Supplementary Methods

Animals

*Aplysia californica* (90-370 g) were obtained from Alacrity Marine Biological Specimens (Redondo Beach, CA) and Marinus Scientific (Long Beach, CA). Animals were housed individually in perforated plastic cages floating in aerated seawater tanks at a temperature of 15 °C. Animals were fed ~1 g of dried seaweed three times per week. The animals used in the behavioral experiments were food deprived for 3-6 days before training to help ensure that the animals were in a similar motivational state.

Two distinct but complementary approaches were used in this study: 1) preparations of ganglia dissected from behaviorally trained animals, and 2) reduced preparations of ganglia dissected from naïve animals, which were trained *in vitro* with a classical conditioning protocol. Protocols used for *in vivo* and *in vitro* testing and training are described below together with the electrophysiological techniques used to measure the intrinsic and synaptic properties of B51.

Behavioral Training

The protocol for *in vivo* classical conditioning of feeding has been described previously. Briefly, animals were placed in clear plastic cylinders (20 cm × 6.5 cm) submerged inside an aquarium (*Fig. 1a*) during testing and training. The tubes were used to maintain the head toward the top of the tube, which facilitated the application of the CS and US to the lips and the observation of feeding behavior. Small pieces of dried seaweed (~15 mg) were used as the US. The seaweed pieces were presented directly to the lips of the animal with blunt forceps. This stimulus reliably elicited feeding. The US presentation lasted either until the food was ingested, or for a maximum
of 60 s, if ingestion failed to occur. Biting was defined by the opening of the jaws, the protraction of the odontophore/radula (a tongue-like structure) past the jaws, subsequent retraction of the odontophore/radula and closure of the jaws (for details, see supplemental video in ref. 4). Tactile stimulation of the lips with a paintbrush (size number 3) was used as the CS (Fig. 1a). The duration of the CS was 8 s. For paired training, the interstimulus interval (ISI) between the onset of the CS and the US was 3 s. For unpaired training, the ISI was 120 s.

The training protocol is illustrated in Figure 1a. Animals were placed in the tubes in a head-up position. After a 10-min rest phase, the response of the animal to the paintbrush application to the lips (CS) was measured (pre-test). During pre-test, the animals received four CS applications separated by 60-s intervals and the number of bites occurring during a 60-sec period following each CS was counted. The total observation time of the pre-test was 4 min. The animals were assigned to either the paired or unpaired group, such that their pre-test scores were balanced. This procedure controlled for any naïve bias toward the CS among the animals.

Training consisted of ten trials with a 4-min intertrial interval (ITI; Supplementary Fig. 1a). Sixty min after training, four CSs were delivered 60 s apart (post-test). As in the pre-test, the number of bites that occurred during a 60-sec period following each CS was counted. The total observation time of the post-test was 4 min. The post-test was conducted by an observer who was blind to whether the animal received paired or unpaired training. For each animal, the number of bites produced during the pre-test was subtracted from the number of bites produced during the post-test (i.e., difference in number of bites). Animals that failed to ingest the seaweed more than once during the training phase or before training were excluded from analysis. Any animal that inked during handling or training was also excluded from analysis. The animals that successfully completed the training were then dissected and the buccal and cerebral ganglia were
removed to enable the measurement of neurophysiological correlates of *in vivo* classical conditioning in B51 (see below).

**Neurophysiological Correlates of *In Vivo* Classical Conditioning in Neuron B51**

Ganglia from animals that received *in vivo* training were then prepared for extracellular nerve stimulation and intracellular measurements of intrinsic properties and synaptic inputs to B51, which were made following standard procedures\(^2,5,6\). Briefly, once the behavioral post-test was concluded, animals were anesthetized by injecting a volume of isotonic MgCl\(_2\) equivalent to 50% of the weight of the animal. Buccal and cerebral ganglia were removed and pinned on a Sylgard-coated Petri dish containing artificial seawater with a high concentration of divalent cations (high divalent ASW). The high divalent ASW was used to decrease neural activity during further dissection\(^7\) and its composition was (in mM): NaCl 210, KCl 10, MgCl\(_2\) 145, MgSO\(_4\) 20, CaCl\(_2\) 33, and HEPES 10 (pH adjusted to 7.4 with NaOH).

The most medial and ventral branch of the right anterior tentacle nerve (denoted AT4) was retained, as it innervates the lip region that received the CS during behavioral testing and training\(^1,2\). Electrical stimulation of AT4 (**Fig. 1b**) was used in all reduced preparations to mimic the mechanical stimulation of the lips, which represented the *in vivo* CS during behavioral testing and training\(^2\). For stimulation, bipolar electrodes were placed on AT4 and isolated from the bath with Vaseline.

The right buccal hemi-ganglion was desheathed on the rostral side to access the soma of neuron B51 (**Fig. 1b**). After desheathing, the high divalent ASW was exchanged for normal ASW, which was composed of (in mM): NaCl 450, KCl 10, MgCl\(_2\) 30, MgSO\(_4\) 20, CaCl\(_2\) 10, and HEPES 10 (pH adjusted to 7.4 with NaOH). The temperature of the bath was maintained at 15
³C. Conventional two-electrode current-clamp techniques were used for intracellular recordings (Axoclamp-2A, Axon Instruments, Burlingame, CA). Fine-tipped glass microelectrodes (resistance 10-15 MΩ) were filled with 2 M potassium acetate. The soma of neuron B51 was identified based on its relative size and position within the right hemiganglion and by the occurrence of its characteristic plateau potential.

On average, about 100 min transpired between the conclusion of the behavioral post-test and the impalement of B51. Beginning five min after impalement, the resting membrane potential, the input resistance, the burst threshold, the magnitude of the CS-evoked synaptic input to B51 and the number of CS-evoked plateau potentials in B51 were measured. If a spontaneous motor pattern occurred while recording the intrinsic and synaptic properties of B51, measurements were halted and then resumed 60 sec after the cessation of the pattern. The input resistance, the burst threshold of B51 and the magnitude of the CS-evoked synaptic input to B51 were measured while the cell was current-clamped at –60 mV. The input resistance of B51 was determined by injecting a hyperpolarizing current of 5 nA for 5 s. The burst threshold of B51 was defined as the minimum amount of depolarizing current necessary to elicit sustained activity in B51 that outlasted the duration of the current pulse. The burst threshold was determined by delivering a series of successively greater amplitude depolarizing current pulses (pulse duration = 5 s). A 10-s rest period between the end of one pulse and the start of another was used.

The magnitude of the CS-evoked synaptic input to B51 was next quantified by delivering a single 5-sec duration train (5 Hz, 0.5-msec pulses, 10 V) to AT4 while the membrane potential of B51 was current clamped at –60 mV, which matched the parameters of the electrical stimulus that was previously used to test the ability of AT4 to evoke BMPs in vitro following behavioral training. Electrical stimulation of AT4 elicits a complex postsynaptic potential (cPSP) in B51.
with two consistently observed components; an early hyperpolarizing phase followed by a late depolarizing phase (Fig. 2a,c). Fast IPSPs are also frequently observed and are likely from neuron B52. The peak amplitude of both components was measured for each preparation. After these properties were measured, the cell was released from current clamp and the resting membrane potential was determined. Next, the number of CS-evoked plateau potentials in B51 was measured by delivering four trains of electrical shocks to AT4 (5 s, 5 Hz, 0.5-ms pulses, 10 V) presented 60 s apart and counting the number of plateau potentials in B51 occurring during a 60-sec period following each CS. The total observation time was 4 min. The experimenter performing the intracellular measurements was unaware of the experimental history of the animals.

**Procedures for In Vitro Classical Conditioning**

The second complementary approach used in this study consisted of measuring the intrinsic properties of B51, the magnitude of the CS-evoked synaptic input to B51, and the number of CS-evoked plateau potentials in B51 in ganglia from naïve animals prior to and after the delivery of a training protocol *in vitro*³ (i.e., *in vitro* analogue of classical conditioning). The procedures for isolation of the cerebral and buccal ganglia, removal of the connective sheath from the right hemiganglion, placement of the extracellular electrodes for recording/stimulation as well as identification and recording of the intrinsic and synaptic properties of B51 were identical to those described in the previous section. Intracellular recordings were performed using conventional two-electrode current-clamp techniques (Axoprobe-1A, Axon Instruments, Burlingame, CA). In the configuration used for the *in vitro* analogue of classical conditioning, electrical stimulation of AT4 (8 s, 5 Hz, 0.5-ms pulses) served as the analogue of the CS during *in vitro* testing and
training\(^3\) and was also used to measure the magnitude of the CS-evoked synaptic input in B51 and the number of CS-evoked plateau potentials. The anterior branch of the esophageal nerve (denoted E n.2) was also retained as electrical stimulation of this nerve (4 s, 10 Hz, 0.5-ms pulses) served as the analogue of the US during *in vitro* training\(^3\). In addition, the radula nerve 1 (R n.1), the nerve of the intrinsic buccal muscle 2 (I2 n.), and buccal nerve 2,1 (n.2,1) of the right hemi-ganglion were also retained (*Fig. 1b*). Electrodes for extracellular recording were placed on these three nerves and the nerve signals were amplified with a differential AC amplifier (model 1700, A-M Systems, Everett, WA). Recording from these nerves enables the monitoring of buccal motor programs (BMPs), which are *in vitro* correlates of feeding behavior\(^9\)\(^{-13}\). Activity in I2 n. corresponds to the protraction of the radula, whereas activity in n.2,1 corresponds to the retraction of the radula, and large-unit activity in R n.1 corresponds to closure of the radula. In the present study, only patterns that consisted of activity in all three buccal nerves clustered in a complete protraction/retraction cycle were classified as BMPs. Patterns consisting of trains of activity in only one or two of the three nerves were classified as incomplete patterns and were not included in the study.

In the experiments with the *in vitro* analogue of classical conditioning, the intrinsic properties of B51 (i.e., resting membrane potential, input resistance and burst threshold) and the magnitude of the CS-evoked synaptic input to B51 were measured prior to and after *in vitro* training (i.e., before the pre-test and the post-test, respectively, see below). To ensure that the preparations were healthy, neurons with an initial resting membrane potential more depolarized than \(-60\) mV or those that exhibited amplitudes of the initial CS-evoked peak depolarization less than \(0.5\) mV were discarded. The changes in the intrinsic properties of B51 and the magnitude of
the CS-evoked synaptic input to B51 as a result of *in vitro* training were expressed as the difference between the post-test and pre-test values normalized to the pre-test value.

The *in vitro* protocol for classical conditioning was described previously\(^3\). Briefly, electrical stimulation of AT4 (8 s, 5 Hz, 0.5-ms pulses) served to mimic the CS and electrical stimulation of E n.2 (4 s, 10 Hz, 0.5-ms pulses) served to mimic the US (Fig. 1b). Paired and unpaired protocols were used. In the paired protocol, the CS preceded the US with an ISI of 4 s, and the CS and the US overlapped for 4 s. In the unpaired procedure, the ISI between CS and US was 120 sec. In both protocols, training consisted of ten trials with 4 min ITI (Supplementary Fig. 1a). Once the extracellular electrodes were in place, the high divalent ASW was exchanged for normal ASW. Preparations were rinsed for 10 min in normal ASW and then neuron B51 was impaled and identified. The intensity for stimulation of E n.2 was adjusted so that each US reliably elicited a BMP (9.7 ± 0.1 V, *n* = 15 paired group; 9.4 ± 0.1 V, *n* = 15 unpaired group; Mann-Whitney *U* test, *P* = 0.110). The intensity used for AT4 electrical stimulation was adjusted for each preparation so that a single CS induced only sporadic large-unit activity in the buccal nerves, but was not sufficient to elicit a BMP (7.8 ± 0.8 V, *n* = 15 paired group; 7.2 ± 0.6 V, *n* = 15 unpaired group; Mann-Whitney *U* test, *P* = 0.663). However, occasional BMPs were expressed by the presentation of four CSs during the pre-test (see below), but the average number of CS-evoked BMPs during the pre-test was modest both in the paired (0.33 ± 0.13 BMPs, *n* = 15) and unpaired (0.47 ± 0.16 BMPs, *n* = 15) groups, and did not differ significantly (Mann-Whitney *U* test, *P* = 0.692). Similarly, the average number of plateau potentials in B51 during the pre-test was modest both in the paired (0.46 ± 0.14 plateau potentials, *n* = 13) and unpaired (0.23 ± 0.12 plateau potentials, *n* = 13) groups, and did not differ significantly (Mann-Whitney *U* test, *P* = 0.327). With the intensities used in this study, the US reliably elicited
plateau potentials in B51 during in vitro training (Supplementary Fig. 1b). During unpaired training, the average number of US-evoked plateau potentials was 8.33 ± 1.30 and the average latency of the first plateau potential evoked by the US over the 10 trials was 51.4 ± 3.8 sec. During paired training, the average number of plateau potentials evoked by the CS-US pairing was 11.86 ± 2.07 and the average latency of the first plateau potential evoked by the CS-US pairing over the 10 trials was 48.2 ± 4.2 sec. Stimulus intensities for the CS and US remained fixed for the remainder of the experiment. Preparations were included if, during training, the US stimulation elicited a BMP in more than half of the ten training trials. To test the ability of the CS to evoke BMPs, a pre-test and a post-test were performed 10 min prior to and 60 min after training, respectively. The pre-test and the post-test were identical and both consisted of four CSs, which were delivered 60 s apart. For both pre-test and post-test, the number of BMPs and the number of plateau potentials in B51 occurring during a 60-sec period following each CS were counted. The total observation time of the pre-test and post-test was 4 min. The difference in the number of CS-evoked BMPs was compared between the paired and unpaired groups. The difference in the number of CS-evoked BMPs was defined as the number of BMPs elicited by four CSs during the post-test minus the number of BMPs elicited by four CSs during the pre-test. Similarly, the difference in the number of CS-evoked plateau potentials (i.e., the number of plateau potentials elicited by four CSs during the post-test minus the number of plateau potentials elicited by four CSs during the pre-test) was calculated for each preparation.
**Statistical Analysis**

All values were expressed as means ± s.e.m. Statistical significance was set at $P < 0.05$. Two-tailed Mann-Whitney tests ($U$) were used for all statistical comparisons between groups of animals or reduced preparations trained with the paired and unpaired protocols. Statistics were performed using SigmaStat 2.0 (Jandel Scientific, San Rafael, CA).

**References**