Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 8266–8269, December 1985
Neurobiology

Monosynaptic connections made by the sensory neurons of the gill- and siphon-withdrawal reflex in *Aplysia* participate in the storage of long-term memory for sensitization

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Contributed by Eric R. Kandel, August 1, 1985

ABSTRACT **We have found that in the gill- and siphon-withdrawal reflex of *Aplysia*, the memory for short-term sensitization grades smoothly into long-term memory with increased amounts of sensitization training. One cellular locus for the storage of the memory underlying short-term sensitization is the set of monosynaptic connections between the siphon sensory cells and the gill and siphon motor neurons. We have now also found that these same monosynaptic connections participate in the storage of the memory underlying long-term sensitization. We examined the amplitudes of the direct synaptic connections produced by siphon sensory neurons on the gill motor neuron L7 in nervous systems removed from control and from long-term sensitized animals 1 day after the end of long-term sensitization training. The connections were significantly larger in long-term sensitized animals than in control animals. The finding that long-term memory occurs at the same synaptic locus as the short-term memory should facilitate study of the cellular and molecular mechanisms involved in the conversion of short-term to long-term memory.**

Recent studies on invertebrate learning have enhanced our knowledge about the neural loci and mechanisms underlying short-term memory, a memory that lasts minutes to hours (1). By contrast, little is known about long-term memory, a memory lasting days to weeks, or about the relationship between short-term and long-term memory. Two questions need to be addressed: first, where in the nervous system is long-term memory stored? Is it stored at the same or at different sites than short-term memory? Second, what is the mechanism for long-term memory? Is it maintained by covalent modifications of existing proteins, as short-term memory has been found to be, or by an alternative mechanism? If different mechanisms are involved for short- and long-term memory, do the mechanisms operate in series or in parallel?

A first step in the analysis of the relationship between short- and long-term memory is to localize, within the neural circuit for a behavior, a storage site for each form of memory. This has previously been examined for habituation in the marine mollusk *Aplysia* (2–4) and for associative learning in the marine mollusk *Hermissenda* (5, 6). We have here explored the locus for long-term sensitization.

Sensitization is a form of nonassociative learning in which an animal's responses to one stimulus are enhanced after presentation of a separate noxious stimulus. The gill- and siphon-withdrawal reflex of *Aplysia* acquires the memory for sensitization in response to noxious stimuli such as electrical shock administered to the tail or head. After this noxious stimulus, the animal learns about the presence of a dangerous stimulus and enhances its defensive responses for various periods of time. The duration of the memory for this type of learning is dependent on the amount of training. A single noxious stimulus produces short-term sensitization, the memory for which lasts from several minutes to >1 hr (7, 8), whereas repeated stimuli produce long-term sensitization in which the memory lasts days to weeks (9).

Previous work (for review, see ref. 8) has shown that the short-term memory for sensitization involves an enhancement in the efficacy of the synapses between the siphon sensory cells and the gill and siphon motor neurons. Sensitizing stimuli excite facilitating interneurons that activate an adenylate cyclase in the sensory neurons, leading to increased levels of cAMP. The cAMP, in turn, acts by means of cAMP-dependent protein kinase to close a specific class of K^+ channels (the serotonin-sensitive K^+ channels). The closure of these channels produces a prolongation of the action potential in the sensory neuron and causes more transmitter release per action potential. In addition, the increase in cAMP alters the handling of Ca^{2+} within the cell, a process that is thought to be important for the mobilization of transmitter (10, 11).

The present study had two goals. First, we sought to examine more closely the behavioral procedures necessary to produce long-term sensitization, and to determine whether long-term sensitization emerges in an abrupt or graded manner with increased training. Second, we attempted to determine whether the synaptic connections between the siphon sensory neurons and motor neurons, the cellular locus that stores short-term memory for sensitization, also store long-term memory. We report here that long-term sensitization is graded and that long- and short-term memory share a common synaptic locus.

METHODS

Behavioral Experiments. *Aplysia californica* (150–300 g) were obtained from Pacific Biomarine (Venice, CA). Animals were housed individually and fed daily in a 200-gallon aquarium for at least 4 days before an experiment was begun. Animals that did not eat for two consecutive days were removed from the experiment. Each experiment consisted of three phases: (*i*) a pretest, (*ii*) training, and (*iii*) one or more post-tests. The pretest and each of the post-tests consisted of determining the mean duration of 10 consecutive siphon withdrawals elicited by Water Pik pulses of 800 msec duration, 2 g intensity, delivered a minimum of 30 sec apart, and directed down the siphon funnel from a distance of ½ inch (see ref. 9). Testing was carried out in the animals' home cages. Siphon withdrawal was measured with a stopwatch from stimulus onset until the siphon reappeared from between the parapodia. A cutoff score of 180 sec was used for all withdrawals above that value. All responses were mea-

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Abbreviation: EPSP, excitatory postsynaptic potential.

sured by a blind procedure. Animals were included in the study only if their mean pretest scores were >2.5 sec and <60 sec, to ensure that they were healthy and not already strongly sensitized. Eighty percent of the animals passed this selection step. In some experiments, the pretest was preceded by 4 days of siphon stimulation as described above, in order to produce long-term habituation (12).

Animals were assigned to control and experimental groups to match their pretest scores. Experimental animals were then trained with a series of 1.5-sec shocks (50 mA; 60 Hz, ac) delivered either to the neck or tail via capillary electrodes placed against the skin. We used three different training protocols: (*i*) four single shocks (intershock interval 100 sec to 2 hr) delivered in 1 day, (*ii*) four trains of four shocks (3.0 sec between shocks in a train, 30 min between trains) delivered in 1 day, and (*iii*) four trains of four shocks a day for 4 days. Animals were removed from their home cages to a separate pan for training. Control animals were housed and tested with experimental animals but were not shocked. Post-tests were carried out 1, 4, or 7 days after the end of training.

Electrophysiological Experiments. Animals used for electrophysiological experiments received four trains of four tail shocks per day for 4 days. Because the degree of effectiveness of the long-term sensitization training varied greatly, the experimental animals used in the cellular analysis did not constitute a random sample of that population. Instead, we selected animals that were highly sensitized on the basis of a two-trial post-test, conducted blind, 1 day after training was completed. Animals were determined to be sensitized if both withdrawal responses in the post-test were >100 sec, or if either response was longer than the cut-off score of 180 sec. Across all training groups, ≈60% of the experimental animals met these criteria. Control animals never met these criteria and thus were admitted to the cellular study indiscriminantly.

Immediately after the post-test, animals were anesthetized by injection of one-third to one-half of their body weight of isotonic $MgCl_2$, and the abdominal ganglion connected to the gill, siphon, and mantle shelf was dissected out and transferred to a recording chamber that was maintained at 15°C. The ganglion was partially desheathed in a solution prepared by mixing equal volumes of isotonic $MgCl_2$ and artificial seawater. The preparation was then perfused with artificial seawater for at least ½ hr before we attempted to locate the gill motor neuron L7. L7 was identified on the basis of its size, location, axonal distribution, the unique gill movement it produces, and the occurrence of a stereotypic spontaneous inhibitory synaptic input associated with the respiratory pumping reflex (13).

Once L7 was identified, the chamber was perfused with an artificial seawater solution containing increased levels of Mg (100 mM) and Ca (50 mM) to reduce the recruitment of interneurons by the sensory cells. Siphon sensory cell candidates were identified on the basis of their size, location, characteristic pigmentation, and the absence of both spontaneous activity and synaptic inputs (14). The monosynaptic connection between a sensory cell and L7 was assessed by impaling the cell with an intracellular electrode, waiting 2 min if it fired upon entry, and then eliciting three single action potentials using a 30-sec interstimulus interval. If the resulting excitatory postsynaptic potentials (EPSPs) in L7 were of short (<10 msec) and constant latency, the amplitude of the first one was measured and recorded. If the first action potential elicited a complex EPSP in L7, then the first monosynaptic EPSP produced by the series of three action potentials was used to back-extrapolate to a value for the first EPSP, using the average homosynaptic depression kinetics obtained from the control population (the rate of homosynaptic depression was not significantly different for the two populations; see *Results*). As many sensory cell candidates as possible were sampled per preparation. During sampling, a cell map was made and afterwards all cells were reimpaled and tested for the presence of an antidromic spike and no synaptic potential in response to siphon nerve shock. Those passing this test were determined to be siphon sensory cells and their EPSP values were entered as experimental data. In each preparation, we demanded a minimum of three sensory cells (the average was seven). A median EPSP value was obtained for each preparation. Twenty controls and 20 experimental preparations were analyzed in this manner.

RESULTS

Behavioral Sensitization Is Graded: The Duration of the Memory Is a Function of the Amount of Training Administered. To determine whether long-term sensitization emerges in an abrupt or graded manner, we systematically explored the relationship between the amount of training and the amount and duration of the sensitization produced. The major conclusion from 10 separate experiments is that the amplitude and duration of long-term sensitization are a graded function of the number of shocks delivered. On average, four single shocks produced sensitization lasting 1 day, four trains of four shocks produced greater sensitization lasting 4 days, and four trains a day for 4 days produced even greater sensitization lasting at least 7 days (Fig. 1). These conclusions are supported by comparison of the different protocols both between experiments (the average results shown in Fig. 1) and within individual experiments in which experimental animals were submitted to different amounts of training. The sensitization produced was roughly the same regardless of whether the animals were prehabituated or not, or whether the neck or tail was shocked. Finally, the amount

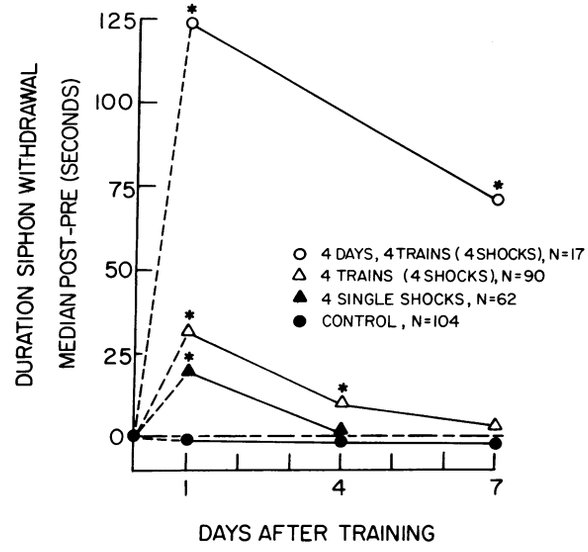

FIG. 1. Summary of behavioral experiments on long-term sensitization. Experimental animals received either four single shocks in 1 day (▲), four trains of four shocks in 1 day (△), or four trains of four shocks a day for 4 days (○). Control animals were not shocked (●). The mean duration of siphon withdrawal in response to 10 Water Pik stimulations was determined for each animal before training (Pre) and again 1, 4, or 7 days after the end of training (Post). The median pretest scores for the different groups of animals ranged from 9.4 to 12.3 sec. The figure shows the median change in duration of siphon withdrawal (Post-Pre) for all animals receiving the same number of shocks in 10 separate experiments. The asterisks indicate a significant difference between experimental and control animals (Mann–Whitney U tests; $P < 0.01$). *N* indicates the starting number of animals per group; subsets of animals were tested at different times after training.

of sensitization varied from experiment to experiment with the same procedure, and in a few individual experiments there was no significant sensitization.

Long-Term Sensitization Produces Changes in the Monosynaptic Connections Between the Sensory and Motor Neurons. We used the most effective training protocol, consisting of four trains of tail stimuli for 4 days, and examined the connections between the sensory neurons and the motor cells 1 day later. We found that after long-term sensitization training, the amplitude of the monosynaptic EPSP between the siphon mechanoreceptor and the gill motor neuron (L7) was significantly increased (Fig. 2). The median monosynaptic EPSP amplitude for the long-term sensitized group was 2.2 times that for the control group (1.1 mV versus 0.45 mV; $P < 0.025$, Mann–Whitney U test).

Animals exposed to long-term sensitization training showed a trend for a higher percentage of cells per sensory cluster that made detectable connections onto L7 (96% versus 75%) and a higher percentage of cells per cluster that recruited interneurons (23% versus 4%) (Table 1). Two postsynaptic factors, L7 input resistance and resting membrane potential, were examined and found not to be significantly different between the two groups.

Since sensory neurons fire repetitively to siphon stimulation (14), and repetitive activity in these cells produces a rapid homosynaptic depression, a decrease in the kinetics of homosynaptic depression with long-term sensitization might act to strengthen the physiological connection between the sensory neurons and L7. To check for this possibility, we assessed the kinetics of homosynaptic depression (ISI, 30 sec) for the two groups but found no difference. Normalized EPSP amplitudes over three trials were 100%, 70%, and 55% in the sensitized group and 100%, 73%, and 60% in the control group.

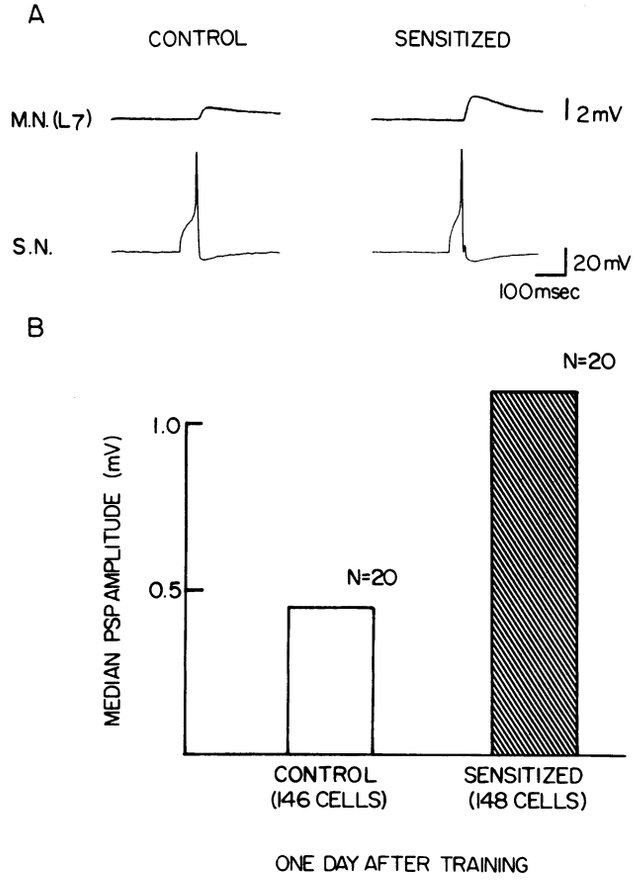

FIG. 2. EPSP data from control and long-term sensitized animals. (*A*) Representative monosynaptic EPSPs from siphon sensory neurons (S.N.) to gill motor neuron L7 (M.N.). The synaptic connection on the left is from a control animal. The connection on the right is from a long-term sensitized animal examined 1 day after the end of training. (*B*) Comparison of median EPSP values from control and long-term sensitized animals examined 1 day after the end of training. The median value from the sensitized population is significantly larger than the median value from the control population. (Mann–Whitney U test; $P < 0.025$.)

Table 1. Synaptic strength

	Control	Sensitized
Median EPSP amplitude, mV	0.45	1.1*
% detectable connections	75%	96%
% complex PSP	4%	23%
Depression kinetics	100% 73% 60%	100% 70% 55%

Summary of the cellular study. Only the median EPSP values were significantly different between the two populations. PSP, postsynaptic potential.
*$P < 0.025$ (Mann–Whitney U test).

DISCUSSION

Previous experiments demonstrated that a single shock of intensity similar to the ones used in this study produces short-term sensitization lasting from several minutes to >1 hr (ref 8; unpublished data), and four shocks a day for 4 days produce long-term sensitization lasting weeks (9). We have found that intermediate levels of training produce intermediate amplitudes and durations of sensitization. The fact that long-term sensitization is a graded function of the amount of training places some restrictions on possible cellular mechanisms. Furthermore, the finding that sensitization lasting several days can be produced in a single 1.5-hr training session consisting of four trains of stimuli separated by 30 min should be useful for future *in vitro* studies of the mechanisms of the long-term memory (15).

Our data also indicate that the same synaptic locus in the neural circuit mediating the gill- and siphon-withdrawal reflex can store both short-term and long-term memory for sensitization. The synaptic enhancement underlying short-term sensitization has been shown to be presynaptic in origin (16), involving an increase in the amount of transmitter released per sensory neuron action potential. The present data do not resolve whether the memory producing the long-term synaptic enhancement is located in the pre- or postsynaptic neurons (or both). Although the input resistance and membrane potential were not significantly different between the experimental and control groups, other postsynaptic factors, such as transmitter receptor properties, were not examined.

That the increase in synaptic strength may be at least in part presynaptic in locus is supported by the study of Bailey and Chen (17), who found morphological changes in the sensory neuron presynaptic terminals of long-term sensitized animals. In their study, serial electron microscope section reconstructions of the varicosities from sensory neurons of control and long-term sensitized animals revealed significant increases in the number of active zones per varicosity and in the number of vesicles in close apposition to each active zone in the sensitized animals. Moreover, the active zone areas from sensitized animals were larger than the active zone areas from control animals. Thus, the combined electrophysiological and morphological data suggest that, as with short-term memory, long-term memory for sensitization may involve enhanced transmitter release from sensory neuron terminals. To explore this possibility directly will require a quantal analysis of the strengthened synaptic connection.

Long-term habituation involves a prolonged decrease in the percentage of siphon sensory cells per ganglion that make

detectable connections onto L7 (87% to 31%; see ref. 4). In the present study, we therefore looked not only at EPSP amplitude but also at the percentage of sensory cell connections onto L7, predicting that perhaps the percentage would show an increase. While we indeed found an upward trend (75% to 96%) in this aspect of synaptic function, the trend was not statistically significant.

Our results do not exclude the participation of other sites in the neuronal circuit for the storage of long-term memory. Indeed, recent work has shown that, in addition to changes at the connections of the sensory neurons, short-term sensitization also involves a reduction in efficacy of a set of identified inhibitory interneurons (18) and an increase in the tonic firing rate of a subclass of siphon motor neurons (19). Furthermore, Eberly and Pinsker (20) have shown that during reflex activation long-term sensitization is correlated with a prolongation of motor neuron firing and an increase in the number of cycles of activity in cells of the interneuron II complex, which produces respiratory pumping movements of the gill and siphon. It has not yet been determined whether the cells Eberly and Pinsker monitored are involved in the actual storage of memory or only in its readout.

Based on this circuit localization of both types of memory, it should now be possible to begin cellular studies of the mechanisms underlying acquisition and retention of long-term memory and the relationship of such processes to those already identified for short-term memory. For example, one should now be able to explore whether consolidation of long-term sensitization involves covalent modifications of preexisting proteins, as does short-term memory, or alterations in gene expression (8). The coexistence of both short- and long-term memory in single identifiable cells simplifies experiments designed to differentially disrupt their respective consolidation processes.

Sensitization shares certain formal features with classical conditioning (21). Hawkins *et al.* (22) have recently shown that the cellular mechanism underlying classical conditioning of the gill–siphon-withdrawal reflex in *Aplysia* involves an activity-dependent enhancement of the sensitization mechanism. Our findings on sensitization may therefore also pertain to the long-term memory underlying classical conditioning.

We thank James Sliney for his indispensable work in maintaining the animals, Emilie Marcus for assistance in training the animals, and Louise Katz and Kathrin Hilten for the illustrations. This research was supported in part by a grant from the McKnight Foundation.

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