Supplemental Data

Experimental Procedures

Cell Cultures

Adult *Aplysia* californica were obtained from local supplier (Alacrity Marine Biological, Redondo Beach, CA, USA). Animals were housed in a 50 gal aquarium filled with cooled (12-14ºC), aerated seawater (Catalina Water Company, Long Beach, CA, USA. All of the experiments used cocultures of pleural sensory neurons and small siphon (LFS-type) motor neurons, which were individually dissociated from central ganglia of *Aplysia* (60-100 g) and placed into cell culture together as described previously [1]. The culture medium contained 50% *Aplysia* hemolyph and 50% Leibowitz-15 (L-15, Sigma, St Louise, MO, USA). The cultures were maintained at 18° C for 3-4 d before the start of the experiments to allow them to form robust monosynaptic connections.

Electrophysiology

The electrophysiological methods used in the experiments on sensorimotor cocultures have been previously detailed [2]. During electrophysiological recording, cultures were perfused with perfusion medium consisting of 50% sterile artificial seawater (ASW) and 50% L-15. All experiments were performed at room temperature. The presynaptic sensory neuron and postsynaptic motor neuron in each coculture were impaled with sharp microelectrodes (20-30 MΩ). In order to prevent spontaneous firing of the motor neurons during testing, the motor neuron was held at -80 to -85 mV by passing negative current (0.3-0.8 nA) into the cell via the

bridge circuit of the microelectrode amplifier. Synaptic strength was determined on Day1 by eliciting a monosynaptic excitatory postsynaptic potential (EPSP) in the motor neuron using brief intracellular stimulation (20 ms, 0.2-0.8 nA positive current) of the sensory neuron (the Pretest). After synaptic assessment the sharp microelectrodes were removed from the sensory and motor neurons. 30-60 min prior to 5-HT treatment the postsynaptic motor neurons were loaded with BAPTA (200 mM in the injection solution, Sigma, St. Louis, MO), the mRNA cap analog m7GppG (2.5 mM in the injection solution), gelonin (10-20 μM in the injection solution, Aczon, Bologna, Italy), or the injection solution (3M KCL, 10 mM HEPES, and 0.2% of the dye Fast Green) alone (in Control cocultures). This was done by impaling a motor neuron with a beveled microelectrode (6-12 MΩ), and then pressure injecting the compound into the cell using 5-10 pulses (50 ms, 10 psi) from a pico-injector. 5-HT was prepared fresh daily as a 2 mM stock solution in ASW, and then diluted to the final concentration of 5-20 μM in the perfusion medium immediately before the first application. To induce long-term facilitation (LTF), cocultures were treated with repeated, spaced applications of 5-HT (five 5-min applications of 5-HT, 20-min interval between applications). After each 5-min application the 5-HT was rapidly washed out with normal perfusion medium for 15 min. Other (Control) cocultures were treated with just the perfusion solution alone. Following the 5-HT or control treatment the cell cultures were rinsed with culture medium and returned to an incubator (18°C). 24 h later the sensory and motor neurons of each coculture were reimpaled with microelectrodes, and the strength of the synaptic connection was reassessed (Posttest).

Immunocytochemistry

Immediately after the last application of 5-HT or control solution, the cocultures were rinsed briefly in ASW, and fixed in 4% paraformaldehyde for 10 min at room temperature, then blocked by 10% donkey serum and 0.5% Tween-20 in 0.01M PBS for 30-60 min. After blocking, the cells were first labeled with rabbit polyclonal antibody specific for sensorin (1:1000) diluted in 1% donkey serum in 0.01 M PBS with 0.5% Tween 20 at 4° C over night, washed twice with PBS for 30 min each, and then labeled with Cy-3-conjugated donkey anti-rabbit IgG antibodies (1:2000, Jackson ImmunoResearch Laboratories, Inc., Suffolk, UK). Negative controls consisted of cocultures that were processed identically, but with the primary antibody omitted. After having been washed in 0.01 M PBS, the cocultures were viewed at 100X with an inverted microscope (Axiovert 25; Carl Zeiss, Germany) attached to a Mercury arc lamp (100 W; HBO 100 AttoArc, Zeiss) for fluorescence excitation. Images were acquired with a cooled-CCD digital camera (ORCA-ER, Hamamatsu Photonics, Hamamatsu City, Japan). The intensity of fluorescent illumination, exposure times and digital gains were kept constant for cocultures processed for immunohistochemistry at the same time. The immunostaining results were analyzed using digital image processing software (AxioVision, Zeiss). The intensity of immunostaining was quantified by measuring the average intensity of the fluorescence in four selected regions of the main axon of the sensory neuron (**Figure S1**). Negative controls showed little immunostaining. The phase contrast image of a coculture shown in **Figure 3A** was obtained with a confocal microscope (LSM 410, Carl Zeiss), and adjusted for brightness and contrast using Adobe Photoshop CS2 (Adobe Systems, San Jose, CA).

Statistical Analyses

For electrophysiological experiments, the peak amplitude of the Posttest EPSP was measured and normalized to the amplitude of the Pretest EPSP for the same coculture. The sensorin staining in the experimental groups was normalized to the average fluorescence intensity in the Control group. The normalized data was expressed as means ± SEM. Initially, a Bartlett's test for homogeneity of variances was performed on the overall data for a given experiment. If this test indicated that the data came from a normal population, analyses of variance (ANOVAs) were performed on the overall group data; these were followed by Student-Newman-Keuls (SNK) post-hoc tests for pair wise comparisons. If, however, the Bartlett's test on the data for an experiment indicated that the data were non-normally distributed (this was the case for the experiments shown in **Figures 1** and **3**), they were log-transformed for subsequent analysis with parametric tests.

Supplemental Results

The mean amplitudes of the Pretest EPSPs for the four groups shown in **Figure 1B** were: Control = 28.7 ± 3.3 mV (n = 14); BAPTA = 28.2 ± 3.8 mV (n = 11); 5-HT = 28.6 ± 3.1 mV (n = 18); and 5-HT-BAPTA = 31.3 ± 3.3 mV (n = 10). These values were not significantly different, as indicated by a one-way ANOVA ($p > 0.9$). The mean amplitudes for the Pretest EPSPs for the experiment presented in **Figures 2A** and **2B** were 23.9 ± 2.7 mV for the Control group (n = 15), 30.0 ± 3.5 mV for the Cap analog group (n = 14), 28.6 ± 1.5 mV for the 5-HT group (n = 20), and 33.0 ± 2.4 for the 5-HT-cap analog group (n = 18). The differences among the groups were not statistically significant (one-way ANOVA, $p > 0.08$). The mean amplitudes of the Pretest

EPSPs for the experiment presented in **Figures 2C** and **2D** were 28.5 ± 2.7 mV in the Control group (n = 25), 23.8 ± 2.2 in the Gelonin group (n = 26), 26.7 ± 2.3 mV in the 5-HT group (n = 23), and 31.0 ± 2.6 mV in the 5-HT-gelonin group (n = 25). A one-way ANOVA indicated that these differences were not significant ($p > 0.1$).

Supplemental Discussion

A problem in evaluating the results of the Sherff and Carew study [3] is that the identity of the postsynaptic neurons that were used is uncertain. The study was performed using pleural-pedal ganglia of *Aplysia*, and the postsynaptic neurons were identified by their presence in a non-homogeneous cluster of cells in the pedal ganglion. Some of these cells have been shown to be tail motor neurons [4]; but this cluster comprises neurons of different sizes and, perhaps, different classes. Therefore, it is possible that a heterogeneous group of synapses were used in the study. An additional complication is that Sherff and Carew did succeed in blocking LTF with postsynaptic gelonin using a different 5-HT treatment protocol. In this protocol, termed the "asymmetric" protocol, 5-HT was applied for 25 min to the pleural ganglion, which contains the cell bodies of the tail sensory neurons, as well as for 5 min to the pedal ganglion, which contains the cell bodies of motor neurons and interneurons, and is also the site of the sensorimotor synaptic connections. The application of 5-HT to the pedal ganglion coincided with the last 5 min of the 5-HT treatment of the pleural ganglion. It is unclear why the two methods used by Sherff and Carew to induce LTF should differ with respect to the requirement for postsynaptic protein synthesis. An intriguing possibility is that the two methods of 5-HT treatment might

have produced changes at different sensorimotor synaptic sites. For example, the asymmetric treatment might have predominately facilitated sensorimotor synapses proximal to the cell bodies of the postsynaptic motor neurons [see 5], whereas the repeated applications of 5-HT applied to the entire pleural-pedal ganglia (the "symmetric" protocol) might have facilitated distal, as well as proximal, synapses. If so, then an injection of a protein synthesis inhibitor into the cell body of the motor neuron would be expected to have a greater effect on facilitation produced by the asymmetric, than the symmetric, protocol.

Supplemental References

1. Lin, X.Y., and Glanzman, D.L. (1994). Long-term potentiation of *Aplysia* sensorimotor synapses in cell culture: regulation by postsynaptic voltage. Proc. Biol. Sci. *255*, 113–118.
2. Li, Q., Roberts, A.C., and Glanzman, D.L. (2005). Synaptic facilitation and behavioral dishabituation in *Aplysia*: dependence upon release of Ca^{2+} from postsynaptic intracellular stores, postsynaptic exocytosis and modulation of postsynaptic AMPA receptor efficacy. J. Neurosci. *25*, 5623-5637.
3. Sherff, C.M., and Carew, T.J. (2004). Parallel somatic and synaptic processing in the induction of intermediate-term and long-term synaptic facilitation in *Aplysia*. Proc. Natl. Acad. Sci. USA *101*, 7463-7468.

4. Walters, E.T., Byrne, J.H., Carew, T.J., and Kandel, E.R. (1983). Mechanoafferent neurons innervating tail of *Aplysia*. I. Response properties and synaptic connections. J. Neurophysiol. *50*, 1522-1542.

5. Zhang, H., Wainwright, M., Byrne, J.H., and Cleary, L.J. (2003). Quantitation of contacts among sensory, motor, and serotonergic neurons in the pedal ganglion of *Aplysia*. Learn Mem *10*, 387-393.

6. Glanzman, D.L., Kandel, E.R., and Schacher, S. (1989). Identified target motor neuron regulates neurite outgrowth and synapse formation of *Aplysia* sensory neurons in vitro. Neuron *3*, 441-450.

Supplemental Figure Legend

Figure S1. Illustration of the Method Used to Quantify the Immunostaining of Sensorin in Sensorimotor Cocultures

(**A**) Four focal regions of staining were selected by drawing four circles of fixed size (~65 μm diameter) along the main axon of sensory neuron that contacted the major neurite of motor neuron. The placement of the circles was designed to maximally cover the major postsynaptic neurite in each coculture, which represents the main site of synaptic contact between the sensory and motor neurons [6]. Consequently, the focal regions most likely corresponded to the area containing the majority of the sensorimotor synapses in each coculture. The intensity of immunostaining was quantified by measuring the average intensity of the fluorescence in each selected circular area using image processing software. Background intensity was determined by

measuring four areas of equivalent size, one at each corner, which contained no neural processes. Scale bar, 20 μm.

(**B**) Net mean intensity of sensorin staining was calculated by subtracting mean background intensity from that of each of the four circular regions covering the probable area of major synaptic contact between the sensory axon and the major motor neurite. The average net staining intensity of the four circular regions represented the sensorin immunostaining intensity for a single coculture. The sensorin measurements for each coculture were then normalized to the overall mean staining intensity of the Control cocultures.