Dissociation of Morphological and Physiological Changes Associated With Long-Term Memory in *Aplysia*

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**INTRODUCTION**

Neuronal outgrowth and modification of synaptic structure have long been correlated with the long-term synaptic plasticity underlying memory (Agnihotri et al. 1998; Bailey and Kandel 1993; Yuste and Bonhoeffer 2001). One simple hypothesis is that learning leads to neurite outgrowth and consequently to the formation of new synapses, which in turn strengthen the neural circuit involved in the behavior. Conversely, learning could also lead to retraction of neurites, decreasing the strength of the circuit. One system to test this hypothesis is the mollusk *Aplysia californica*. In this animal, sensitization is the form of learning that has been studied in greatest detail. Sensitization has a variable time course depending on the training regimen (Frost et al. 1985; Goldsmith and Byrne 1993; Pinsker et al. 1973; Scholz and Byrne 1987; Sutton et al. 2002; Walters 1987). Different phases of sensitization appear to be mediated by different cellular mechanisms (Byrne and Kandel 1996; Byrne et al. 1991; Kandel 2001; Sutton and Carew 2000). Of these, only the long-term form has been correlated with changes in neuronal structure (Bailey and Chen 1988a; Wainwright et al. 2002).

Previous evidence suggests that extensive long-term sensitization training of defensive withdrawal reflexes is correlated with structural changes in sensory neurons forming the afferent limb of the reflex (Bailey and Chen 1988a; Wainwright et al. 2002). Therefore sensory neuron outgrowth could contribute to enhancement of the sensorimotor connection through new synapse formation (Bailey and Chen 1988b). Indeed, long-term sensitization training does lead to an increased number of synapses onto motor neurons (Bailey and Chen 1988b), but the source of the new synapses is unknown. Consequently, despite the appeal of the hypothesis, there is no direct evidence that long-term sensitization in the intact animal affects the number of synapses between individual pairs of sensory and motor neurons.

An in vitro analogue of long-term sensitization has been used to examine features of neurite outgrowth and varicosity formation in cultured neurons. Twenty-four hours after pulsed application of serotonin, synaptic strength is enhanced, and neurite outgrowth and varicosity formation are increased (Glanzman et al. 1990). This process is blocked by inhibitors of transcription or translation (Bailey et al. 1992). Activation of protein kinase A and MAP kinase appears to be required, as well as downregulation of apCAM from the surface of sensory neurons (Bailey et al. 1996; Michael et al. 1998). It should be pointed out, however, that behavioral training experiments with the same time course did not produce observable changes in sensory neuron structure (Wainwright et al. 2002).

Synapse formation cannot, however, be the only mechanism underlying long-term sensitization. For example, both long-term sensitization and long-term facilitation of sensorimotor connections is produced by either a single day of training or 4 days of training. Structural changes are only induced when animals are trained for 4 days, however (Wainwright et al. 2002). Therefore long-term sensitization produced by 1 day of training does not induce observable structural changes. Moreover, recent experiments in sensorimotor co-cultures suggest that synaptic strength can be enhanced in the absence of structural changes (Casadio et al. 1999; Sun and Schacher 1998). Thus modification of preexisting synapses may also play a role in long-term sensitization produced by 4 days of training.

One way to address this issue is to examine the effect of behavioral training on the number of sensorimotor synapses and compare that with the effect on synaptic strength. In practice, this is difficult to accomplish because of the rigid criteria for demonstrating functional synaptic contacts. Recently, however, we demonstrated that confocal microscopy can be used to assess the number of appositions between sensory and motor neurons and thereby estimate synaptic strength (Zhang et al. 2003). We used this technique to examine the effects of long-term sensitization training on the number of sensorimotor appositions and on two electrophysiological correlates, synaptic strength and sensory neuron excitability.

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Animals were exposed to long-term sensitization training as described previously (Wainwright et al. 2002). The tail-siphon withdrawal reflex was elicited by weak AC electrical stimulation of electrodes implanted bilaterally in the tail 1 cm rostral to the tip of the tail and 0.5 cm lateral to the midline. The duration of the stimulating pulse was 20 ms, and the intensity was twice the threshold for eliciting the reflex. After a pre-training assessment of the strength of the reflex, four trains (1 Hz, 10 s) of sensitizing AC stimuli (500 ms, 60 mA) were delivered at intervals of 30 min (Fig. 1A). Stimuli were delivered diffusely to the lateral body wall outside of the receptive field of tail sensory neurons. This procedure was repeated for three additional days at 24-h intervals. The stimuli were delivered to just one side of the animal; a balanced number of animals received stimulation on left and right sides. The posttraining assessment was performed 24 h after the last day of training, on day 5.

After each test stimulus, the duration of siphon withdrawal was measured. Averages of the five test scores before and after training were calculated, and the percent change in the average test score after training relative to the average score before training was calculated for each side (ipsilateral and contralateral). For untrained animals, scores from the two sides were randomly assigned into two groups. The values between the two untrained groups did not differ significantly in any experiments and were pooled for analysis.

In all experiments, different investigators carried out testing and training, and the investigator testing the animals was unaware of their prior treatment. All behavioral experiments were conducted at 15°C. Immediately after the posttest, animals were anesthetized, and pleural-pedal ganglia were removed. Intracellular recordings were made from sensory and motor neurons as described previously (Cleary et al. 1998; Zhang et al. 2003). Dextran-conjugated tetramethylrhodamine (Rhn-Dextran; 3,000 MW, Molecular Probes; 2% in 0.9% KCl) was injected by pressure into the sensory neuron, and dextran-conjugated Alexa 488 (10,000 MW, Molecular Probes; 2% in 0.9% KCl) was injected by pressure into the motor neuron; ganglia were prepared for microscopy as described previously (Zhang et al. 2003).

Sections were examined with a confocal scanning laser microscope (Bio-Rad, Hercules, CA; MRC1024ES attached to an Olympus BX50WI upright microscope). Sections were imaged under a ×60 oil-immersion lens (NA 1.4) as 800 × 800 pixel images with a zoom factor of 1. Serial optical sections at increments of 0.5 μm were collected through the depth of each 40-μm physical section (z series). The fluorophores were imaged sequentially, using laser wavelengths of 568 and 488 nm. Digital images were captured using Laser-Sharp software (Bio-Rad) and analyzed using the Metamorph software package (Universal Imaging, Downingtown, PA). Data were averaged for each group and compared by either Student’s unpaired t-test (for instances where 2 groups were compared) or one-way ANOVA using Prism 3.0 (GraphPad Software, San Diego, CA). When significant differences were found, the Newman-Keuls test was performed for multiple comparisons. All data are represented as means ± SE.

Animals were trained for four consecutive days and tested 24 h after training (Fig. 1A). This treatment produced a significant change in the duration of the siphon withdrawal reflex \([F(2,66) = 13.33; P < 0.0001]\). As reported previously (Wainwright et al. 2002), training produced an enhanced reflex response on the side of the animal that received sensitization training (ipsilateral) but not on the contralateral side (Fig. 1B). The enhancement was significantly different from the behavioral change on the contralateral side (225 ± 32 vs. 93 ± 8%; means ± SE; \(q = 6.72; P < 0.001\)) and from untrained control animals (225 ± 32 vs. 109 ± 8%; \(q = 5.77; P < 0.001\)).

After the behavioral tests, ganglia were removed from the animals and two electrophysiological correlates were examined, the strength of the sensorimotor synapse and sensory neuron excitability (Cleary et al. 1998). The 4-day training protocol induced a significant facilitation of the sensorimotor synapse \([F(2,21) = 8.09; P < 0.005]\; Fig. 2A). The EPSPs in ipsilateral ganglia were significantly larger compared with the contralateral ganglia (8.7 ± 2.1 mV; \(q = 4.73; P < 0.001\)) and ganglia from untrained controls (8.7 ± 2.1 vs. 2.7 ± 0.4 mV; \(q = 5.29; P < 0.01\)). This unilateral enhancement parallels the behavioral enhancement.

A 1-day training protocol produced an increase in sensory neuron excitability (Cleary et al. 1998) as well as decreases in membrane outward currents (Scholz and Byrne 1987). Similar changes in membrane excitability have been observed in several in vitro analogues of long-term sensitization (Dale et al. 2005).

**FIG. 1.** Sensitization training enhanced the tail-elicited siphon withdrawal response. A: schematic of the 4-day training protocol. B: 4 days of sensitization training produced a robust sensitization of the reflex. Twenty-four hours after the end of training, the duration of the siphon withdrawal in response to mild tail stimulation was significantly longer on the side of the animals ipsilateral to the sensitizing stimuli (I, \(n = 23\)) than on the contralateral side (C, \(n = 23\)) or in untrained control animals (U, \(n = 21\)). All values are expressed as the percent increase of posttest values over pretest values. (*, \(P < 0.001\)).
Inc. Excitability was measured as the number of action potentials elicited by a single depolarizing current pulse (1 s, 2 nA). The excitability of sensory neurons on the trained side of the animal seemed to increase over untrained animals. This effect was not statistically significant, however \( F(2,37) = 3.0, P = 0.08 \). This result suggests that enhanced excitability in the sensory neuron may be less important in mediating the effects of long-term sensitization as the duration of the training period is increased. This result is consistent with the idea that the 1- and 4-day training protocols induce mechanistically distinct forms of long-term memory (Wainwright et al. 2002).

After physiological measurements were taken, both the sensory neuron and its follower were labeled by injecting different fluorophores, and the ganglia were prepared for analysis by confocal microscopy as described previously (Zhang et al. 2003). The entire arborization of the sensory neuron in the pedal ganglion was imaged at high resolution, but only the processes of the follower that were in those microscopic fields were imaged. The microscopist did not know the behavioral history of the animal. Four days of sensitization training led to a significant change in the number of varicosities in the tail sensory neuron arborization within the pedal ganglion \( F(2,18) = 27.78, P < 0.0001 \); Fig. 3A and B1. After training, the number of varicosities in sensory neuron arborizations from ipsilateral ganglia was 2.5-fold greater than untrained controls \( (181 \pm 12 \text{ vs. } 71 \pm 7; q = 2.75; P < 0.001) \). The number of varicosities in contralateral ganglia was more than threefold higher than the number in ganglia from untrained controls \( (224 \pm 24 \text{ vs. } 71 \pm 7; q = 10.13; P < 0.001) \). This result is consistent with the earlier demonstration of an increased number of varicosities after 4 days of sensitization training (Wainwright et al. 2002). The previous analysis did not explicitly compare neurite outgrowth and varicosity formation on the two sides of trained animals. The experiment reported here demonstrates that the number of varicosities is increased on both sides of trained animals compared with untrained animals. Thus a structural change was observed in the absence of a behavioral change.

A more functionally significant question is whether long-term sensitization results in an increased number of synapses between physiologically connected neurons. Although we could not conclusively identify functional synapses, we did find that training led to a significant change in the number of appositions between sensory neuron varicosities and motor neuron processes \( F(2,18) = 10.57, P < 0.005 \); Fig. 3C). The number of appositions in ipsilateral ganglia was 1.5 times greater than the number observed in contralateral ganglia \( (Ipsi, 82 \pm 11 \text{ vs. Contra, } 50 \pm 10; q = 2.75; P < 0.001) \) and three times greater than the number observed in untrained controls \( (Ipsi, 82 \pm 11 \text{ vs. Untrained, } 26 \pm 4; q = 10.13; P < 0.001) \). Although the number of appositions on the contralateral side was nearly double the number of appositions in untrained animals, the difference was not statistically significant \( \text{Contra vs. Untrained, } q = 2.768, P > 0.05 \). The induction of new appositions in parallel with enhanced synaptic strength and behavioral response is consistent with, but does not prove, the hypothesis that sensory neuron outgrowth contributes to enhancement of the reflex through the formation of new synapses with follower neurons (Bailey and Chen 1988b).

These results suggest that multiple steps are required to convert new outgrowth into functional synapses. Training induced an increase in the number of varicosities on both trained and untrained sides of the animal (Table 1). Based on
previous results (Wainwright et al. 2002), we infer that there was an increase in neurite outgrowth as well. On the trained side, this outgrowth would result in an increased number of appositions with follower neurons, paralleling the increased synaptic strength and behavioral enhancement. On the untrained side, however, outgrowth would not result in a significant increase in the number of appositions with follower tail motor neurons. Consequently, there is no enhancement of synaptic strength or behavioral response. It is worth noting that although not significant, there was a trend toward an enhanced number of appositions, whereas there was no such trend for EPSP amplitude. Future experiments should examine this point more closely to test the hypothesis that appositions formed on the contralateral side are ineffective or silent. Silent synapses have not been thoroughly characterized in comparison to untrained animals.

There was no difference between the number of varicosities from the ipsilateral and contralateral sides of trained animals. C: training led to a significant increase in the number of appositions between SN varicosities and MN processes. The number of appositions in ipsilateral ganglia (I, n = 6) was 1.5 times greater than the number observed in contralateral ganglia (C, n = 6) and 3 times greater than the number observed in untrained controls (U, n = 7). Although the number of appositions on the contralateral side was nearly double the number in untrained animals, this difference was not statistically significant.

On the trained side of the animal, outgrowth does seem to lead to new contacts and enhanced synaptic strength, suggesting that there is a causal relationship. Nevertheless, additional experiments will be necessary to demonstrate conclusively that newly formed contacts are indeed functional. The time course over which these changes occur remains to be investigated. No changes were observed 24 h after a single training session (Wainwright et al. 2002), suggesting that structural changes are induced slowly. Nevertheless, it is possible that the number of appositions peaked some time before the end of training and was declining by the time we examined the tissue. Moreover, the time course for changes on the ipsilateral and contralateral sides of the animal could be different.

In conclusion, the results of the present study suggest that although structural plasticity is induced by extensive sensitization training, structural changes are not sufficient to account for long-term sensitization. Although these changes appear to increase the capacity for sensory neurons to make contacts with follower cells, other mechanisms must be in place to control the contribution of structural plasticity to long-term memory.

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