Long-term sensitization in *Aplysia* increases the number of presynaptic contacts onto the identified gill motor neuron L7

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**ABSTRACT** We have used the gill and siphon withdrawal reflex of *Aplysia* to study the morphological basis of the persistent synaptic plasticity that underlies long-term sensitization. One critical focus for storage of the memory for sensitization is the set of monosynaptic connections between identified siphon sensory neurons and gill and siphon motor neurons. To complement previous morphological studies of the presynaptic terminals of identified sensory neurons, we examined the effects of long-term sensitization on the structure of an identified postsynaptic target—the gill motor neuron L7. We found an increase in the frequency, size, and vesicle complement of presynaptic contacts onto L7 processes in sensitized compared to control animals. Combined, these data indicate a striking increase in the percentage of the surface area of L7 that is occupied by synaptic contacts after long-term training. These results are consistent with our observations that sensitization produces an increase in the synapses that the sensory neurons make on their target cells and provide additional support for the hypothesis that changes in synapse number may represent a mechanism underlying long-term memory.

How information is stored in the brain is a question central to both neurobiology and psychology. Recent studies on several higher invertebrates have enhanced our knowledge about the synaptic loci and mechanisms that are involved in the acquisition and retention of various elementary forms of learning and memory (1, 2).

One such model system—the gill and siphon withdrawal reflex of *Aplysia californica*—has proven to be a particularly accessible behavioral preparation for cellular and molecular studies of learning and memory (3). This reflex can be modified by a simple type of nonassociative learning—sensitization—the memory of which can exist in a short-term form lasting minutes to hours (4) and a long-term form persisting for days to weeks (5, 6). Previous work (for review, see ref. 7) has shown that the short-term memory for sensitization involves an enhancement in the effectiveness of the synapses between siphon sensory neurons and gill and siphon motor neurons (8–11). More recent electrophysiological studies examining the amplitude of the monosynaptic excitatory postsynaptic potentials between sensory neurons and the gill motor neuron L7 have shown that the same synaptic loci are involved in the storage of the long-term memory for sensitization (6). This enduring change in the functional expression of sensory to follower cell connections is accompanied by two classes of morphological change: (i) increases in active zone morphology at identified sensory neuron synapses (12) and (ii) an increase in the total number of presynaptic varicosities per sensory neuron (13, 14).

In the present study we have examined further the morphological consequences of long-term sensitization by determining its effect on a postsynaptic target of the sensory neurons—the identified gill motor neuron L7. We report here that long-term sensitization is accompanied by an increase in the frequency, size, and vesicle complement of presynaptic active zones onto the dendritic tree of L7 with a resultant increase in the percentage of its receptive surface that is occupied by synaptic contact.

**METHODS**

Animals were trained for long-term sensitization by the protocol of Pinzer *et al.* (5). Animals were individually housed for a minimum of 5 days in circulating seawater before behavioral training. To assess their responsiveness, we delivered two jets of seawater to the siphon with a Water Pik. Animals were accepted for the experiment if the mean duration of their first two test responses (interstimulus interval, 30 sec) was 10 sec or longer. The scores (total of 10 stimuli) were ranked and the animals were randomly distributed into a control (untrained) group and a group for long-term sensitization. There were no significant differences between the groups before training. Long-term sensitization was produced by giving animals training sessions on four consecutive days. Each session consisted of exposure to four electrical stimuli (100 mA for 2 sec), each separated by 1.5 hr. All electrical stimuli were delivered to the neck region through bipolar capillary electrodes.

Retention of sensitization was tested 1 day after completion of the last training session. The behavioral performance of animals—control and long-term sensitized—was estimated by comparing their behavioral scores 24 hr after the completion of training with their pretraining scores. The ratios after training/pretraining were as follows: long-term sensitized animals were significantly different from control [5.3 ± 1.1 (mean ± SEM, n = 4) vs. 0.91 ± 0.12 (mean ± SEM, n = 4); t = 4.01, P < 0.01].

Within 48 hr of the end of training, animals were anesthetized by intracoelomic injection of isotonic MgCl2. The abdominal ganglion connected to the gill and abdominal aorta was removed from each animal and transferred to a solution of supplemented artificial seawater containing a high Mg2+ content (200 mM) and a low Ca2+ content (1 mM) to block synaptic transmission during pinning and desheathing. After desheathing, the ganglion was bathed in seawater with a normal Mg2+ (55 mM) and Ca2+ (10 mM) content for a minimum of 30 min before we attempted to identify L7. L7 was identified on the basis of its size, location, electrophysiological characteristics, the occurrence of stereotypic spontaneous synaptic input, and, most importantly, the unique gill movements (15) and contractions of the wall of the abdominal aorta (16) it produces when caused to fire by the intracellular injection of current. Once L7 was identified, its soma was slowly pressure-injected with horseradish peroxidase (type V1, purchased from Sigma) at a concentration of 4% in 0.5 M potassium chloride. After a 20-hr incubation period to allow
the horseradish peroxidase to fill the neuropil arbor of L7 as well as its axons in the branchial, genital-pericardial, and siphon nerves, ganglia were fixed, histochemically processed, and embedded in Epon (17). Each L7 was serially sectioned using 20-μm slab-thick sections.

To analyze the fine structure of the L7 processes and quantitate the frequency of presynaptic contacts onto its dendritic arbor, 20 slab-thick sections from similar regions of the left hemiganglion neuropil in control (n = 4) and experimental (n = 4) animals were re-embedded and thinly sectioned. Complete sets of 100–200 serial thin sections were taken from each re-embedded thick section. All horseradish peroxidase-labeled L7 profiles on each of 10 equally spaced thin sections from each re-embedded slab-thick section were photographed and analyzed through a blind procedure. To examine in detail the fine structure and extent of synaptic input onto the postsynaptic surface of each neuron, we analyzed a total of 1267 L7 profiles taken from eight animals.

Quantitation of L7 processes and presynaptic contacts onto them was made using a Bioquant II digitizing tablet (R&M Biometrics, Nashville, TN) interfaced with an Apple IIe microcomputer running the Bioquant II morphometry software. The surface areas of relevant structures were computed by multiplying the thickness of the section (estimated to average 0.1 μm as judged by interference color) by the linear values obtained by tracing the perimeter (L7 profiles) or length (presynaptic active zones) on prints enlarged to a final magnification of 60,000. The number of vesicles associated with each active zone was determined by counting the total number of vesicle profiles in each section that fell within 30 nm of the presynaptic active zone membrane (12, 18).

RESULTS

Cell L7 is a primary motor neuron for the gill and mantle shelf in *Aplysia* and is involved in the defensive reflex of these mantle organs. It receives both direct and indirect (by way of interneurons) input from the mechanoreceptor sensory neurons of the siphon skin and these connections are critical loci for the changes in synaptic effectiveness that underlie behavioral sensitization (3).

The three-dimensional morphology of L7 has been examined by Winlow and Kandel (19). Using cobalt chloride as a marker substance, this light microscopic study revealed the distribution and extent of the major processes of L7 within the abdominal ganglion. Since the finest ramifications of the cell were most likely not visualized, this study primarily provided a low-resolution view of the architecture of L7.

To address this issue in more detail, we have begun a combined light and electron microscopic examination of the morphology of L7 using horseradish peroxidase and serial 20-μm slab-thick sections of Epon-embedded material. This approach has permitted the visualization of the finest processes of the neuron and thereby facilitated quantitative analysis of the synaptic organization of the dendritic arbor of L7.

Long-Term Sensitization Increases the Number of Presynaptic Contacts onto the Dendritic Tree of L7. In the present study we have examined the effects of long-term sensitization on the fine structure of the dendritic field of L7 and on the extent of the synaptic input it receives. Using random thin sections taken through the horseradish peroxidase-labeled secondary and tertiary branches of the proximal dendritic tree of L7, we quantitated changes in the receptive surface of L7 in control and behaviorally modified animals.

We began by examining the incidence of presynaptic active zones onto L7 processes in both behavioral groups. Quantitative ultrastructural analysis of 1267 L7 profiles revealed an increase in the frequency of presynaptic contacts onto L7 processes in sensitized compared to control animals (0.45 ± 0.03 (mean ± SEM, n = 4) vs. 0.197 ± 0.02 (n = 4), t = 7.43, P < 0.001; Table 1). The number of synaptic contacts per square micrometer of L7 surface area increases (1.72 ± 0.06 vs. 0.61 ± 0.05, t = 13.36, P < 0.001) as does the incidence of multiple synaptic contacts onto the same postsynaptic profile (0.06 ± 0.008 vs. 0.01 ± 0.004, t = 4.8, P < 0.01; Fig. 1). Both the size (0.49 ± 0.01 μm vs. 0.4 ± 0.02 μm, t = 3.1, P < 0.05) and vesicle complement (4.1 ± 0.1 vs. 2.8 ± 0.2, t = 5.9, P < 0.01) of the active zones at these presynaptic contacts onto L7 dendritic processes are larger in sensitized animals than in controls (Table 2). Combined, these data indicate a striking increase in the percentage of the surface area of L7 that is occupied by synaptic contacts after long-term training (0.084 ± 0.002 vs. 0.025 ± 0.003, t = 16.86, P < 0.001; Fig. 2).

In addition to quantitating the effects of long-term sensitization on the nature and extent of presynaptic contacts onto L7, we have also begun to characterize the morphology of the postsynaptic components at these synapses. We have found an increase in the frequency of small (<0.5 μm), spine-like postsynaptic L7 processes at these synapses in sensitized compared to control animals (0.72 ± 0.06 vs. 0.40 ± 0.09, t = 3.04, P < 0.05; Fig. 1C).

DISCUSSION

Previous studies of long-term sensitization of the gill and siphon withdrawal reflex in *Aplysia* have indicated that a common locus—the synapses between identified sensory neurons and follower cells—undergoes both functional and structural alterations. Electrophysiological examination has shown a 2-fold increase in the amplitude of monosynaptic excitatory postsynaptic potentials between siphon mechanoreceptors and the gill motor neuron L7 in sensitized compared to control animals (6). Similarly, quantitative morphological analysis of the identified presynaptic varicosities of sensory neurons has shown an increase in the number, size, and vesicle complement of their active zones (12) as well as a near doubling of the total number of varicosities per sensory neuron (13, 14). The nature and extent of both the functional

<table>
<thead>
<tr>
<th>Animal</th>
<th>L7 profiles, n</th>
<th>No. presynaptic active zones onto L7</th>
<th>Ratio of L7 profiles with presynaptic contact, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>190</td>
<td>89</td>
<td>47</td>
</tr>
<tr>
<td>2</td>
<td>275</td>
<td>130</td>
<td>47</td>
</tr>
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<td>3</td>
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</tr>
<tr>
<td>4</td>
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<td>13</td>
<td>17</td>
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</tr>
<tr>
<td>4</td>
<td>244</td>
<td>55</td>
<td>23</td>
</tr>
</tbody>
</table>

Table 1. Number of L7 profiles, number of presynaptic profiles with active zones onto L7 profiles, and ratio of L7 profiles receiving presynaptic contact in sensitized and control animals.

As one control for possible nonspecific effects of the training protocol, we examined the incidence, size, and vesicle complement of presynaptic active zones onto unidentified postsynaptic profiles in regions of the neuropil contiguous to the dendritic arbor of L7. Approximately 1500 μm² of tissue was sampled and no significant differences were found with respect to the number per square micrometer (0.068 ± 0.003 vs. 0.074 ± 0.003), length (0.28 ± 0.007 μm vs. 0.34 ± 0.06 μm) or vesicle complement (1.8 ± 0.14 vs. 2.4 ± 0.5) of presynaptic active zones onto unlabeled postsynaptic profiles between sensitized and control animals.
Fig. 1. Increased synaptic contact onto L7 processes after long-term sensitization training. The incidence of multiple presynaptic contacts (*) onto the same L7 profile is increased in sensitized compared to control animals. This convergence can occur on intermediate-size processes (A) or on smaller profiles (B and C). Long-term sensitization also increases the frequency of narrow, spine-line postsynaptic L7 processes at these synapses (C). All profiles belonging to L7 are easily identified by a blanket of electron-dense horseradish peroxidase reaction product. (Bar = 0.5 μm.)

Table 2. Length of active zones and number of associated vesicle profiles in presynaptic terminals contacting L7 processes in sensitized and control animals

<table>
<thead>
<tr>
<th>Animal</th>
<th>Active zones analyzed, n</th>
<th>Active zone length, μm</th>
<th>No. vesicle profiles associated with each active zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitized</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>89</td>
<td>0.486 ± 0.02</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>130</td>
<td>0.523 ± 0.02</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>3</td>
<td>54</td>
<td>0.466 ± 0.03</td>
<td>4.3 ± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>38</td>
<td>0.473 ± 0.04</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>13</td>
<td>0.352 ± 0.03</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>0.367 ± 0.04</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td>3</td>
<td>29</td>
<td>0.439 ± 0.02</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>4</td>
<td>55</td>
<td>0.450 ± 0.03</td>
<td>3.1 ± 0.2</td>
</tr>
</tbody>
</table>

Results represent mean ± SEM.

and structural changes at identified sensory neuron synapses are consistent with the known behavioral efficiency of long-term sensitization (5, 6).

In the present study we have examined the effects of long-term sensitization on the structure of an identified postsynaptic target of the sensory neurons—the identified gill motor neuron L7. Our initial quantitative survey was focused primarily on the extent of synaptic contacts onto the receptive surface of L7. The results indicate a dramatic increase in the number and percentage of occupancy of synapses onto the dendritic tree of L7. In addition, there appears to be an increased convergence of presynaptic contacts onto the same postsynaptic profile after long-term training.

The increase in presynaptic input during long-term sensitization is accompanied by an increased percentage of synapses occurring on small (<0.5 μm) L7 processes. This could be due to a shift in the position of synapses onto the L7 neuropil arbor or perhaps to an increased outgrowth in the number of small processes from L7. Preliminary qualitative light microscopic observations (data not presented) support the second possibility, with an apparent increase in focal outpocketing of finger-like spines from L7 in sensitized compared to control animals. Further analysis will be necessary to determine whether such an increase is a consistent postsynaptic correlate of long-term sensitization in Aplysia and whether it contributes to an increase in postsynaptic surface membrane area, as has been reported in vertebrates (refs. 20, 21; for review, see ref. 22).

That the increases in morphological features of presynaptic contacts onto L7 are likely to result, at least in part, from the behavioral training protocol is supported by our analysis of active zones at presynaptic terminals that synapse onto unlabeled postsynaptic profiles. If, for instance, long-term training was producing a general, nonspecific increase in the number or size of presynaptic contacts, such an increase should be evident in a quantitative analysis of neuropil contiguous to the territory of L7. By contrast, our data suggest that the alterations in presynaptic features are differentially expressed at a known postsynaptic locus for the changes in efficacy that underlie sensitization—the gill motor neuron L7. This finding provides additional support for the hypothesis that the morphological alterations we have observed share some specificity to the learning experience.

Although the neurons of origin for the unlabeled presynaptic terminals contacting L7 are not known in the present study, it is attractive to think that some percentage of these contacts belong to sensory neurons. This would be consistent with the results of biophysical studies indicating a trend toward a higher percentage of sensory cell connections onto L7 after long-term training (6). To address directly the structural correlates of this will require simultaneous exam-
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![Graph](image)

**Fig. 2.** Percentage of the surface area of L7 that is occupied by presynaptic contacts in control and long-term sensitized animals. Long-term training increases both the frequency and size of presynaptic contacts onto L7 with a resultant increase in the percentage of receptive surface of L7 that is occupied by synaptic contact.

...indicates that long-term sensitization may resemble a form of neuronal growth across a broad segment of the animal kingdom and suggests that the stability required for a long-term memory may be provided by alterations in the number of synaptic connections.

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