory. To this aim, a number of simplified vertebrate and invertebrate animal preparations are being investigated, and some of these studies are reviewed.

II. *Non associative learning.*

The processes of learning can be analyzed either in behaving organisms or in neuronal networks taken from vertebrate or invertebrate nervous system.

Most of progress has come from studies on the changes observed in relatively simple invertebrates nervous systems. This reductionistic approach has allowed to enlighten common feature linking all the learning processes.

At the aim of studying the basic mechanisms underlying changes of behavior, it has been necessary to extend the cellular investigations to various neural circuits in order to select the common mechanisms of different learning processes.

The last cellular studies have singled out common unifying themes in the study of plasticity such as the modulation of transmitter release, the formation of phosphorylation process in short-term memory and the synthesis of novel proteins in long-term changes. Therefore, different forms of modulation represent functional building blocks that in various combinations set up the "language of learning" subserving the more complicated synaptic plasticity.

In invertebrates, it has been possible to identify single neurons of the networks underlying specific behavioral acts. Therefore, we can analyze, when learning and behavior changes occur, the "molecular loci" and the kind of modifications at the level of single neuron of the circuit.

This investigation has been carried out in the marine snail *Aplysia californica*,

which has a relatively simple nervous system with well identified cells packaged in distinctive clusters in ganglionic structures. These cells are among the largest neurons and have peculiar shape and function.

The cellular mechanisms of elementary forms of non associative learning such as habituation and sensitization, have been studied taking as behavioral model the gill and siphon withdrawal reflex (WR). A mild tactile stimulus delivered to the siphon elicits withdrawal of both the siphon and the gill. After repeated stimulation, WR habituates (18). A strong nociceptive stimulus to the head or the tail of the animal produces a potentiation of the WR (sensitization); this potentiation is called dishabituation when the WR has been previously habituated. WR has been studied in detail at the level of neuronal circuit. The stimulus brought onto the siphon, activates a cluster of sensory neurons which generates excitatory synaptic potentials in motor cells. When the stimulus is repetitively presented, the synaptic potentials become progressively smaller. This decrease in synaptic transmission in the sensory neuron results from a reduction in the amount of chemical transmitter released from the presynaptic terminal (homosynaptic depression) (52, 18). Quantal analysis has shown that homosynaptic depression reflects a decrease in the number of transmitter or quanta released from the presynaptic terminals (19, 69).

A part of this reduction seems due to inactivation of N-type Ca^{2+} channel in the presynaptic terminal. As a result less Ca^{2+} flows into the terminals at each action potential and therefore less transmitter is released. In addition there is a less ability of transmitter vesicles to be mobilized to the active zone so as to be available for release. This reduction can last many minutes (3, 4).

This synaptic depression accounts for habituation of escape responses in crayfish (69) and cockroaches and in the startle reflex in vertebrates.

Not all synapses are plastic and adaptable. Some synaptic connections in the nervous system do not change their strength, even with repeated activation.

Similar results of homosynaptic depression have been obtained in simplified systems of co-culture "in vitro" of sensory and motor neurons (55). In habituated sensory neurons, it has been observed, by means of voltage clamp experiments, that repeated stimulation of the sensory neuron resulted in a decrease in the inward Ca^{2+} current.

Several molecular steps for habituation are still unknown: various hypothesis can be taken in consideration for the homosynaptic depression: 1) reduction of Ca^{2+} current by increase of cytosolic Ca^{2+}; 2) activation of second messenger cascade of phospholipid/arachidonic acid type, due to the binding of the transmitter released by sensory neuron with autoreceptors onto the presynaptic terminals. 3) In the neuronal circuitry of WR, inhibitory interneurons and their aminergic and peptidergic transmitters have already been identified. They might have a consistent relevance in the habituation acting through an heterosynaptic mechanism.

During habituation, Bailey and Chen (3, 4) have shown ultrastructural changes in the vesicles of sensory neuron terminals.

Sensitization is a more complex form of non associative learning than habituation. In *Aplysia* a strong stimulus to the neck or tail greatly enhances the WR. A

number of synaptic connections between sensory and motor neurons in the neuronal circuit becomes modified. While habituation is considered an example of homosynaptic depression, sensitization involves a presynaptic alteration in transmitter release between sensory and motor neurons. Sensitizing stimuli from the tail excite a group of modulatory interneurons that enhance transmitter release from sensory neurons, a heterosynaptic process called presynaptic facilitation (20, 38, 48). These facilitating neurons are serotonergic (17, 48).

With a pioneering investigation Brunelli, *et al.* (17) have shown that serotonin (5HT) mimics the facilitatory effect onto the sensory-motor neuron synapse. In addition, intracellular injection of cAMP potentiates the monosynaptic EPSP recorded in the motor neurons.

Hence the molecular model outlined from these findings is the following: 5HT activates on the sensory neuron terminal receptors that engage a GTP binding protein (Gs), which activates an adenylate cyclase and increases the concentration of cAMP. This in turn activates the protein kinase A (cAMP-dependent protein kinase, PKA) which phosphorylates a number of substrate proteins, including the S-type K^+ channel (K_s), or a possible protein acting on this channel. Phosphorylation leads to the closure of this channel and broadens the action potentials (43, 44, 62, 63). The direct evidence of the cAMP mediated phosphorylation and of the activity of K_s channel emerges from single-channel recordings worked out by membrane patches still attached to the whole cell (63) or in membrane patches detached from the cell (62). In cell-attached membrane patches, serotonin application or intracellular injection of cAMP causes lasting closure of K_s channel. In cell-free membrane patches from sensory neurons the catalytic subunit of mammalian PKA produces all-or-none closure of individual K_s channels. In addition, the kinase A modulates the kinetics of a second K^+ channel, a voltage-gated transient K^+ channel which further enhances broadening of the action potential (6, 41). This broadening of the action potential is thought to contribute to presynaptic facilitation by allowing more Ca^{2+} to enter the presynaptic terminal via voltage-gated Ca^{2+} channels. 5HT selectively increases the magnitude of L-type Ca^{2+} current: the monoamine has, on the contrary, no direct effect on the N-type current. Blocking Ca^{2+} influx via the L-type channel by dihydropyridines does not cause alteration of presynaptic facilitation (28).

Eliot (29) measured the Ca^{2+} influx during facilitation, by using FURA-2 to image Ca^{2+} in the presynaptic region of a single sensory neuron innervating a single motor neuron in dissociated cell culture. After blocking L-type channel the enhancement of Ca^{2+} influx through N-type channel produces an increase of transmitter release.

Since N-type Ca^{2+} channel is not regulated by 5HT, this modulation of Ca^{2+} influx is attributable to the broadening of the action potential by serotonin, owing to its action on K_s channel.

5HT, through the PKA, modulates L-type Ca^{2+} channel, that in turn increases the mobilization of transmitter vesicles to the releasing sites (40). In this effect the cAMP-dependent protein kinase works in parallel with protein kinase C which is

also activated by serotonin, likely through a different receptor. This activation leads to enhancement in spontaneous release that is largely independent of changes in free Ca^{2+} (15, 35, 56).

The investigations carried out in *Aplysia* have greatly contributed to the knowledge of the neural basis of learning, but have opened novel questions such as:

1) is the mechanism involved in non associative learning invariant along the phylogenetic scale? 2) Do serotonin and other neuromodulators play the same pivotal role in short-term changes of different neural systems of vertebrates and invertebrates alike? 3) Are the same molecular mechanisms underlying short-term plastic changes in *Aplysia* valid for more complex neural networks (and therefore more complex behavioral activity), or can further mechanisms gain relevance in other systems?

Recently, in addition to the modifications of efficacy of sensory-motor synapses, the importance of interneurons and their modulation in the behavioral expression of learning has been pointed (67).

In order to give an answer to these questions more recently the analyses have been performed on other invertebrates models.

III. *The leech model.*

The leech *Hirudo medicinalis* has proven to be an ideal system in which it is possible to explore the basic principles of learning. The nervous system of this annelid is made up of a chain of segmental interconnected ganglia. Several cellular networks underlying elementary behavioral acts such as shortening, swimming, etc., have been extensively investigated. Many studies have been worked out by different groups focusing on the following simple behaviors which exhibit non associative learning such as habituation and sensitization: a) movements in response to light and water currents; b) local bending (46); c) shortening reflex to repeated photic (2, 45) or tactile stimulation (2, 14).

It has been observed that the two variables which influence the rate of habituation are the intensity of habituating stimulus and the interstimulus interval; they operate in the same way both in the leech and in the vertebrates models. Repeated stimulation ranging from 10 sec to 2 min produces habituation, and an intense stimulation brought in other part of the body surface increases the responsiveness to the text stimulus (dishabituation).

In a first series of experiments on the shortening reflex of the leech, it has been demonstrated that the plastic changes of habituation and sensitization type occur at the level of the pathway from mechanosensory neurons onto a chain of electrically coupled neurons, the S cells (2). This S cell chain is often called the *fast conducting system* (FCS) because the S cell axon is the largest in the leech and has the fastest conduction velocity. The ablation of S cells disruptes sensitization of FCS and regeneration of these cells restores the facilitation.

On the isolated nerve cord it has been demonstrated that the potentiation observed during the sensitization (or dishabituation) of the FCS response is mediated by 5HT, through an increase of cAMP (8). 5HT mimics the dishabituation which

is blocked both by preincubation with imidazole, a cAMP phosphodiesterase activator, and by methysergide, a 5HT antagonist. Depletion of 5HT eliminates sensitization to noxious stimuli (30).

Stimulation of 5HT-containing neurons or application of 5HT agonists dishabituates the local bending reflex (46).

More recently the search for learning mechanisms has been focused on the swimming of the leech. This behavioral activity has been extensively studied at the level of the cellular network. The rhythmic swimming oscillation is set up by a chain of interconnected neurons initiating from the fast oscillatory cells, which trigger pattern generating neurons that, in turn, excite the dorsal and ventral motoneurons. Mechanosensory stimulation onto the body wall produces in each segmental ganglion the activation of the swimming network.

In animals with a cut between cephalic and first segmental ganglion, tactile or light electrical stimulation of the skin produces constantly a swim cycle with a predictable latency between the application of the stimulus and the onset of the response.

Low frequency (1/min) repeated stimulation induces a progressive increase in latency (habituation) (Fig. 1A, open squares). Nociceptive stimuli brought onto various segments of the body surface give rise to a rapid shortening of the latency, either in the habituated (dishabituation) or in non habituated response (sensitization). This facilitation of latency lasts up to 1 hour. The investigation performed in this model has provided the following useful information: 1) 5HT blocking agent methysergide application inhibits the behavioral dishabituation; 2) 5HT injection into the whole animal mimics the dishabituation and sensitization; 3) treatment with 5,7-DHT, a neurotoxic agent of 5HT neurons, abolishes the sensitization; 4) an adenylate cyclase blocking agent RMI 12330A blocks the sensitization.

These data taken together suggest two major conclusions:

a) 5HT mediates short-term changes underlying sensitization; *b*) the potentiation accompanying sensitization is mediated by second messengers of nucleotide type.

The analogy with the *Aplysia* model is remarkable: they do share a common framework.

In order to evaluate whether cellular and molecular mechanisms underlying habituation and sensitization of swim induction in this model are similar to those ones found out in *Aplysia*, several experiments have been performed. The analysis of the cascade of molecular events subserving these short term changes has shown that additional mechanisms are present in the leech nervous system.

The investigation has been carried out onto the first cellular station of the circuitry of the swim represented by the mechanosensory neurons located in each segmental ganglion.

IV. *Molecular mechanism of non associative learning in the leech: modulation of an electrogenic pump.*

In each segmental ganglion of the leech three types of sensory neurons have been identified: T (touch), P (pressure) and N (nociceptive) cells (50).

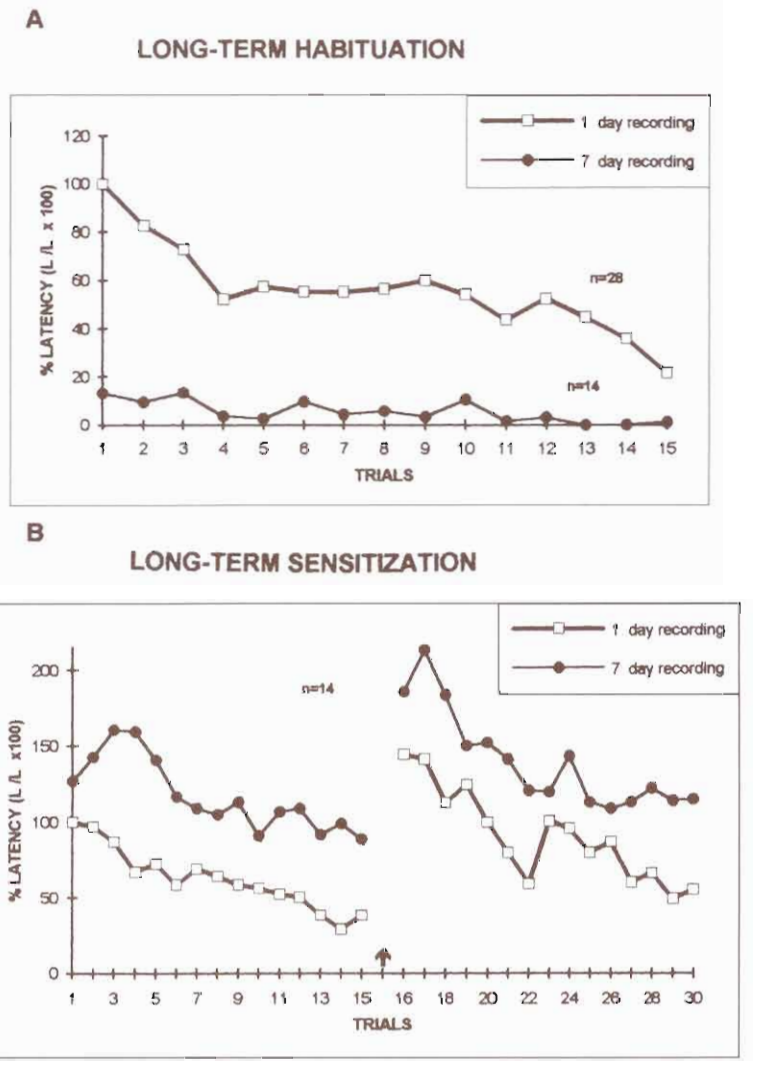

Fig. 1. - *Long term habituation and sensitization in the swim induction behavioral model in the leech.*

In animals with a cut between the cephalic and the first segmental ganglion swimming activity is triggered by electrical stimulation of the caudal portion. The latency between the starting of the stimulus and the onset of a swim cycle has been measured. The latency value of the first response has been taken as 100% and all the following responses have been normalized to the first one. The values in the ordinate represent the reciprocal of the latency measurements. The stimulation has been applied at low frequency (1/min). The curves on the top (A) show the results of experiments on LT habituation. In the first day of recording repetitive stimulation induces a gradual increase of the latency (open squares graph). Daily habituating session led to a long lasting and consistent increase of the latency as shown by black circles curve.

The curves on the bottom (B) illustrate the results obtained from animals which received daily sensitizing stimuli. The curve with open squares represents the recordings made on the first day, whereas the curve with black circles reports the data collected after seven days of application of sensitizing stimuli. This curve is higher than that one of the first day (LT sensitization). The graphs on the right represent the short term dishabituation of both the first day and the seventh day.

A burst of action potentials elicited in these neurons produces a lasting afterhyperpolarization (AHP) that in T neuron is mainly due to an electrogenic pump and in a less extent to an increase in gK^+/Ca^{2+} while in P and in N neurons it is prevalent the Ca^{2+}-dependent K^+ current. One of the major function of AHP is to produce a conduction block at the branching points where small axonal processes join the main neurite (7, 68).

At the aim of enlightening the cellular mechanisms underlying sensitization in a first group of experiments, Belardetti *et al.* (9) have observed that 5HT application depresses, for a prolonged period of time (30 up to 60 min) the AHP amplitude in T neurons. The effect is reversible and is blocked by methysergide. Similar effects have been obtained following the electrical activation of Retzius cells. The effect is not observed in P and in N neurons.

Later two groups of experiments have been worked out (21). In the first one the K^+/Ca^{2+}-dependent channel has been blocked either with $BaCl_2$ or $CdCl_2$. In these experimental conditions 5HT is still capable of further depressing AHP. In a second series of investigations it has been studied the effect of 5HT after direct activation of the Na^+/K^+ electrogenic pump. The activation has been established by means of two different experiments: 1) intracellular injection of Na^+ into T sensory neurons produces an ouabain-sensitive hyperpolarization for ATPase stimulation. Perfusion with 5HT generates a depolarization because the ions could not be driven out by the block of the pump. 2) 5HT application brings about a decrease in the repolarization normally obtained with Na^+/K^+ pump activator Cs^+ after "K^+ free" perfusion.

Changes in the AHP amplitude produce a control of the traffic of impulses travelling along the sensory fibers. When the AHP amplitude decreases, a relief from block in branching points occurs and more action potentials are transported along the neurites and more synapses are invaded by the impulses.

The feature of this scheme is more sophisticated than the *Aplysia* model. In the leech nervous system by controlling specific loci where the fibers are branching it is possible to modulate a synaptic territory below the branching points. Subsequently it has been established that cAMP enhancement represents the intermediate step which bridges neurotransmitter action with the inhibition of the Na^+/K^+ electrogenic pump.

This emerges from the recent findings of Catarsi *et al.* (22): 1) application of 5HT in the presence of RMI 12330A does not reduce the AHP amplitude; 2) application of cAMP analogs, 8Br-cAMP, and phosphodiesterase inhibitor IBMX, provokes a reversible reduction of the AHP amplitude; 3) intracellular iontophoretic application of cAMP produces a clear-cut reduction of the AHP amplitude; 4) intracellular injection of Na^+ ions activates the pump and generates an hyperpolarization of the resting potential. In presence of 8Br-cAMP and IBMX the hyperpolarizing effect disappears and a depolarization arises; 5) the physiological relevance of cAMP-dependent phosphorylation, triggered by 5HT is further demonstrated by neurochemical experiments in which it has been demonstrated that 5HT raises cAMP levels in the leech CNS and activates cAMP-dependent protein kinase (13).

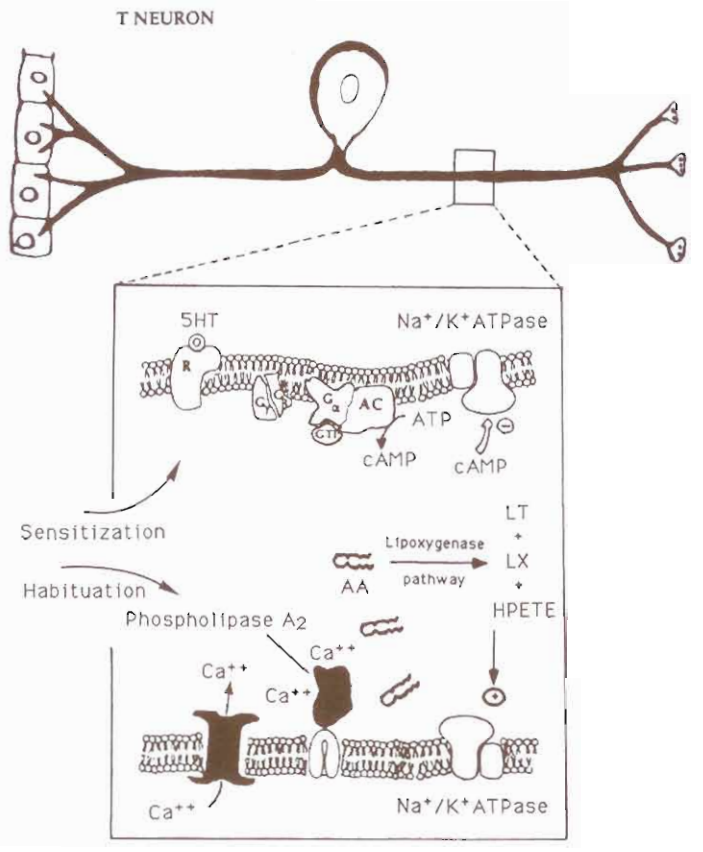

Fig. 2. - *A schematic drawing of the molecular machinery for plastic changes operating inside the mechanosensory T neuron in the leech ganglion.*

Low frequency repetitive intracellular stimulation of a T sensory cell produces (after 15 trials) an increase of the amplitude of the AHP.

The burst of action potentials activates a L-type Ca^{2+} channel which triggers the lipoxygenase pathway of arachidonic acid whose metabolites in turn activate the Na^+/K^+ electrogenic pump. These events lead to the increase in AHP amplitude and to a block of impulses travelling along the neurite. As a consequence there is a reduction of efficacy of synaptic contacts with the follower cells.

In contrast, 5HT (or substances acting in the same way) binds a receptor that by means of G proteins increases the cAMP level and through PKA produces an inhibition of the Na^+/K^+ electrogenic pump. The reduction of AHP, which facilitates the traffic of impulses along the T axon, is set up.

A group of proteins of 79 kDa was phosphorylated in the presence of 5HT, 8Br-cAMP and phorbol ester, a protein kinase C activator (32, 33, 34)(Fig. 2).

More recently it has been demonstrated that low rate intracellular stimulation (L.R.I.S.) of T cell produces after 15 stimuli a lasting increase of AHP amplitude. This effect is still present when the gK^+/Ca^{2+} is blocked by $CdCl_2$. Application of NDGA, a blocking agent of the lipoxygenase pathway, or nifedipine, a blocking substance of Ca^{2+} channel, inhibits the AHP potentiation. In addition, the perfusion with 5HT prevents the increase of the AHP after repetitive stimulation. On the

contrary, it is still possible to obtain AHP increase after application of indomethacin, a blocking agent of cycloxygenase pathway. These data demonstrate that the increase in AHP amplitude after L.R.I.S. is due to a potentiation of the Na^+/K^+ pump, is triggered by voltage-dependent L-type Ca^{2+} channels and is mediated by lipoxygenase metabolites of arachidonic acid. The increase in AHP amplitude may provide an interesting cellular mechanism underlying the habituation: it might block the traffic of action potentials along the sensory fibers, thus decreasing the synaptic efficacy of T neuron (Fig. 2).

These findings have singled out a completely novel mechanism: the modulation of an electrogenic pump which is responsible for non associative learning processes. In this model the plastic changes are correlated to biophysical properties of single neurons.

The investigations performed on other invertebrate models have emphasized changes in the synaptic connections between sensory cells and their target neurons; in this system each sensory neuron has in its molecular machinery the capabilities to exhibit plastic changes when the adaptive mechanisms require changes in behavior.

It is reasonable to suppose that it exists a kind of hierarchy in the neuronal elements regarding their capabilities to be plastic. It is possible that more than one basic mechanism works in the regulation of learning process, but one of them might be the presence of "specific learning neurons" which change their functioning in relationship with adaptive situations and store this modification and translate it in a message (biophysical or biochemical) that leads to changes in the circuitry in which is operating.

Are the effects observed in the electrophysiological and neurochemical experiments the real mechanisms operating during the changes in behavior underlying non associative learning? To give an answer to this question behavioral experiments have been performed in the leech.

First of all it has been observed that ouabain injected in the whole animal prevents the dishabituation; in addition 5HT effect of sensitization is blocked by preincubation with RMI 12330A. These behavioral findings are in accordance with data gained at electrophysiological and neurochemical experiments (Fig. 2): 5HT, through the cAMP increase, would inhibit the Na^+/K^+ ATPase in the sensory neuron membrane, reducing the AHP, relieving the block in the branching points and allowing the increase of traffic of action potentials which can potentiate many synaptic fields, facilitating the interconnections between sensory cells and follower neurons.

By utilizing the experimental model of swim induction we have demonstrated that the leeches subjected to repetitive tactile or electrical stimulation exhibit habituation; this reduction of the response to tactile stimulation disappears when nifedipine is injected into the animals. In addition, to determine the involvement of arachidonic acid metabolites, experiments with specific blockers have been carried out. Injection of the cycloxygenase inhibitor indomethacin does not produce any change in the rate of habituation, while injection of a lipoxygenase blocker, NDGA, strongly affects the habituation curve (Fig. 2).

Application of 5HT through cAMP causes a prolonged reduction in the electrotonic coupling between the two Retzius cells. The effect is reversible and is present also with high Mg^{2+} solution, suggesting that the monoamine acts directly on the gap junction which bridges the two cells (23). Thus, 5HT would modulate, for lasting period of time, the electrical synapses. It is still unknown whether this effect of modulation of ephaptic connection after neurotransmitter application is generalized and widespread or is restricted to specific synapses. Further investigations are needed to elucidate the role of these plastic changes in non associative learning processes.

V. *Long-term learning process and enduring memory.*

It is a general knowledge that retention of learned information has two major components: short-term (ST) learning process lasting minutes to hours and long-term (LT) learning process in which the changes remain for days or weeks or longer. Behavioral and cellular studies performed in *Aplysia* have suggested that short-term and long-term memory are two stages of a graded process. They share many common peculiarities but have also distinctive features.

What are the set of events which are involved in the genesis of persistent engrams? One of the most suggestive hypothesis is represented by the idea that in LT memory a bunch of newly synthesized proteins is activated keeping stabilized the mechanisms which operate in short-term memory. Many researches were oriented to perform experiments in this direction and it has been demonstrated that inhibitors of messenger RNA or protein synthesis may block or disrupt the long-term modifications, leaving the short-term unchanged. This occurs when the inhibitors are administered 1 or 2 hours after training (5, 54). But many of the results obtained in the past are questionable for the difficulty to control all the variability of the studied system. Only recently the withdrawal reflex system studied in *Aplysia* has exhibited powerful experimental advantages. The monosynaptic sensory-motor connection of the reflex has been rebuilt in culture.

Several successive applications of 5HT within about two hours result in a potentiation of the EPSP recorded in the motoneurons 24 hours later. This is regarded as long-term changes (49). Inhibitors of protein synthesis (anisomycine and emetine) and inhibitors of mRNA synthesis (actinomycin D and α-amanitine) block LT sensitization without affecting the ST one (49). There is a critical "time window" for the inhibitory action; the drugs are effective when applied during the serotonin treatment. This "time window" is present also in various forms of memory in invertebrate and perhaps also in man. In addition parallel experiments have been performed at behavioral level. A single trial (or a single application of 5HT) given every day for one week produces a sensitization that persists for a week or longer. In both cellular and behavioral approach the long-term process of sensitization resembles the short-term one in several features: *a*) there are changes in synaptic strength at the same locus: the sensory motoneuron connection; *b*) there is an increase in transmitter release; *c*) there is a modulation of K_s type channels; *d*) it can be induced by the same transmitter (5HT) and second messenger (cAMP) (25, 26, 59, 60, 61).

These similarities result from the fact that 5HT leads to phosphorylation of some of the proteins which are involved in the ST process. This phosphorylation is persistent in LT and seems caused by a decrease in the amount of regulatory subunit of protein kinase A, which remains long active (11, 12, 36). While the ST does not need neoformation of substances, but is generated by modifications of a preexisting proteins through a mechanism of phosphorylation, LT depends on macromolecular synthesis.

Recent experiments (27) have been performed in which a sequence of somatostatine CRE (a cAMP responsive element) has been injected into the nucleus of sensory neuron (in culture with motoneurons).

These molecular studies indicate that with repeated training (or repeated application of 5HT) the cAMP-dependent protein kinase (PKA) induces phosphorylation of a transcriptional activator which binds CRE. This, in turn, binds a protein, CREBP (CRE binding protein), which increases transcription when phosphorylated by PKA. These events lead to the activation of two classes of proteins which have distinct functions: one protein, an ubiquitine hydrolase, is a component of a specific protease that induces a down-regulation of the regulatory subunit which brings about the kinase to be persistently active, even though the level of cAMP has returned to its basal value. The second set of proteins is important for the growth of active zones and the development of new synaptic connections. Bailey and Chen (3, 4) have shown that in sensitized animals: *a*) the number of synaptic terminals of the sensory neurons increases significantly, so as the number of the postsynaptic receptive area of the motoneurons; *b*) the active zones of sensory terminal increase; *c*) the total number of vesicles associated at the releasing pool in sensory terminal is enhanced; *d*) the dendrites of motoneuron grow to link the novel additional synaptic increment. In addition, it has been demonstrated that in LT trained preparations a reduction of adhesion proteins of N-CAM type, which usually maintain rigid and solid the synaptic contact, has been observed; this down-regulation might induce a decrease of the synaptic knobs in order to rearrange the synaptic array and to allow accommodation of the structural changes. By means of low light level fluorescent videomicroscopy which permits the visualization of a living neuron structure repetitively over the course of several days, it has been demonstrated that repeated application of 5HT produces an increase in the number of processes or varicosities of sensory neurons. In this preparation the presence of motoneurons is necessary.

Morphological changes also occur during long-term plasticity in *Hermissenda* and in crayfish (1). In these cases as well, the sites involved in short-term plasticity are also used for long-term change, and the long-term process requires new protein synthesis (24, 51). This LT process appears similar to that one operating during development and maturation. During the development the neurons grow and take contacts with target cells generating the synaptic networks with tight ultrastructural connections. Once reached the maturation those mechanisms are stopped and kept silent. When a learning process arises, through the activation of phosphoproteins, a chain of events in the genoma produces temporarly activation of the factors which are active during the development, likely by means of the block of a

repressor which inhibits the development. This theory exhibits undoubtedly a beautiful unity of the cellular processes which are functioning both during learning and during growth. In this sense, the formation of a stable engram of memory might be analogous to what is occurring during developmental process. But a criticism cannot be neglected. Almost all the investigations have been carried out "in vitro", in neuronal networks made up by cells in developing stages. A doubt exists that the structural changes and the synaptic modifications observed might be correlated mostly with the developing or rearranging processes rather than with the stabilization of engrams. For this reason many data deserve confirmation in alternative models.

Experiments performed in *Aplysia* have shown short term behavioral depression of the gill-withdrawal reflex upon stimulation of the tail (47). This effect is due to a presynaptic inhibition of the sensory neurons by inhibitory interneurons which release a neuropeptide, FMRFamide (64). Voltage-clamp and patch-clamp analyses in sensory neurons have demonstrated that FMRFamide brings about an increase of the K_s current (10, 16) and a decrease in the dihydropyridine-insensitive N-type calcium current (28). These effects lead to a decrease of total Ca^{2+} influx in the terminal. FMRFamide direct action onto K_s is mediated by a pertussis toxin-sensitive G protein and is mediated by arachidonic acid metabolites of the lipoxygenase enzymatic pathway, such as 12-HPETE (53). FMRFamide also antagonizes the increase in protein phosphorylation by 5HT or exogenous application of cAMP analogues, either by activating a phosphatase or inhibiting a protein kinase (42, 66).

In contrast to sensitization, a few data are available on LT habituation. It has been observed a reduction of the number of terminals per neuron (*pruning*) (4).

In order to clarify the molecular cascade of events which bridges ST to LT processes, several experiments on LT habituation and sensitization in the behavioral model of swim in the leech have been performed.

By applying every day a session of stimulation it is possible to obtain a LT habituation: after 4-5 days the latency between the starting of the stimulus and the onset of the response increases, reaching the maximum after 7-9 days and persists for some weeks. In a similar manner, it is possible to induce LT sensitization by stimulating the animals with daily nociceptive stimulation for 7 consecutive days: the experimental group exhibits a shortening of the latency for inducing swim that is markedly lower than both that one of the control group and that one measured on the first day (Fig. 2). This potentiation persists for several days with a slow gradual comeback to the normal latency. It is possible to obtain a kind of "massive potentiation" by applying the same amount of stimuli at regular intervals just only in 24 hours instead of in 7 days.

Inhibitors of protein synthesis (cycloheximide) and mRNA blocking agents (actinomycin D) block significantly the LT facilitation of swimming induction whereas the ST modifications were unchanged. Analogous treatments with protein synthesis and mRNA inhibitors (cycloheximide, actinomycin D and emetine) have been carried out for LT habituation: also in this case the inhibitors block the

formation of the LT learning process. In preliminary experiments with a guanylate cyclase inhibitor, LY-83583, it has been demonstrated an inhibition of the rate of habituation. This finding opens a new field of investigation: to explore the possibility that messengers of cGMP type may trigger the molecular cascade that leads to the formation of transcriptional regulators which activate genes whose protein products have LT consequences in habituation.

The data obtained in the leech confirm and extend the framework of the *Aplysia* model, even if many steps of cellular and molecular mechanisms of the two systems differ substantially.

Following the unitary theory of growth and learning, it is only possible to speculate about the changes occurring during LT processes in the leech. One possible explanation is provided by the neurogenesis: when LT is building up, a sustained change of the branchings of the neuritic tree occurs. In addition (or in alternative) to these structural modifications some other putative hypotheses can be exploited:

A) *for habituation*: 1) during the repetitive sessions a peptide or some other substances can be released, exerting the potentiation of AHP; 2) protein phosphorylation, through cAMP mediation, can trigger the activation of tubulin and fibrillogenesis which increase the branching points and therefore the passage of impulses; 3) an increase of autoreceptors binding the neurotransmitters released by the sensory neurons themselves.

B) *For sensitization*: 1) the formation of substances which keep depressed the AHP of sensory neurons or keep inactivated the phosphatase complex; 2) a substance which potentiates the electrical coupling among the sensory cells so that they can work as a single unit.

The putative formation of one or more proteic substances which can exert multiple actions, represents an appealing hypothesis which deserves more appropriate investigations. This or these substances may act at different levels: *a*) intracellularly by controlling the gating of ionic channels, or the biochemical regulation of phosphorylation of molecules important for the reorganization of various branching, or changing in vesicles formation and release, or in modification of active zones; *b*) extracellularly by the formation of a peptide which spreads around to inform neighbouring cells in order to synchronize synergically the activity of a common functional cellular pool.

It is also possible that during LT second light messengers like NO or prostaglandines generate a bridge among various cells and trigger either the modulation of synaptic efficacy or the control of electrogenic pump in order to transform the plastic changes originated in one "memory neuron" in a diffusible message that can form a kind of synchronous concert of cell population functioning as a whole.

VI. *Associative learning.*

The siphon and gill-withdrawal reflex of *Aplysia* can be enhanced by classical conditioning as well as sensitization. These reflexes can be elicited, according to the classical paradigms, by stimulating two separate spots of the siphon area which

are separately innervated by distinct populations of sensory neurons. By pairing a stimulus to an appropriate area with an unconditioned one, like a strong shock on the tail, the response of the conditioned pathway is significantly stronger than that one of the unconditioned pathway. Timing is critical for this associative learning. The conditioned stimulus (CS) must precede, of a critical interval, the unconditioned stimulus (US) (39). Investigations on this model have suggested a mechanism of amplification of presynaptic facilitation as responsible for classical conditioning. US on the tail activates interneurons which interact at the level of sensory neurons with CD originating presynaptic facilitation.

If the two stimuli are timed so that the interneurons are activated by the US immediately after the sensory neurons begin to fire in response to CS, a greater facilitation is produced. This property has named activity-dependence.

The molecular mechanism of this activity-dependent facilitation might be outlined as follows: Ca^{2+} which enters into the sensory terminal binds calmodulin and potentiates the activation of the adenylate cyclase or other modulators. This, in turn, generates much more cAMP and, as a consequence, more facilitation of transmitter release. Another interesting model for studying associative learning at genetic level is that one of the fruit fly *Drosophila*. This insect can be conditioned in an avoidance response test to recognize different odors. Single-gene mutants deficient in learning have been isolated. They are called "dunce", "rutabaga" and "amnesiac". They have been studied in detail showing interesting features: 1) all of the mutants that do not exhibit classical conditioning also fail to show sensitization; 2) all three mutants have a defect in cAMP cascade.

The "dunce" mutant lacks a phosphodiesterase, an enzyme that degrades cAMP. Therefore this mutant has abnormally high levels of cAMP. The "rutabaga" mutant has a defect in the Ca^{2+}/calmodulin-dependent adenylate cyclase and a low basal level of cAMP. The "amnesiac" mutant lacks a peptide which controls the activity of adenylate cyclase (31). These animals learn normally, but forget rapidly. These findings give new interest to the role of peptides in memory store. The gene "amnesiac" encodes a novel neuropeptide homologous to mammalian pituitary adenylate cyclase-activating peptide (PACAP), member of a family of polypeptide hormones that includes VIP and others. PACAP appears to interact with two types of seven transmembrane-domain receptors: one is positively coupled with adenylate cyclase, and recognizes VIP, too; the other is linked to phospholipase C and to adenylate cyclase (37, 65). PACAP increases not only cAMP but also intracellular Ca^{2+} concentration and promotes neurite outgrowth. Thus, what emerges from studies on *Aplysia* and *Drosophila* indicates that cAMP cascade is the fundamental mechanism also for elementary forms of associative learning.

In the leech, associative learning has been reliably demonstrated (57), mostly on the shortening model induced by either photic or tactile stimulation. The association between tactile stimuli and electrical shocks occurs also in a semi-intact preparation, which is amenable to simultaneous cellular analysis and behavioral measurements of the shortening reflex. 5HT plays an important role in the expression of this associative learning in the leech as well (58). After the application of the neurotoxin

5,7-DHT which destroys 5HT neurons, a significant impairment of conditioning occurs, even if a residual positive performance in learning survives (58).

This finding suggests that: 1) learning-dependent modifications of neural circuits emerge as potentially important mechanisms for learning and 2) associative learning involves more than one elaboration of sensitization: there are mechanisms 5HT-independent and sensitization-independent.

This short trip along the studies executed on single forms of learning processes in elementary animal models provides a fantastic new insight in the neurobiology of memory. A survey of the data collected from various models singles out some basic principles which can represent the elementary cellular mechanism subserving plastic changes: 1) one solid point is that in many models it has been observed that synaptic changes in efficacy certainly play a fundamental role in both non associative and associative learning. But the experiments performed in the leech have demonstrated that inside a single specific neuron is present a molecular machinery which can adapt the activity of the cell to the environmental stimuli and integrate them in the network in order to build up lasting behavioral changes. In this view the specific neural cells are capable of learning by themselves. We can speak about "learning neurons". All the changes in the activity of the cells are translated in a modulation of the synaptic activity that links the neuron with its followers. 2) The role of 5HT is widespread in all kind of studied learning. This transmitter is a strong modulator of lasting sensitizing actions. 3) cAMP is the ubiquitary intracellular messenger that, leading to PKA activation and protein phosphorylation, represents the simplest molecular alphabet for plastic changes. 4) The protein phosphorylation seems to be present in all the short-term learning processes. 5) In various models (e.g. *Aplysia* and *Hermissenda*) the modulation of K^+ channels and Ca^{2+} channels represents the basic mechanism. In leeches the modulation of the membrane electrogenic pump is the pivotal point for all the plastic modifications. This new finding enlarges the capabilities of cells to change their activity. 6) In long-term plastic changes the most important finding is that newly synthesized proteins are formed. It is possible that one or more of these proteins might be a peptide which spreads around acting as an extracellular messenger informing the neighbouring cells.

Finally one has to consider whether the more complex implicit or explicit memory is built up by networks in which the basic principles observed (or still unknown) in invertebrates are assembled together in each complex circuitry underlying complex learning processes. Novel mechanisms are set up just adapting the environmental stimulation to the activity of the neurons. It is likely that in all complex languages single letters of the alphabet lose their unicity to form a new word with its specific meaning, also in the learning process the molecular mechanisms are invariant along the phylogeny, but when they are assembled together build something new.

In conclusion, it is clear that in the memory process exists a molecular alphabet represented by the linkage neurotransmitter-chain of second messengers, modification of function of cellular membrane or changes in genomic factors. But the real

expression of such mechanisms can vary from different systems in relationship to specific various adaptive mechanisms that all the species have to set up in the continuous dialogue with the environment and also in consideration of the increasing complexity of the behavioral act of the higher vertebrates.

SUMMARY

An increasing flow of evidences collected on elementary forms of learning processes in selected animal models evidentiates some mechanisms which can represent the basic cellular principles underlying plastic changes:

1. 5HT and second messengers of nucleotide type (like cAMP) have a pivotal role in the learning process.
2. In almost all short-term learning processes the modifications are subserved by a mechanism of protein phosphorylation.
3. In various animal models the modulation of K^+ and Ca^{2+} channels is the molecular mechanism for learning. Experiments performed in sensory T neuron of the leech indicate that the modulation of Na^+/K^+ electrogenic pump is one of the fundamental mechanism for learning.
4. In long-term plastic changes, the most important finding is that newly synthesized proteins are formed.
5. In addition to what has been observed in the *Aplysia* model, where changes in synaptic efficacy represent the basic principles of memory storage, in the leech it has been demonstrated that a molecular machinery present in a single neuron can adapt the activity of the cell to environmental stimuli.

REFERENCES

1. Alkon, D. L., Ikeno, H., Dworkin, Mcphie, D. L., Olds, J. L., Lederhendler, I., Matzel, L., Schreurs, B. G., Kuzirian, A., Collin, C. and Yamoah, E. Contraction of neuronal branching volume: an anatomic correlate of Pavlovian conditioning. *Proc. Natl. Acad. Sci. USA*, **87**: 1611-1614, 1990.
2. Bagnoli, P., Brunelli, M., Magni, F. and Pellegrino, M. The neuron of the fast conducting system in *Hirudo medicinalis*: identification and synaptic connections with primary afferent neurons. *Arch. Ital. Biol.*, **113**: 21-43, 1975.
3. Bailey, C. H. and Chen, M. Long-term memory in *Aplysia* modulates the total number of varicosities of single identified sensory neurons. *Proc. Natl. Acad. Sci. USA*, **85**: 2372-2377, 1988.
4. Bailey, C. H. and Chen, M. Long-term sensitization in *Aplysia* increases the number of presynaptic contacts onto the identified gill motor neuron L7. *Proc. Natl. Acad. Sci. USA*, **85**: 9356-9359, 1988.
5. Barondes, S. H. and Jarvik, M. E. The influence of Actinomycin D on brain RNA synthesis and on memory. *J. Neurochem.*, **11**: 187-195, 1964.
6. Baxter, D. A. and Byrne, J. H. Serotonergic modulation of two potassium currents in the pleural sensory neurons of *Aplysia*. *J. Neurophysiol.*, **62**: 665-679, 1989.

7. Baylor, D. A. and Nicholls, J. G. After effects of nerve impulse on signaling in the central nervous system of the leech. *J. Physiol. Lond.*, **203**: 571-589, 1969.
8. Belardetti, F., Biondi, C., Colombaioni, L., Brunelli, M., and Trevisani, A. Role of serotonin and cAMP on facilitation of the fast conducting system activity in the leech *Hirudo medicinalis*. *Brain Res.*, **246**: 89-103, 1982.
9. Belardetti, F., Brunelli, M., Demontis, G. C. and Sonetti, D. Serotonin and Retzius cell depress the hyperpolarization following impulses of the leech touch cell. *Brain Res.*, **300**: 91-102, 1984.
10. Belardetti, F., Kandel, E. R. and Siegelbaum, S. Neuronal inhibition by the peptide FMRFamide involves opening of S K^+ channel. *Nature*, **325**: 153-156, 1987.
11. Bergold, P. J., Sweatt, J. D., Winicov, I., Weiss, K. R., Kandel, E. R. and Schwartz, J. H. Protein synthesis during acquisition of long-term facilitation is mediated for the persistent loss of regulatory subunits of the *Aplysia* cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA*, **87**: 3788-3791, 1990.
12. Bergold, P. J., Beushausen, S. A., Saktor, T. C., Cheley, S., Bayley, H. and Schwartz, J. H. A regulatory subunit of the cAMP-dependent protein kinase down-regulated in *Aplysia* sensory neurons during long-term sensitization. *Neuron*, **8**: 387-397, 1992.
13. Biondi, C., Belardetti, F., Brunelli, M., Portolan, A. and Trevisani, A. Increased synthesis of cAMP and short term plastic changes in the segmental ganglia of *Hirudo medicinalis*. *Cell Molec. Neurobiol.*, **2**: 81-92, 1982.
14. Boulis, N. M. and Sahley, C. L. Behavioral analysis of habituation and sensitization of shortening in the semi-intact leech. *J. Neurosci.*, **8**: 4621-4627, 1988.
15. Braha, O., Dale, N., Hochner, B., Klein, M., Abrams, T. W. and Kandel, E. R. Second messengers involved in the two processes of presynaptic facilitation that contribute to sensitization and dishabituation in *Aplysia* sensory neurons. *Proc. Natl. Acad. Sci. USA*, **87**: 2040-2044, 1990.
16. Brezina, V., Eckert, R. and Erxleben, C. Modulation of potassium conductances by an endogenous neuropeptide in neurons of *Aplysia californica*. *J. Physiol. Lond.*, **382**: 267-290, 1987.
17. Brunelli, M., Castellucci, V. F. and Kandel, E. R. Synaptic facilitation and behavioral sensitization in *Aplysia*: possible role of serotonin and cAMP. *Science*, **194**: 1178-1181, 1976.
18. Castellucci, V. F., Pinsker, H., Kupfermann, I. and Kandel, E. R. Neuronal mechanism of habituation and dishabituation of the gill-withdrawal reflex in *Aplysia*. *Science*, **167**: 1745-1748, 1970.
19. Castellucci, V. F. and Kandel, E. R. A quantal analysis of the synaptic depression underlying habituation of the gill-withdrawal reflex in *Aplysia*. *Proc. Natl. Acad. Sci. USA*, **71**: 5004-5008, 1974.
20. Castellucci, V. F. and Kandel, E. R. Presynaptic facilitation as a mechanism for behavioral sensitization in *Aplysia*. *Science*, **194**: 1176-1178, 1976.
21. Catarsi, S. and Brunelli, M. Serotonin depresses the afterhyperpolarization through the inhibition of the Na^+/K^+ electrogenic pump in the sensory neurons of the leech. *J. exp. Biol.*, **155**: 261-273, 1991.
22. Catarsi, S., Scuri, R. and Brunelli, M. Cyclic AMP mediates inhibition of the Na^+/K^+ electrogenic pump by serotonin in T sensory neurons of the leech. *J. Physiol., Lond.*, **462**: 229-242, 1993.
23. Colombaioni, L. and Brunelli, M. Neurotransmitter-induced modulation of an electrotonic synapse in the central nervous system of *Hirudo medicinalis*. *Exp. Biol.*, **47**: 139-144, 1988.
24. Crow, T. and Forrester, J. Inhibition of protein synthesis blocks long-term enhance-

ment of generator potentials produced by one-trial in vivo conditioning in *Hermissenda*. *Proc. Natl. Acad. Sci. USA*, **87**: 4490-4494, 1990.

25. DALE, N., KANDEL, E. R., and SCHACHER, S. Serotonin produces long-term changes in the excitability of *Aplysia* sensory neurons in culture that depend on new protein synthesis. *J. Neurosci.*, **7**: 2232-2238, 1987.
26. DALE, N., SCHACHER, S. and KANDEL, E. R. Long-term facilitation in *Aplysia* involves increase in transmitter release. *Science*, **239**: 282-285, 1988.
27. DASH, P. K., HOCHNER, B. and KANDEL, E. R. Injection of cAMP-responsive element into the nucleus of *Aplysia* sensory neurons blocks long-term facilitation. *Nature*, **345**: 718-721, 1990.
28. EDMONDS, B., KLEIN, M., DALE, N. and KANDEL, E. R. Contribution of two types of calcium channels to synaptic transmission and plasticity. *Science*, **250**: 1142-1147, 1990.
29. ELIOT, L. S., BLUMENFELD, H., EDMONDS, B. W., KANDEL, E. R. and SIEGELBAUM, S. A. Imaging $[Ca]_i$ transients at *Aplysia* sensorimotor synapses: contribution of direct and indirect modulation to presynaptic facilitation. *Soc. Neurosci., Abstr.*, **17**: 1485, 1991.
30. EHRLICH, J. S., BOULIS, N. M., KARRER, T. and SAHLEY, C. L. Differential effects of serotonin depletion on sensitization and dishabituation in the leech *Hirudo medicinalis*. *J. Neurobiol.*, **23**: 270-279, 1992.
31. FEANY, M. B. and QUINN, W. G. A neuropeptide gene defined by the *Drosophila* memory mutant amnesiac. *Science*, **268**: 869-873, 1995.
32. GARCIA-GIL, M., TONGIORGI, E., CATTANI, M., CIPOLLINI G. and BRUNELLI, M. Cyclic AMP dependent phosphorylation of a 79 kDa protein in segmental ganglia of the leech *Hirudo medicinalis*. *Neurochem. Int.*, **15**: 161-168, 1989.
33. GARCIA-GIL, M., BERTON, F., TONGIORGI, E. and BRUNELLI, M. Effects of cyclic nucleotides and calcium/calmodulin on protein phosphorylation in the CNS of *Hirudo medicinalis*. *J. Neurochem.*, **52**: 699-704, 1989.
34. GARCIA-GIL, M., BOTTAI, D., ZACCARDI, M.L., CANNIZZARO, M. and BRUNELLI, M. Effect of serotonin on protein phosphorylation in the central nervous system of the leech. *Comp. Biochem. Physiol. C*, **104**: 125-131, 1993.
35. GHIRARDI, M., BRAHA, O., HOCHNER, B., MONTAROLO, P. G., KANDEL, E. R. and DALE, N. E. The contributions of PKA and PKC to the presynaptic facilitation of evoked and spontaneous transmitter release at depressed and nondepressed synapses in the sensory neurons of *Aplysia*. *Neuron*, **9**: 479-489, 1992.
36. GREENBERG, S. M., CASTELLUCCI, V. F., BAYLEY, H. and SCHWARTZ, J. H. A molecular mechanism for long term sensitization in *Aplysia*. *Nature*, **329**: 62-65, 1987.
37. HASHIMOTO, H., ISHIHARA, T., MORI, K. and NAGATA, S. Molecular cloning and tissue distribution of a receptor for Pituitary Adenylate Cyclase-Activating Polypeptide. *Neuron*, **11**: 333-342, 1993.
38. HAWKINS, R. D., CASTELLUCCI, V. F. and KANDEL, E. R. Interneuron involved in mediation and modulation of gill-withdrawal reflex in *Aplysia*. II. Identified neurons produce heterosynaptic facilitation contributing to behavioral sensitization. *J. Neurophysiol.*, **45**: 315-326, 1981.
39. HAWKINS, R. D., ABRAMS, T. W., CAREW, T. J. and KANDEL, E. R. A cellular mechanism of classical conditioning in *Aplysia*: activity-dependent amplification of pre-synaptic facilitation. *Science*, **219**: 400-405, 1983.
40. HOCHNER, B., KLEIN, M., SCHACHER, S. and KANDEL, E. R. Additional component in the cellular mechanism of presynaptic facilitation contributes to behavioral dishabituation in *Aplysia*. *Proc. Natl. Acad. Sci. USA*, **83**: 8794-8798, 1986.
41. HOCHNER, B. and KANDEL, E. R. Modulation of a transient K^+ current in the pleural

sensory neurons of *Aplysia* by 5-HT and cAMP: implications for spike broadening. *Proc. Natl. Acad. Sci. USA*, **89**: 11476-11480, 1992.

42. Ichinose, M. and Byrne, J. H. Role of protein phosphatase in the modulation of neuronal membrane currents. *Brain Res.*, **549**: 146-150, 1991.
43. Klein, M. and Kandel, E. R. Mechanism of calcium current modulation underlying presynaptic facilitation and behavioral sensitization in *Aplysia*. *Proc. Natl. Acad. Sci. USA*, **77**: 6912-6916, 1980.
44. Klein, M., Camardo, J. S. and Kandel, E. R. Serotonin modulates a specific potassium current in the sensory neurons that show presynaptic facilitation in *Aplysia*. *Proc. Natl. Acad. Sci. USA*, **79**: 5713-5717, 1982.
45. Lockery, S. R., Rawlins, J. M. P. and Gray, J. A. Habituation of the shortening reflex in the medicinal leech. *Behav. Neurol.*, **99**: 333-341, 1985.
46. Lockery, S. R. and Kristan, W. B. Jr. Two forms of sensitization of the local bending reflex of the medicinal leech. *J. Comp. Physiol.*, **168**: 165-177, 1991.
47. Mackey, S. L., Glanzman, D. L., Small, S. A., Dyke, A. M., Kandel, E. R. and Hawkins, R. D. Tail shock produces inhibition as well as sensitization of the siphon-withdrawal reflex of *Aplysia*: possible behavioral role for presynaptic inhibition mediated by the peptide Phe-Met-Arg-Phe-NH_2. *Proc. Natl. Acad. Sci. USA*, **84**: 8730-8734, 1987.
48. Mackey, S. L., Kandel, E. R. and Hawkins, R. D. Identified serotonergic neurons LCB1 and RCB1 in the cerebral ganglia of *Aplysia* produce presynaptic facilitation of siphon sensory neurons. *J. Neurosci.*, **9**: 4227-4235, 1989.
49. Montarolo, P. G., Goelet, P., Castellucci, V. F., Morgan, J., Kandel, E. R. and Schacher, S. A critical period for macromolecular synthesis in long-term eterosynaptic facilitation in *Aplysia*. *Science*, **234**: 1249-1254, 1986.
50. Nicholls, J. G. and Baylor, D. A. Specific modulation and receptive fields of sensory neurons in CNS of the leech. *J. Neurophysiol.*, **31**: 740-756, 1968.
51. Nguyen, P. V. and Atwood, H. L. Expression of long-term adaptation of synaptic transmission requires a critical period of protein synthesis. *J. Neurosci.*, **10**: 1099-1109, 1990.
52. Pinsker, H., Kupfermann, I., Castellucci, V. F. and Kandel, E. R. Habituation and dishabituation of the gill-withdrawal reflex in *Aplysia*. *Science*, **167**: 1740-1742, 1970.
53. Piomelli, D., Volterra, A., Dale, N., Siegelbaum, S.A., Kandel, E.R., Schwartz, J.H. and Belardetti, F. Lipoxygenase metaholites of anachidonic acid as second messangers for presynaptic inhibition of *Aplysia* sensory cells. *Nature*, **328**: 38-43, 1987.
54. Rainbow, T. C. Role of RNA and protein synthesis in memory formation. *Neurochem. Res.*, **4**: 297-312, 1979.
55. Rayport, S. G. and Schacher, S. Synaptic plasticity *in vitro*: cell culture of identified *Aplysia* neurons mediating short-term habituation and sensitization. *J. Neurosci.*, **6**: 759-763, 1986.
56. Sacktor, T. C. and Schwartz, J. H. Sensitizing stimuli cause translocation of protein kinase C in *Aplysia* sensory neurons. *Proc. Natl. Acad. Sci. USA*, **87**: 2036-2039, 1990.
57. Sahley, C. L. and Ready, D. F. Associative learning modifies two behaviors in the leech, *Hirudo medicinalis*. *J. Neurosci.*, **8**: 4612-4620, 1988.
58. Sahley, C. L. Serotonin depletion impairs but does not eliminate classical conditioning in the leech *Hirudo medicinalis*. *Behav. Neurosci.*, **108**: 1043-1052, 1994.
59. Schacher, S., Castellucci, V. F. and Kandel, E. R. Cyclic AMP evokes long-term facilitation in *Aplysia* sensory neurons that requires new protein synthesis. *Science*, **240**: 1667-1669, 1988.
60. Scholz, K. P. and Byrne, J. H. Long-term sensitization in *Aplysia*: biophysical correlates in tail sensory neurons. *Science*, **235**: 685-687, 1987.

61. SCHOLZ, K. P. and BYRNE, J. H. Intracellular injection of cAMP induces long-term reduction of neuronal K^+ currents. *Science*, **240**: 1664-1666, 1988.
62. SHUSTER, M. J., CAMARDO, J. S., SIEGELBAUM, S. A. and KANDEL, E. R. Cyclic AMP-dependent protein kinase closes the serotonin-sensitive K^+ channels of *Aplysia* sensory neurons in cell-free membrane patches. *Nature*, **313**: 392-395, 1985.
63. SIEGELBAUM, S., CAMARDO, J. S. and KANDEL, E. R. Serotonin and cAMP close single K^+ channels in *Aplysia* sensory neurons. *Nature*, **299**: 413-417, 1982.
64. SMALL, S. A., COHEN, T. E., KANDEL, E. R. and HAWKINS, R. D. Identified FMRF-amide-immunoreactive neuron LP16 in the left pleural ganglion of *Aplysia* produces presynaptic inhibition of siphon sensory neurons. *J. Neurosci.*, **12**: 1616-1627, 1992.
65. SPENGLER, D., WAEBER, C., PANTALONI, C., HOLSBOER, F., BOCKAERT, J., SEEBURG, P. H. and JOURNOT, L. Differential signal transduction by five splice variants of the PACAP receptor. *Nature*, **365**: 170-175, 1993.
66. SWEATT, J. D., VOLTERRA, A., EDMONDS, B., KARL, K. A., SIEGELBAUM, S. A. and KANDEL, E. R. FMRFamide reverses protein phosphorylation produced by 5-HT and cAMP in *Aplysia* sensory neurons. *Nature*, **342**: 275-278, 1989.
67. TRUDEAU, L. E. and CASTELLUCCI, V. F. Contribution of polysynaptic pathways to the mediation and plasticity of *Aplysia* gill and siphon withdrawal reflex: evidence for differential modulation. *J. Neurosci.*, **12**: 3838-3848, 1992.
68. VAN ESSEN, D. C. The contribution of membrane hyperpolarization to adaptation and conduction block in sensory neurons of the leech. *J. Physiol., Lond.*, **230**: 509-534, 1973.
69. ZUCKER, R. S. Crayfish escape behavior and cervical synapses. II. Physiological mechanisms underlying behavioral habituation. *J. Neurophysiol.*, **35**: 621-637, 1972.