

logical, and psychophysical data suggests that the visual processing of form, color, depth, and motion is segregated into separate pathways and structures in the visual cortex (27). We have shown that high spatial resolution optical imaging can be used to visualize many of these components of visual processing in vivo, using activity-dependent intrinsic signals. We have also shown that these subdivisions of visual processing (for example, the blobs of V1 and the thick and thin stripes of V2) can be distinguished by their specialized functional properties and can now more easily be studied by the use of optical maps for guiding single-unit recording and tracer injections (26).

Note added in proof: We have found that by imaging with a shallow depth of field below the cortical surface, blood vessel artifacts can be nearly eliminated (28).

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17. Monkeys (*Macaca fascicularis*) were initially anesthetized with ketamine HCl (20 mg per kilogram of body weight, intramuscular) followed by sodium pentothal (20 mg/kg, intravenous supplemented by further injections as needed). The animal was then cannulated through a tracheotomy, paralyzed with vecuronium bromide ($0.1 \text{ mg kg}^{-1} \text{ hour}^{-1}$), and artificially respired. The electrocardiogram, electroencephalogram, temperature, and expired CO_2 were monitored throughout the entire experiment.
18. A hole was made in the skull above the striate cortex and a stainless steel optical chamber was cemented over the hole. After the dura was opened, the chamber was sealed with a glass cover plate and filled with silicone oil. Single-unit electrical recordings were made through a rubber gasket in the glass cover plate.
19. A slow-scan CCD camera (Photometrics, Ltd.) was

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21. Data analysis began with the summation of frames acquired for each type of visual stimulus. We then divided each of these summed images by the sum of the blank stimulus trials, to minimize the effects of uneven illumination and other common mode features. The resultant summed, blank-adjusted images were subtracted from each other as appropriate for the particular functional property under study (for example, for ocular dominance, the summed, blank-adjusted images from the left eye were subtracted from the right-eye images). The color map of the display was set for either linear 8-bit gray scale translation, or sometimes, pseudo-color mapping designed to facilitate interpretation.
22. In a typical experiment, adequate orientation maps were obtained after cortical images were averaged for 30 min. The signal-to-noise ratio (S/N) of our maps seems at least as good as similar maps from 2-DG studies. For example, a comparison between the densitometry analysis of a 2-DG map of orientation tuning and the optical imaging map of orientation showed that the 2-DG map had a S/N of roughly 5 and the optical imaging map had a S/N of roughly 7 (for 45 min of data acquisition). We estimate that the spatial resolution of this imaging technique is 100 to 150 μm . The functional maps obtained with optical imaging are very reproducible. For example, a pixel-by-pixel comparison between two separate

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Long-Term Synaptic Changes Produced by a Cellular Analog of Classical Conditioning in *Aplysia*

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A change in synaptic strength arising from the activation of two neuronal pathways at approximately the same time is a form of associative plasticity and may underlie classical or Pavlovian conditioning. A cellular analog of a classical conditioning protocol produces short-term associative plasticity at the connections between sensory and motor neurons in *Aplysia*. A similar training protocol produced long-term (24-hour) enhancement of excitatory postsynaptic potentials (EPSPs). EPSPs produced by sensory neurons in which activity was paired with a reinforcing stimulus were significantly larger than unpaired controls 24 hours after training. Thus, associative plasticity at the sensory to motor neuron connection can occur in a long-term form in addition to the short-term form. In this system, it should be possible to analyze the molecular mechanisms underlying long-term associative plasticity and classical conditioning.

CHANGES IN SYNAPTIC STRENGTH are believed to be the basis for short- and long-term memory. A particularly important form of synaptic modification is associative plasticity. Associative synaptic plasticity occurs when activation of a neuron or of a neural pathway at approximately the same time as the activation of a second neural pathway induces changes in synaptic efficacy not observed when the

same pathways are activated noncontiguously. Neuronal changes resulting from associative plasticity are of particular interest, because they are likely to underlie classical or Pavlovian conditioning (1) and because theoretical work indicates that associative plasticity may function as a fundamental "learning rule" for more complex phenomena such as higher order forms of classical conditioning, associative memories, and self-organization of neural networks (2). Although various instances of short-term (minutes to hours) associative synaptic plasticity have

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In *Aplysia* a cellular analog of a classical conditioning paradigm produces a short-term form of associative plasticity (3, 4), termed activity-dependent neuromodulation, at the synapse between the sensory and motor neurons of the siphon and tail withdrawal circuits. In this neural analog of classical conditioning, activation of a sensory neuron represents the conditioned stimulus, electrical stimulation of the tail or of a peripheral nerve represents the reinforcing or unconditioned stimulus, and the excitatory postsynaptic potential (EPSP) in the motor neuron produced by stimulation of a sensory neuron represents the conditioned response. EPSPs produced by sensory neurons in which activity was paired with a reinforcing stimulus exhibited significantly more short-term enhancement than those EPSPs elicited by sensory neurons that were activated in an unpaired fashion with a reinforcing stimulus (3, 4). Although activity-dependent neuromodulation exists in a short-term form at the sensory neuron to follower neuron connection, it is not known whether it also exists at the same site in a long-term form. A long-term form of activity-dependent neuromodulation would provide a neural substrate for the long-term memory of classical conditioning as well as a basis for molecular analyses of the mechanisms underlying long-term associative plasticity and classical conditioning.

To examine whether activity-dependent neuromodulation exists in a long-term form, we applied a cellular analog of a classical conditioning paradigm (4) to the isolated pleural-pedal ganglia of *Aplysia californica* (5). Changes in the amplitude of monosynaptic EPSPs elicited by two sensory neurons in a common motor neuron (6) were used as the measures of associative and nonassociative plasticity. Training consisted of five trials with an intertrial interval of 5 min. Within a training trial (Fig. 1A), each sensory neuron was activated with a train of ten suprathreshold pulses. Onset of the activation of one sensory neuron (paired; SN+) was followed 400 ms later by a reinforcing stimulus composed of a train of electric shocks to a peripheral nerve (nerve shock) (7). The other sensory neuron (unpaired; SN-) was activated 2.5 min before or after activation of SN+ (Fig. 1A).

Stimulation of SN- and SN+ produced EPSPs of similar amplitude in the motor neuron before training (Fig. 1B) (8). Training (Fig. 1A) was initiated immediately after the baseline test. Although the EPSPs elicited by both SN- and SN+ were enhanced 5 min after training (Fig. 1C), those produced

facilitation. The greater enhancement observed in the EPSP produced by SN+ represents short-term activity-dependent neuromodulation (3, 4). After the 5-min test, neurons that neighbored SN-, SN+, and the motor neuron were injected with Fast Green (9) and the chamber was placed in an incubator at 15°C for 24 hr. The following day the same three cells were reimpaired, and the 24-hr test was performed (Fig. 1D). Both SN- and SN+ cells elicited enhanced EPSPs relative to their amplitude before training. The EPSP produced by SN+, however, exhibited a larger increase in amplitude than that of the SN- cell. Thus the training procedure leads to a long-term pairing-specific enhancement of the connection between the sensory neuron and its follower motor neuron.

Data from 14 experiments are illustrated in Fig. 2 (10). Short-term associative plasticity is illustrated by the difference in the amplitude of the EPSPs produced by the SN- and SN+ cells (SN-, $140 \pm 14\%$; SN+, $203 \pm 21\%$) during the 5-min test. Moreover, long-term associative enhancement is evident by the difference in the amplitude of the EPSPs (SN-, $245 \pm 39\%$;

revealed a significant effect of training [$F(1,13) = 13.58$; $P < 0.01$]. In contrast, the analysis of variance revealed that neither the effect of time nor of the time-treatment interaction was significant (11). Thus a classical conditioning procedure that produces short-term associative plasticity leads to the induction of long-term associative plasticity in the same cells.

The degree of associative plasticity (that is, the ratio of the amplitudes of the EPSPs produced by SN+ and SN-) was 1.4 both at the 5-min and 24-hr tests, indicating that the associative plasticity is expressed to the same degree in both its short- and long-term forms. The degree of short-term associative plasticity was similar to that observed previously (4, 12). No significant changes were observed in the input resistance of the motor neuron during either the 5-min or 24-hr test (13). This observation is consistent with previous data on short-term plasticity of these synapses (14) and indicates that generalized postsynaptic changes are unlikely to contribute to long-term plasticity.

The mechanism underlying short-term associative plasticity in *Aplysia*, termed activity-dependent presynaptic facilitation (3) or

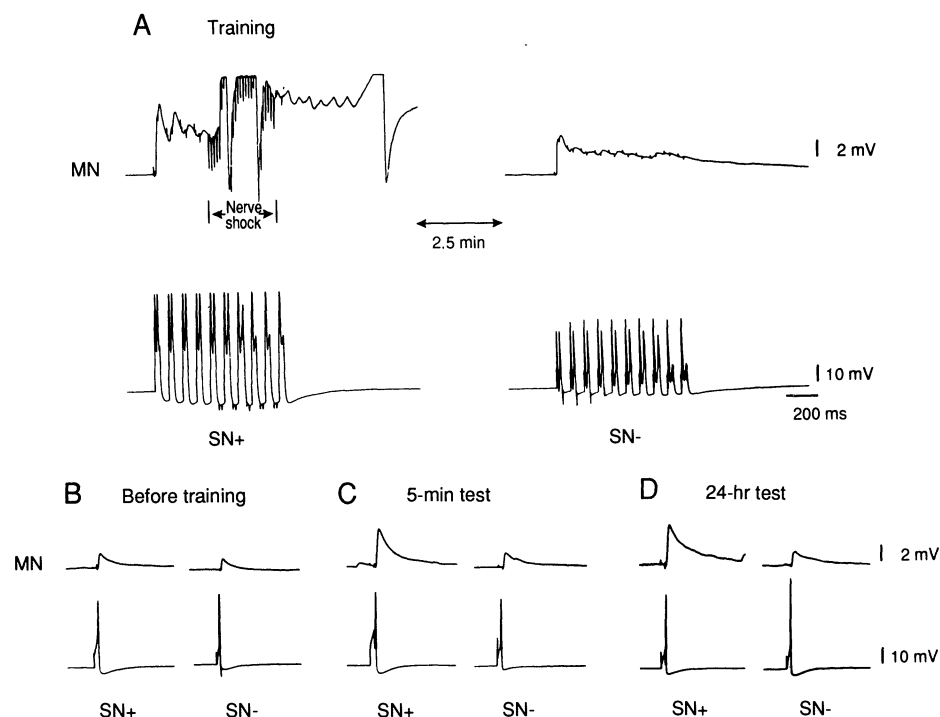


Fig. 1. Training and testing during conditioning of the inputs of two sensory neurons to a motor neuron (MN). (A) Training procedure (illustrated by trial 2). Both sensory neurons were activated with a train of ten depolarizing pulses, eliciting on average 17 spikes. One sensory neuron (SN+) was activated 400 ms before a train of electric shocks to the nerve (nerve shock). The other sensory neuron (SN-) was activated 2.5 min after stimulation of the nerve. (B) Amplitudes of the EPSPs produced by both sensory neurons before training. (C) Amplitude of the EPSPs 5 min after training. (D) Amplitude of the EPSPs produced by the same sensory neurons 24 hr after training. During each test phase three action potentials were elicited in each sensory neuron (the third EPSP of each test is shown).

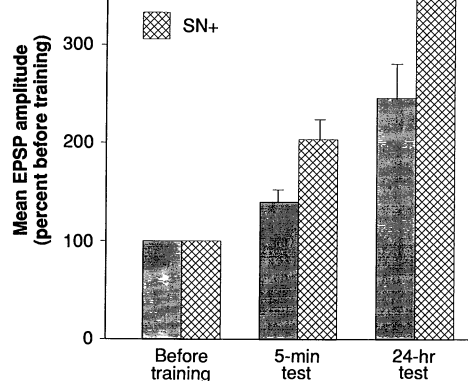


Fig. 2. Long-term associative plasticity of the synaptic connections between sensory and motor neurons. There was a significant difference in the amplitudes of the EPSPs produced by the SN- and SN+ group both at the 5-min and 24-hr test. Error bars represent the SEM.

activity-dependent neuromodulation (4), appears to be an elaboration of the mechanisms of short-term nonassociative plasticity, that is, heterosynaptic facilitation. The mechanism for long-term associative plasticity may rely on the same mechanisms as long-term nonassociative plasticity. The mechanisms contributing to both short- and long-term forms of nonassociative plasticity have been analyzed extensively. Reinforcing stimuli lead to the release of modulatory transmitters that induce an increase in adenosine 3',5'-monophosphate (cAMP) within the sensory neurons (15). Cyclic AMP in turn contributes to short-term facilitation through a cAMP-dependent phosphorylation (16), as well as the induction of long-term facilitation through cAMP-dependent regulation of gene expression (17). Although both short- and long-term nonassociative plasticity appear to use common cellular mechanisms for induction and expression (18), only the latter depends on new protein synthesis (19).

In short-term associative plasticity, the amount of cAMP in the cells is increased above that observed during nonassociative plasticity (20). Calcium influx associated with action potentials in the sensory neurons is thought to interact synergistically with the activation of adenylate cyclase by modulatory transmitters to amplify the synthesis of cAMP (3, 4, 20, 21) and therefore enhance cAMP-dependent effects beyond those produced by modulatory transmitters alone. Thus, for long-term associative plasticity, increased concentrations of cAMP might lead to an enhancement of cAMP-dependent regulation of the same genes involved in long-term nonassociative plasticity. In this case, long-term associative plas-

ticity would be qualitatively different from differences between long-term nonassociative plasticity and long-term associative plasticity (22). The modulatory transmitter and Ca^{2+} signaling pathways could interact synergistically downstream from adenylate cyclase. For example, cAMP and Ca^{2+} could interact to regulate the expression of genes specific for long-term associative plasticity (23). Our results provide an experimental basis for analyzing the molecular mechanisms underlying long-term associative plasticity and long-term forms of classical conditioning.

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5. Before dissection, animals (150 to 300 g) were anesthetized by intracoeleomic injection of a volume of isotonic MgCl_2 equivalent to approximately one-half their body weight. The right pleural-pedal ganglia were removed and placed in a chamber containing equal parts of isotonic L15 medium (Flow) and isotonic MgCl_2 . The L15 medium was made up with additional salts added to bring the medium to seawater conditions [final concentrations: 460 mM NaCl, 11 mM CaCl_2 , 10 mM KCl, 27 mM MgSO_4 , 27 mM MgCl_2 , 2 mM NaHCO_3 , and 15 mM Hepes; modified from S. Schacher and E. Proshansky, *J. Neurosci.* **3**, 2403 (1983)]. Both ganglia were then desheathed to expose the cluster of sensory neurons in the pleural ganglion and the tail motor neurons in the pedal ganglion. Nerves P8 and P9, which carry input from the tail, were drawn into separate suction electrodes so that they could be stimulated electrically. After the dissection and nerve placement were completed, the solution used during the dissection was replaced with 100% L15 medium. The chamber was then placed on a cooling plate and maintained at $15^\circ \pm 1^\circ\text{C}$ during the electrophysiological procedures.
6. Two sensory neurons and one motor neuron were impaled with intracellular electrodes, each of which was filled with 3 M potassium acetate. The sensory and motor neurons were identified as described [E. T. Walters, J. H. Byrne, T. J. Carew, E. R. Kandel, *J. Neurophysiol.* **50**, 1543 (1983)]. The two sensory neurons were always adjacent or separated by a single cell. A necessary condition for an experiment to proceed was that both sensory neurons made monosynaptic connections onto a common motor neuron and had axons in the same nerve, usually P9. The remaining nerve, usually P8, could then be used as the reinforcement pathway for nerve stimulation without antidromically activating either sensory neuron. In seven experiments, the sensory neurons were randomly assigned to the paired (SN+) or unpaired (SN-) groups. For the remaining seven experiments, SN+ and SN- assignments were made in a manner to minimize the difference in the baseline amplitudes of the EPSPs. There was no significant statistical difference in the average baseline amplitude of the EPSPs for the SN- and SN+ groups (2.98 ± 0.6 mV and 2.67 ± 0.67 mV, respectively, $t_{13} = 0.61$). Unless otherwise noted, paired t tests were used for statistical analyses.
7. During each trial, the SN+ cell was activated with a train of ten 40-ms depolarizing pulses at 10 Hz. A 500-ms train of electrical shocks of 3-ms pulses (50 training and set at a current that elicited subthreshold EPSPs in the motor neuron with a single 3-ms pulse. SN- was stimulated 2.5 min after or before activation of SN+. SN- was stimulated with the same parameters used for stimulation of SN+. In half of the experiments, SN+ was stimulated first; in the remaining half SN- was stimulated first. The average number of spikes elicited during stimulation of the sensory neurons was 17.6 ± 0.87 and 17.0 ± 0.87 for the SN- and SN+ groups, respectively. The difference in the number of spikes was not significant ($t_{13} = 0.7$).
8. During the baseline phase, three action potentials were elicited in each sensory neuron with an interstimulus interval of 5 s. As is characteristic of these synapses, we observed some synaptic depression during these three stimuli. Similarly, three action potentials were elicited during each of the two test phases. The 5-min test was begun 5 min after the last train of stimuli was delivered to each sensory neuron.
9. After the 5-min test, a group of at least five sensory neurons surrounding SN- and SN+ were iontophoretically injected with a 30-mM solution of Fast Green. Iontophoresis was performed by applying trains of 50-ms hyperpolarizing current pulses at 10 Hz. Similarly, at least two cells neighboring the motor neuron were injected with Fast Green. A drawing of the position of SN-, SN+, and the motor neuron in relation to the injected cells was then made to permit subsequent identification. The chamber was then placed in an incubator at 15°C for 24 hr. The next day SN-, SN+, and the motor neuron were reimpaled. The analysis proceeded if the sensory neurons had a resting membrane potential of between -35 and -55 mV, and the motor neuron had a resting membrane potential of between -45 and -65 mV. For the 24-hr test, three action potentials were elicited in each sensory neuron and the amplitudes of the EPSPs measured.
10. For data analysis, the three EPSPs produced by a sensory neuron during each test phase were averaged. Each average was then normalized to the average EPSP values before training. This procedure was done for both the SN+ and SN- cells. Group means for each test phase were determined. A two-way analysis of variance with repeated measures on both factors (training and time) was used for the statistical analysis [J. L. Bruning and B. L. Kintz, *Computational Handbook of Statistics* (Scott, Foresman, Glenview, IL, 1977)]. A repeated measures analysis was used for the training factor because (i) the control for each paired cell was the unpaired cell in the same ganglia and the EPSPs were measured from the same motor neuron, and (ii) the degree of facilitation in both cells is correlated within experiments.
11. The significant effect of training along with the absence of an interaction effect [$F(1,13) = 1.67$] indicates that there was a significant effect of training at both the 5-min and 24-hr tests. The effect of time revealed a strong but nonsignificant trend [$F(1,13) = 6.37$]. Increases in the response to both the paired and unpaired conditioned stimuli have been associated with the transition from short- to long-term memory in behavioral experiments in *Aplysia* [T. J. Carew, E. T. Walters, E. R. Kandel, *J. Neurosci.* **1**, 1426 (1981)].
12. Whereas the degree of short-term associative plasticity was similar, the absolute magnitude of the short-term nonassociative effect was somewhat smaller than that observed previously (4). This difference could be due to variations in the intensity of the reinforcing stimulus, or to the fact that stimulation of the tail is more effective than stimulation of the nerve. There were also slight differences in the parameters used for stimulation of the sensory neurons and differences in the temperature at which the experiments were performed.
13. The average input resistance of the motor neuron during baseline, 5-min test, and the 24-hr test was 13.34 ± 1.4 , 13.65 ± 1.7 , and 15.26 ± 1.3 megohms, respectively. Although there was a trend for the motor neuron input resistance to increase over the 24-hr period, these effects were not statistically

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Use of Prior Vaccinations for the Development of New Vaccines

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There is currently a need for vaccine development to improve the immunogenicity of protective epitopes, which themselves are often poorly immunogenic. Although the immunogenicity of these epitopes can be enhanced by linking them to highly immunogenic carriers, such carriers derived from current vaccines have not proven to be generally effective. One reason may be related to epitope-specific suppression, in which prior vaccination with a protein can inhibit the antibody response to new epitopes linked to the protein. To circumvent such inhibition, a peptide from tetanus toxoid was identified that, when linked to a B cell epitope and injected into tetanus toxoid-primed recipients, retained sequences for carrier but not suppressor function. The antibody response to the B cell epitope was enhanced. This may be a general method for taking advantage of previous vaccinations in the development of new vaccines.

IT WOULD BE EXTREMELY USEFUL IF highly immunogenic proteins widely used in vaccines [such as tetanus toxoid (TT)] could be used as carriers to develop new vaccines for poorly immunogenic protective epitopes (such as small peptides). Unfortunately, overall effectiveness with this approach has not been generally achieved (1, 2). Since the antibody response to a hapten coupled with a carrier protein can be inhibited when the recipient has been previously immunized with the unmodified protein (3), it is possible that poor immunogenicity of the hapten + carrier is due to prior vaccinations.

The phenomenon, termed epitope-specific suppression, is related in part to the presence of carrier-specific B cells (4) and suppressor T cells (4, 5). The observation

that peptides recognized by suppressor and helper T cells can be distinct (6) suggested that some peptides might only be functionally recognized by the latter cell type in carrier-primed animals. We therefore attempted to identify a peptide with helper but not suppressor function. The involvement of carrier-specific B cells in epitope-specific suppression provided a criterion for peptide selection; a basis for excluding a peptide from consideration as a carrier sequence would be cross reactivity between the peptide and antibody to the carrier.

The worldwide use of TT prompted us to choose this protein as the model. Furthermore, a conjugate of TT and repeats of the sequence Asn-Ala-Asn-Pro (NANP), the immunodominant sequence of the major surface protein of *Plasmodium falciparum* sporozoites (7, 8), has already undergone clinical testing as a vaccine (2). The goal was to prepare a peptide bearing helper T and

To obtain a peptide capable of eliciting the desired T cell activity, we hydrolyzed TT with trypsin after reduction and alkylation. Peptides in the digest were separated by column chromatography and activity was monitored by in vitro T cell proliferation tests with human peripheral blood leukocytes (PBLs) and lymph node cells from mice injected with TT. Since helper T cells proliferate under these conditions, the assays provided an initial screen for appropriate peptides. A peptide from an active fraction was partially sequenced and, on the basis of the published sequence of TT, a peptide containing amino acid residues 73 to 99 (TT73-99) was synthesized.

The peptide was tested for cross reactivity with antisera against TT. Neither mouse nor human antisera against TT reacted with TT73-99 (Table 1, experiment 1, and Table 2). These data suggested that TT-specific B cells reactive with TT73-99 may not have been sensitized after TT immunization in either species and, therefore, that this potential basis for suppression would be absent. The positive T cell proliferation results in initial screening studies indicated that TT73-99 was recognized by helper T cells. To investigate this, we primed mice with TT73-99 and challenged them with (NANP)₃TT. Such animals produced increased titers of antibody to NANP [anti-NANP] and antibody to TT (anti-TT) (Table 1, experiment 1). The use of BALB/c mice, which are genetically unresponsive to NANP at the T cell level (10), and the absence of antibody cross reactivity between NANP or TT and TT73-99 indicated that priming of the helper T cell had occurred.

TT73-99 contained information for carrier function and did not cross-react with antibody against the parent protein. This peptide should not be susceptible to carrier-specific, B cell-mediated suppression. We predicted that this peptide, when linked to a B cell epitope, would not be susceptible to T cell-mediated suppression in TT-primed mice. We tested this prediction by comparing the effect of prior immunization with TT on the subsequent response to a conjugate containing either the entire protein, (NANP)₃TT, or a peptide with only a portion of the protein, (NANP)₄TT73-99.

As expected, TT priming inhibited the anti-NANP response to (NANP)₃TT even though the anti-TT response was elevated in pretreated mice (epitope-specific suppression). Not only did TT priming fail to inhibit the anti-NANP response to (NANP)₄TT73-99, it actually resulted in the enhancement of the anti-NANP response after primary and secondary chal-

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