Changes in neuronal excitability serve as a mechanism of long-term memory for operant conditioning

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Supplementary Fig. 1  Procedures for long-term in vitro operant conditioning.  (a) In vitro preparation consisting of the isolated buccal ganglion (left panel).  Fictive feeding behavior (iBMP) was recorded extracellularly from the peripheral nerves controlling protraction, closure and retraction of the radula (right panel).  Electrical stimulations of the E n.2 nerve was used as reinforcement (black arrow).  Monotonic electrical stimulation of buccal nerve n.2,3 was used to activate the CPG during training and during a 10-min observation time 24 h after training (white arrow).  (b–d) In vitro training protocols.  (b) In the contingent protocol, the reinforcement was delivered immediately after the expression of iBMPs.  (c) In the yoke group protocol, the reinforcement was not contingent with iBMPs.  (d) Preparations in the control group did not experience any reinforcement during training.
Supplementary Fig. 2  Protocol for cAMP injection and measurement of B51 membrane properties and plateau potentials. (a) In naïve ganglia, B51 was impaled with one standard electrode filled with potassium acetate and one electrode filled with a solution containing either cAMP or vehicle. (b) The membrane properties of B51 (i.e., resting membrane potential, input resistance and burst threshold) were measured before injection (pre-test). Then, B51 was injected with either cAMP or vehicle (see below). (c) 24 h after injection, B51 was again identified. Bipolar electrodes were also placed on the n.2,3 nerve for monotonic electrical stimulation (white arrow). (d) B51 membrane properties were again measured (test). In addition, the number of plateau potential elicited by n.2,3 stimulation were counted during a 10-min observation time (c,d). (e) The injection protocol consisted of 7 impulses of hyperpolarizing current (30 sec, 20 nA) delivered over a 10-min period.
Supplementary Methods

Animals

*Aplysia californica* 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. Animals were food deprived for 3-5 days before training to help ensure that they were in a similar motivational state.

Procedures for Long-Term *In Vitro* Operant Conditioning

To study the mechanisms of long-term memory (LTM) of operant conditioning, we slightly modified a previously developed *in vitro* analogue of operant conditioning of feeding behavior of *Aplysia*1-3. As in the original protocol, naïve animals were anaesthetized by injecting a volume of isotonic MgCl₂ equivalent to 50% of the weight of the animal. The buccal ganglia were removed and pinned on the Sylgard-coated bottom of a Plexiglas recording chamber 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 dissection⁴ and its composition was (in mM): NaCl 210, KCl 10, MgCl₂ 145, MgSO₄ 20, CaCl₂ 33 and HEPES 10 (pH adjusted to 7.5 with NaOH). The temperature of the bath was maintained at 15 °C. In the first series of experiments, buccal ganglia were kept intact (i.e., not desheathed) during the entire duration of the experiment. For nerve stimulation, bipolar electrodes were placed on the anterior branch of the esophageal nerve (denoted E n.2) and on the ipsilateral buccal nerve 2,3 (denoted n.2,3) and they were isolated from the bath with Vaseline. 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), contralateral to the stimulated E n.2 and n.2,3, were also retained (Fig. 1a, Supplementary Fig. 1a). 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).
Recording from these nerves were used to monitor the occurrence of buccal motor programs\(^1\) (BMPs, Fig. 1a, Supplementary Fig. 1a). 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. BMPs consist of specific patterns of neural activity, which represent cycles of protraction and retraction of the radula and odontophore\(^5-6\). 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. During a BMP, large-unit activity in I2 n. precedes large-unit activity in n.2,1 and large-unit activity in R n.1 (i.e., radula closure) overlaps to a varying extent with protraction and retraction activity\(^1,5,6\).

The large-unit activity in R n.1 corresponds to action potentials in the radula closure motor neuron B8, which has an axon in R n.1\(^3,6\). BMPs were classified as ingestive BMPs (iBMPs), which are neural correlates of ingestive behavior, if \(\geq 50\%\) of radula closure activity occurred after the termination of the protraction\(^1,5-10\) (Fig. 1a, Supplementary Fig. 1a). The criterion for classification of egestive BMPs (eBMPs), which are neural correlates of rejection behavior, was the occurrence of radula closure activity during the protraction, but no overlap between radula closure activity and retraction activity\(^1,5-10\) (see Fig. 2 in reference [1] for a detailed comparison between ingestive and egestive patterns).

In the first series of experiments, three training protocols were used: contingent, yoke and control (Supplementary Fig. 1b-d). After the nerves were prepared for stimulation and recording, 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 7.5 with NaOH). As in previous studies, electrical stimulation of E n.2 (6 s, 10 Hz, 0.5-ms pulses, 9 V) was used as reinforcement\(^1,11\) (black arrow in Fig. 1a and in Supplementary Fig. 1a). Monotonic electrical stimulation of buccal nerve n.2,3 (2 Hz, 0.5-ms pulses, 7 V) was used to activate the feeding central pattern generator (CPG) during training and during a 10-min observation time 24 h after training\(^1\) (white arrow in Fig. 1a and in Supplementary Fig. 1a). The original protocol used a 10-min training period. However, in an effort to increase the likelihood of inducing
long-term memory, a 15-min training period was used in the present study. A similar approach was successfully used in vivo to induce long-term memory of operant conditioning. Experiments were conducted in blocks of three matched preparations. In a single block, each preparation received one of the three different stimulus protocols: contingent, yoke or control (Supplementary Fig. 1b-d). In the contingent group, the reinforcement was delivered immediately after the expression of iBMPs (Supplementary Fig. 1b). In the yoke group, the reinforcement was applied with the same parameters and the same timing as that in its matched contingent preparation in the block (Supplementary Fig. 1c). Thus, in the yoke preparations the reinforcement was not contingent with iBMPs. Preparations in the control group did not experience any reinforcement during training (Supplementary Fig. 1d). In the original protocol, testing occurred immediately followed training (Supplementary Fig. 1b-d). To maintain the preparations for 24 h, the ganglia were bathed in a modified L-15 ASW once the training was concluded and were stored at 15 °C. The modified L-15 ASW consisted of L-15 medium (Sigma) supplemented with (in mM) NaCl 293.1, KCl 4.6, MgCl2 26, MgSO4 18.9, CaCl2 10.9, HEPES 30 and 0.10 g/l of streptomycin (Sigma) and 0.12 g/l of penicillin-G (Sigma), pH adjusted to 7.5 with NaOH. The nerve electrodes were kept in place after training to allow for n.2,3 stimulation 24 h after training and iBMP recordings. To prevent evaporation, the recording chamber was placed inside a small Styrofoam box with wet paper towels. 24 h after training, ganglia were rinsed in normal ASW and the number of iBMPs was counted during a 10-min observation time and compared among the contingent, yoke and control groups (Supplementary Fig. 1b-d). Preparations were included in the analysis if they were capable of eliciting at least one complete BMP 24 h after training. The percentage of discarded preparations that did not fulfill this criterion was 19%.
Long-Term Effects of *In Vitro* Operant Conditioning on the Membrane Properties of Neuron B51 and on its Ability to Generate Plateau Potentials

The second series of experiments only included two training protocols: contingent and yoke. Procedures for dissection, training, storage and testing of the ganglia were similar to those described above. 24 after training, the ganglia were briefly bathed with high divalent ASW and the buccal hemi-ganglion contralateral to the stimulated E n.2 and n.2,3 was desheathed on the rostral side to access the soma of neuron B51 (Fig. 1a). The solution was replaced with normal ASW after the desheathing. B51 exhibits a characteristic all-or-nothing sustained level of activity (i.e., plateau potential) during the occurrence of iBMPs (Fig. 1a), which is critical for the expression of this motor pattern. It was not possible to desheath the buccal hemi-ganglion in the presence of all extracellular nerve electrodes. Therefore, the stimulating electrode on E n.2 and the recording electrodes on R n.1, I2 n. and n.2,1 were removed before desheathing. Only the stimulating electrode on n.2,3 was retained. Because the occurrence of plateau potentials in B51 correlates with the expression of iBMPs, measuring the number of CPG-elicited plateau potentials in B51 was considered an indirect, but reliable, readout of the occurrence of iBMPs in the absence of nerve recordings. Conventional two-electrode current-clamp techniques were used for intracellular recordings (Axoprobe-1A, Molecular Devices). Fine-tipped glass microelectrodes (resistance 8-10 MΩ) were filled with 2 M potassium acetate. The soma of neuron B51 was identified based on its relative size and position within the hemi-ganglion and by the occurrence of its characteristic plateau potential. Beginning 5 min after impalement, the resting membrane potential (RMP), the input resistance (R\text{in}) and the burst threshold (BT) of B51 were measured. If a spontaneous motor pattern occurred while recording the intrinsic properties of B51, measurements were halted and then resumed 60 sec after the end of the motor pattern. The R\text{in} and the BT of B51 were measured while the cell was current-clamped at –60 mV. The R\text{in} of B51 was determined by injecting a hyperpolarizing current of 5 nA for 5 s. The BT 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 BT 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. 10 min after the end of the measurement of B51 membrane properties, the number of plateau potentials was also counted during a 10-min observation time during which monotonic electrical stimulation of n.2,3 was presented. In this case, a burst of activity in B51 was classified as a plateau potential when it occurred at a frequency > 4 Hz and for > 1 s. The presence of these patterns of activity in B51 was previously associated with the occurrence of iBMPs². As previously noted¹, a slightly stronger stimulation of n.2,3 was used in desheathed preparations (4 Hz, 0.5-ms pulses, 8.5 V). The RMP, R_in, BT of B51 and the number of plateau potentials were compared between the contingent and yoke groups.

**Long-Term Effects of Iontophoretic Injection of cAMP onto B51 on its Membrane Properties and its Ability to Generate Plateau Potentials**

In the third series of experiments, buccal ganglia were removed from naïve animals and one hemi-ganglion was desheathed on the rostral side to access the soma of neuron B51 (Supplementary Fig. 2a). n.2,3 contralateral to the desheathed hemi-ganglion was left intact and prepared for nerve stimulation (Supplementary Fig. 2a). Once B51 was impaled and identified using a conventional potassium acetate electrode, a second electrode filled with either cAMP (150 mM cAMP, 200 mM KOH, 20 mM TRIS, pH 7.5) or vehicle (200 mM KOH, 20 mM TRIS, pH 7.5) solutions was inserted in the soma (Supplementary Fig. 2a). This concentration of cAMP was selected because it produced short-term changes in B51 membrane properties (Lorenzetti, Baxter and Byrne, Society for Neuroscience Abstract 2006). Experiments were conducted in blocks of two preparations, in which the injections of either cAMP or vehicle were alternated. The membrane properties of B51 were measured before the beginning of the injection protocol (pre-test; Supplementary Fig. 2b). Then, cAMP or vehicle was iontophoretically injected into B51 using a protocol, which consisted of seven hyperpolarizing current pulses (20 nA, 30 sec) over a 10-min period (Supplementary Fig. 2b-e). This number of current pulses was selected because seven reinforcements¹⁴, were capable of inducing short-term changes in B51 membrane properties both in the *in vitro* and in the
single-cell analogue of operant conditioning\textsuperscript{3,11}. After injection, buccal ganglia were kept in the incubator at 15 °C, bathed in L-15 ASW for 24 h. 24 h after injection, the solution was replaced with normal ASW and B51 was identified again and its membrane properties were measured (test; Supplementary Fig. 2c,d). 10 min after the end of the measurement of B51 membrane properties, the number of plateau potentials was also counted during a 10-min observation time of n.2.3 monotonic stimulation (4 Hz, 0.5-ms pulses, 8.5 V; Supplementary Fig. 2c,d). The changes in the intrinsic properties of B51 as a result of the injection protocol were expressed as the difference between the test and the pre-test values normalized to the pre-test value and compared between preparations in which B51 was injected with either cAMP or vehicle. In addition, the number of plateau potentials counted during a 10-min observation time was compared between preparations in which B51 was injected with either cAMP or vehicle.

**Single-Cell Analogue of Operant Conditioning**

Culturing procedures followed those previously described\textsuperscript{11,15-17}. Buccal ganglia from adult *Aplysia* were incubated in 1% protease type IX (Sigma) at room temperature for 24 h and then desheathed. B51 neurons were removed from the ganglia by microelectrodes with fine tips and plated on poly-L-lysine coated petri dishes with culture medium containing 50% hemolymph and 50% isotonic L15 (Sigma). The cells were allowed to grow for 4-5 days and the medium was changed on the third day. Culture medium was exchanged for ASW prior to recording. The electrophysiological methods used to record from cultured neurons were similar to those used to record from neurons in the ganglia and the training protocol for the single-cell analogue was identical to the protocol established previously\textsuperscript{11}. The cells were current clamped to -80 mV. Five minutes after impalement, $R_{in}$ and BT were determined. Due to the high input resistance of cultured cells, less current was needed to measure $R_{in}$ and BT. $R_{in}$ was tested by injecting a hyperpolarizing current pulse of 0.5 nA for 5 s and BT was tested in 0.1 nA increments. The cells were then divided into either a contingent reinforcement group or an unpaired control group. Plateau potentials were generated by a 5-s long depolarizing current pulse with an amplitude 0.1 nA higher than the previously determined
threshold. Both groups received 7 evenly spaced supra-threshold depolarizing current pulses over a ten-minute training period. The cells in the contingent reinforcement group received a 6-s iontophoretic pulse of dopamine immediately after the plateau potential, whereas iontophoresis was delayed by 40 s in the unpaired control group. Dopamine was iontophoresed through a fine-tipped glass microelectrode (resistance 10-15 MΩ). A retaining current of -1 nA was used during the course of the experiment. A square wave current pulse of 35 nA for 6 s was used to eject the dopamine. The concentration of dopamine in the electrode was 200 mM. An equimolar concentration of ascorbic acid was added to the electrode to reduce the oxidation of dopamine. The membrane properties were measured 24 h after training and compared to the pre-test levels. Recordings were performed at room temperature.

The membrane permeable competitive cAMP inhibitor Rp-8-Br-cAMPS (Rp-cAMP, Biolog, Bremen, Germany) was used to block the long-term contingent-dependent changes produced by the single-cell analogue. Rp-cAMP was bath applied to cultured B51 neurons to a final concentration of 2 mM. The Rp-cAMP was applied for 30 min prior to the start of the single-cell analogue as described above and the inhibitor was maintained in the bath throughout the experiment. After the 30 min incubation, the membrane properties were measured (pre-test), the single-cell analogue was performed, and then the culture medium was changed to remove the Rp-cAMP from the bath. The membrane properties were measured 24 h after training (test) and compared to the pre-test levels.

Statistical Analysis

All values were expressed as means ± s.e.m. Statistical significance was set at \( P < 0.05 \). Comparisons between three groups (contingent, yoke, control; contingent, unpaired, contingent + Rp-cAMP) were made with the Kruskal-Wallis test (\( H \)). When indicated, post hoc pairwise comparisons were performed using the nonparametric analog to the Student-Newman-Keuls multiple range test (\( q \))\(^{19} \). Two-tailed Mann-Whitney tests (\( U \)) were used for the statistical comparisons of the remaining experiments in which two groups of
preparations were used (contingent vs. yoke, or cAMP vs. yoke). Statistics were performed using SigmaStat
2.0 (Jandel Scientific, San Rafael, CA).

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