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Morphological Basis of Long-Term Habituation and Sensitization in *Aplysia*

Abstract. *The morphological basis of the persistent synaptic plasticity that underlies long-term habituation and sensitization of the gill withdrawal reflex in* Aplysia californica *was explored by examining the fine structure of sensory neuron presynaptic terminals (the critical site of plasticity for the short-term forms of both types of learning) in control animals and in animals whose behavior had been modified by training. The number, size, and vesicle complement of sensory neuron active zones were larger in animals showing long-term sensitization than in control animals and smaller in animals showing long-term habituation. These changes are likely to represent an anatomical substrate for the memory consolidation of these tasks.*

Since the work of Ramón y Cajal at the turn of the century, it has often been suggested that learning and memory produce structural changes at the synapse (*1*). Although many investigators have reported alterations in synaptic morphology after experimental manipulation (*2*), the functional significance of these changes has been difficult to assess because the contribution of the synapses to the learning process has not been known. In recent years, the tractable nervous systems of higher invertebrates have proven useful for correlating changes in cellular function with learning (*3*). One such model system has been the gill and siphon withdrawal reflex of *Aplysia*, in which several forms of learning (short- and long-term and nonassociative and associative) have been studied to advantage on both the cellular and molecular level. This reflex undergoes two simple forms of nonassociative learning—habituation and sensitization—that can exist in a short-term form lasting minutes to hours (*4*) and in a long-term form lasting more than 3 weeks (*5*). The biophysical and biochemical mechanisms of short-term habituation and sensitization are known to involve changes in synaptic effectiveness produced by modulation of the Ca^{2+} current at a common locus—the presynaptic terminals of identified mechanoreceptor sensory neurons (*6*). Less well characterized are the morphological mechanisms that underlie habituation and sensitization, particularly their long-term form. For both habituation and sensitization the critical site of plasticity, the synapses between sensory neurons and follower cells, is shared by the short- and long-term forms (*7*, *8*).

We exploited the cellular specificity of the gill withdrawal reflex to explore the morphological basis of long-term habituation and sensitization in *Aplysia*. Using horseradish peroxidase (HRP) to label the presynaptic terminals (varicosities) of sensory neurons and serial reconstruction to analyze synaptic contacts, we compared the fine structure of identified sensory neuron synapses in control and behaviorally modified animals. Our results indicate that learning can modulate long-term synaptic effectiveness by altering the number, size, and vesicle complement of synaptic active zones.

Aplysia californica (70 to 100 g) were trained for long-term habituation or for long-term sensitization (*5*, *9*). To maximize the chances of detecting morphological differences, only those animals that demonstrated the most profound behavioral changes after training (< 10 percent of the day 1 score for habituated animals and > 200 percent for sensitized animals) were used for subsequent electron microscopic analysis. Within 48 hours of behavioral testing all animals were anesthetized by injection of $MgCl_2$ and the abdominal ganglion was removed and desheathed in seawater containing high Mg^{2+} (220 m*M*) and low Ca^{2+} (1 m*M*). This solution was washed out and the ganglion was bathed in seawater with normal concentrations of Ca^{2+} (10 m*M*) and Mg^{2+} (55 m*M*) for a minimum of 30 minutes before the cells were impaled (*7*). Individual mechanoreceptor sensory neurons were identified (*10*) and intrasomatically pressure-injected with HRP (type VI, Sigma) at a concentration of 20 mg/ml in distilled water. After approximately 2 hours the ganglia were fixed, histochemically processed, and embedded (*11*). Serial thin sections (0.1 μm; 500 to 1000 per set) were made through a region containing labeled sensory neuron processes in each ganglion. Every HRP-labeled profile in each section was photographed and sensory neuron varicosities were then completely reconstructed and analyzed through a

Table 1. Number of varicosities, number of active zones, and ratio of active zones to varicosities in control and behaviorally modified animals. Analysis of variance shows that the means are significantly different [$F(2, 3) = 79.8$, $P < .01$)]. Moreover, individual comparisons (Studentized range tests) show that the mean for habituated animals (12 percent) is significantly less than the mean for control animals (41 percent) ($P < .05$) and that the mean for sensitized animals (65 percent) is significantly greater than that for control animals ($P < .05$).

Group	Varicosities	Active zones	Ratio of zones to varicosities (%)*
Habituated			
Animal 1 (four cells)	63	8	13
Animal 2 (one cell)	48	5	10
Control			
Animal 3 (two cells)	47	17	36
Animal 4 (one cell)	46	21	46
Sensitized			
Animal 5 (two cells)	59	39	66
Animal 6 (four cells)	48	31	65

*(Number of active zones/number of varicosities) (100).

Table 2. Area of active zones and number of associated vesicles. The mean number of vesicles (± standard error) associated with each active zone differs significantly among the three behavioral groups [$F(2, 3) = 32.76$, $P < .01$]. Numbers in parentheses show the number of active zones analyzed.

Group	Active zone area (μm^2)	Number of vesicles associated with each active zone
Habituated		
Animal 1	0.11 ± 0.03 (3)	7.0 ± 1.3 (3)
Animal 2	0.15 ± 0.03 (2)	4.0 ± 2.0 (2)
Control		
Animal 3	0.16 ± 0.02 (14)	13.0 ± 2.1 (14)
Animal 4	0.23 ± 0.04 (15)	13.0 ± 2.1 (15)
Sensitized		
Animal 5	0.28 ± 0.02 (27)	20.0 ± 2.1 (23)
Animal 6	0.42 ± 0.05 (21)	24.0 ± 3.5 (21)

blind procedure. Six animals containing a total of 14 labeled sensory neurons were used in this study: two untrained animals as controls (three cells), two animals trained for long-term habituation (five cells), and two animals trained for long-term sensitization (six cells). To examine in detail the synaptic terminals of the injected cells in each animal, we completely reconstructed 311 sensory neuron varicosities, a task that required the examination of more than 12,000 HRP-labeled profiles.

Active zones (*12*) have been described in a variety of chemical synapses (*13*), and it is generally accepted that these modified sites represent the regions at which neurotransmitter is released, although this has been proved only for the neuromuscular junction (*14*). The active zone at *Aplysia* synapses is similar to that described in other animals. It is characterized by differentiated paramembranous densities that are coextensive with an area of specialized membrane and vesicle accumulation (*15*). When an electron-dense label is present in the presynaptic neuron (as is the case for the HRP-labeled sensory neurons), the full morphology of presynaptic densities can be masked by the blanket of reaction product. Under these circumstances, active zones can be reliably identified by the coexistence of rigidly parallel, apposed membranes bounding a widened synaptic cleft that contains electron-dense material (Fig. 1). As we demonstrated previously (*11, 15, 16*), all these features are identical to active zones found in unlabeled synapses in *Aplysia*. We used them to identify release zones in the present study.

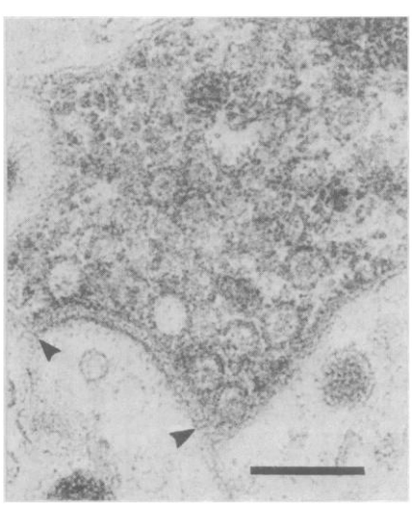

Fig. 1. Sensory neuron varicosity labeled with HRP. The active zone is between the arrowheads. Scale bar, 0.25 μm.

We first examined the incidence of active zones in the sensory neurons of the three behavioral groups. In control animals 41 percent of the varicosities had active zones compared to 65 and 12 percent in sensitized and habituated animals, respectively (Table 1).

We next examined the total surface membrane area of reconstructed sensory neuron active zones and the total number of vesicles associated with each release site in the three groups (*17*). These features paralleled the trend in the number of active zones. In control animals the mean area of active zones was 0.195 μm^2 and was occupied by a mean of 13 vesicles. In sensitized animals the active zone area was 0.35 μm^2 and was occupied by 22 vesicles. By contrast, in long-term habituated animals the active zone area was 0.13 μm^2 and was occupied by only six vesicles (Table 2).

The parallel changes in active zone structure (number, size, and associated vesicles) are complementary and can be expressed in such a way that the overall differences among the three behavioral groups are more apparent than when the active zone parameters are considered individually. Figure 2 illustrates the total number of vesicles that would be associated with all the active zones for a single sensory neuron in habituated, sensitized, and control animals. Since this method of expression may take into account the total number of vesicle quanta available for release per impulse, it should relate most directly to the transmissive capabilities of sensory neurons—the amount of transmitter released at all the terminals of a sensory neuron by a single action potential.

Thus clear structural changes accompany behavioral modification, and these changes can be detected at the level of identified synapses that are critically involved in learning. The nature and extent of the alterations in sensory neuron active zones are consistent with (i) the known behavioral efficacy of long-term habituation and sensitization in *Aplysia* (*5*), (ii) electrophysiological studies showing that long-term habituation results in a significant decrease in the percentage of detectable connections between sensory neurons and follower cells (*7*), and (iii) recent experiments indicating that the strength of the sensory to follower cell connection is enhanced in long-term sensitization (*8*). An additional possibility is that long-term behavioral modifications produce a change in the number of varicosities per sensory neuron. This could be tested by counting the number of varicosities in single HRP-injected sensory neurons in control, habituated, and sensitized animals.

Our results also indicate that active zones are plastic rather than immutable components of the synapse and that the

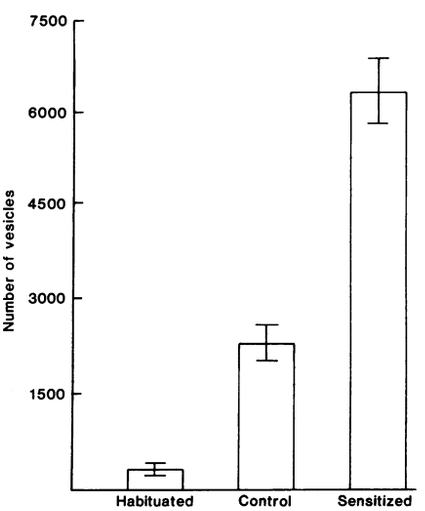

Fig. 2. Total number of vesicles associated with active zones per sensory neuron in control and behaviorally modified animals, as calculated by using the mean values for the incidence of active zones and their vesicle complement. This calculation assumes an average of 435 varicosities per sensory neuron, a value obtained from total reconstructions of HRP-injected sensory neurons in untrained animals (*11*). Each bar represents the mean ± standard error for two animals.

normal set of varicosities in *Aplysia* serves as a mere scaffolding for behavior. Even such elementary learning experiences as habituation and sensitization can build upon this scaffolding by altering the number and size of active zones to modulate the functional expression of neural connections. Since our observations were made on animals that received long-term behavioral training, these morphological changes may represent an anatomical substrate for memory consolidation.

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9. 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 seconds) was 10 seconds or larger. The scores (total of ten stimuli) were ranked and the animals were randomly distributed into a control (untrained) group and groups for long-term habituation and long-term sensitization. There were no significant differences among the groups before training. Long-term habituation was produced by giving animals ten sessions of habituation training per day for 10 days. Long-term sensitization was produced by giving animals training sessions on either four or ten consecutive days. For the 10-day protocol a session consisted of exposure to two electrical stimuli (20 mA for 2 seconds), each separated by a minimum of 2 hours. For the 4-day protocol a session consisted of exposure to four electrical stimuli (100 mA for 2 seconds), each separated by 1.5 hours. All electrical stimuli were delivered to the neck region through bipolar capillary electrodes.
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17. The surface area and vesicle density of active zones were measured only when the full extent of the active zone could be reliably followed throughout the series. This was the case for approximately 70 percent of the active zones found. For the remaining 30 percent a variety of factors, such as smearing of reaction product, oblique sectioning of membranes, or masking of the area by section folds or foreign debris prevented reconstruction. Surface areas of serially reconstructed active zones were measured on prints enlarged to a final magnification of ×59,400 by multiplying the section thickness (estimated by interference color to average 0.1 μm) by the length of the active zone in each section. 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 membrane. This value is the mean height of dense projections at *Aplysia* active zones (*15*). The true number of vesicles was then determined by using a correction factor representing the incidence in adjacent sections of profiles cut from the same vesicle (*11*).
18. This work was supported by NIH grant MH37134-01, NSF grant BNS57824476, an Irma T. Hirschl Career Scientist Award to C.H.B., and Scope B of NIGHS grant GM23540. We thank E. R. Kandel, J. H. Schwartz, V. Castellucci, R. D. Hawkins, and E. Shapiro for helpful comments on the manuscript, L. Katz and K. Hilten for illustrations, and H. Ayers for typing.

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Selection, Outbreeding Depression, and the Sex Ratio of Scale Insects

Abstract. *The black pineleaf scale insect has haploid males and diploid females. Ratios of males to females late in development ranged from 0.005 to 0.320 among insect subpopulations that were infesting different host trees. Demes well adapted to an individual ponderosa pine had a higher proportion of males than did demes that were poorly adapted to the host. Ratios of males to females rose in successive annual samples as natural selection increased insect adaptation. Gene flow between demes on different host trees produced predictable changes in the sex ratio.*

Ponderosa pines (*Pinus ponderosa* Lawson) show great intraspecific variability in their phytochemical defenses (*1*). Because each tree is effectively different (*2*) and because the life history of trees is long relative to the generation time of insect herbivores, pest populations may adapt to individual hosts. Artificial transfer experiments have shown that single pines carry a specialized, differentiated deme or subpopulation of black pineleaf scale insects (*Nuculaspis californica* Coleman) (*3*). We now describe patterns of sex ratio variation in this sedentary insect herbivore which suggest that (i) the differentiation is a genetic process; (ii) drift, or selection adapting scales to the defensive phenotype of an individual host tree, reduces genetic variance within the deme; and (iii) gene flow between scale demes on closely adjacent trees produces a decrease in local adaptation.

Table 1. Male to female (M/F) sex ratios of scale insect demes from 18 different host trees. Samples were taken just before male eclosion in three consecutive years. Tree demes are listed from low to high density (*8*).

1979		1980		1981	
N	M/F	*N*	M/F	*N*	M/F
		327	.073	299	.128
71	.014	409	.073	467	.107
181	.028	347	.089	1037	.101
126	.000	403	.032	192	.091
		327	.052	519	.126
188	.005	412	.068	381	.085
203	.005	337	.113	580	.064
108	.009	368	.098	335	.196
122	.025	382	.055	912	.068
141	.052	208	.087	86	.323
		399	.043	382	.117
147	.007	356	.076	468	.093
76	.101	260	.162	344	.147
		358	.101	462	.079
580	.034	246	.166	398	.223
		366	.109	509	.077
		384	.081	489	.153
		389	.090	414	.092

Eclosion of males, flight, and mating of scale insects occur in mid-May on ponderosa pines in northwestern United States. A census of the insects just before males emerge showed wide variation in the male-female ratios among host trees (*4*). Field observations of insect mortality indicated that fewer males than females survive. Sex is determined by a haplodiploid mechanism with obligate fertilization and elimination of paternal chromosomes early in the development of male progeny (*5*). Males carry half as many chromosomes ($n = 4$) as do females ($2n = 8$) and thus are not heterozygous at any loci. We propose that variability in survivorship and sex ratio reflect selection and change in the genetic composition of the subpopulation; genes that are partially deleterious to