

mediate the biological activities of other natural products with metabolic effects. For example, the activation of hPXR-SXR by the hyperforin present in the herbal antidepressant St. John's Wort results in undesirable effects on drug metabolism (30, 31). It is an intriguing possibility that further characterization of the effects of natural products on such receptors will identify additional agents that, like guggulsterone, have more desirable activities.

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- probe (5' ATGTGCCAGGCCTCCGTGCCT) labeled with 6-carboxy fluorescein (FAM) reporter fluorescent dye, and a 50 nM and 300 nM final concentration of forward (5' GTACCTGAAGGGCAGCATCC) and reverse (5' AGCCTCCTGTGCAGGTGT) primers, respectively. For analysis, 1 ng of total RNA isolated from primary hepatocytes was used per reaction. The cycle parameters included a reverse transcription step at 48°C for 30 min, followed by 40 cycles of 95°C denaturation and 60°C annealing and extension. The 18S rRNA was used for the endogenous control.
39. For FRET analysis, the human FXR ligand-binding domain (LBD) (amino acids 244 to 472) was expressed as a GST-FXR-LBD fusion protein (glutathione S-transferase fused to FXR-LBD) in DH5 $\alpha$  and purified using glutathione beads. The FRET assay was performed by incubating 8 nM of GST-FXR-LBD, 8 nM of Europium-labeled antibody to GST (Wallac, PerkinElmer Life Sciences, Boston, MA), 16 nM biotin-SRC-1 peptide [5'-biotin-CPSSSHSLTERHKILHRLLEQEGSPS-CONH2] (32), 20 nM allophycocyanin conjugated streptavidin (APC-SA) (Wallac) in FRET assay buffer (20 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> (pH 7.3), 150 mM NaCl, 2 mM CHAPS detergent, 2 mM EDTA, 1 mM dithiothreitol (DTT) in the presence of the test compound(s) for 2 to 4 hours at room temperature. Data were collected using an LJI Analyst (Molecular Devices, Sunnyvale, CA). The results are expressed as 1000 $\times$ (665 nm/615 nm).
40. Experimental diets consisted of control diet (TEKLAD 7001, Harlan Teklad, Madison, WI) supplemented

with 2% cholesterol. Male 8- to 12-week-old mice were used for all experiments and were allowed water ad libitum. Z-Guggulsterone was resuspended in 0.2-ml saline and administered to mice by oral gavage. Control animals received the same amount of saline. At the end of the experiment, mice fasted for 4 hours, after which time livers were harvested and snap-frozen in liquid nitrogen and then stored at -80°C until use.

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# Operant Reward Learning in *Aplysia*: Neuronal Correlates and Mechanisms

Björn Brembs,\* Fred D. Lorenzetti,\* Fredy D. Reyes, Douglas A. Baxter, John H. Byrne†

Operant conditioning is a form of associative learning through which an animal learns about the consequences of its behavior. Here, we report an appetitive operant conditioning procedure in *Aplysia* that induces long-term memory. Biophysical changes that accompanied the memory were found in an identified neuron (cell B51) that is considered critical for the expression of behavior that was rewarded. Similar cellular changes in B51 were produced by contingent reinforcement of B51 with dopamine in a single-cell analog of the operant procedure. These findings allow for the detailed analysis of the cellular and molecular processes underlying operant conditioning.

Learning about relations between stimuli [i.e., classical conditioning (1)] and learning about the consequences of one's own behavior [i.e., operant conditioning (2)] constitute the major part of our predictive understanding of the world. Although the neuronal mechanisms underlying appetitive and aversive classical conditioning are well studied (e.g., 3–8), a comparable understanding of operant conditioning is still lacking. Published reports include invertebrate aversive conditioning (e.g., 9–12) and vertebrate

operant reward learning (e.g., 13). In several forms of learning, dopamine appears to be a key neurotransmitter involved in reward (e.g., 14). Previous research on dopamine-mediated operant reward learning in *Aplysia* was limited to in vitro analogs (15–18). In this report, we overcome this limitation by developing both in vivo and single-cell operant procedures and describe biophysical correlates of the operant memory.

The in vivo operant reward learning paradigm was developed using the consummatory phase (i.e., biting) of feeding behavior in *Aplysia*. This model system has several features that we hoped to exploit. The behavior occurs in an all-or-nothing manner and is thus easily quantified (see supplemental video). The circuitry of the underlying central pattern generator (CPG) in the buccal ganglia is well characterized (19). The anterior branch of the esophageal nerve

Department of Neurobiology and Anatomy, W. M. Keck Center for the Neurobiology of Learning and Memory, The University of Texas–Houston Medical School, Houston, TX 77030, USA.

\*These authors contributed equally to this work.  
 †To whom correspondence should be addressed. E-mail: john.h.byrne@uth.tmc.edu

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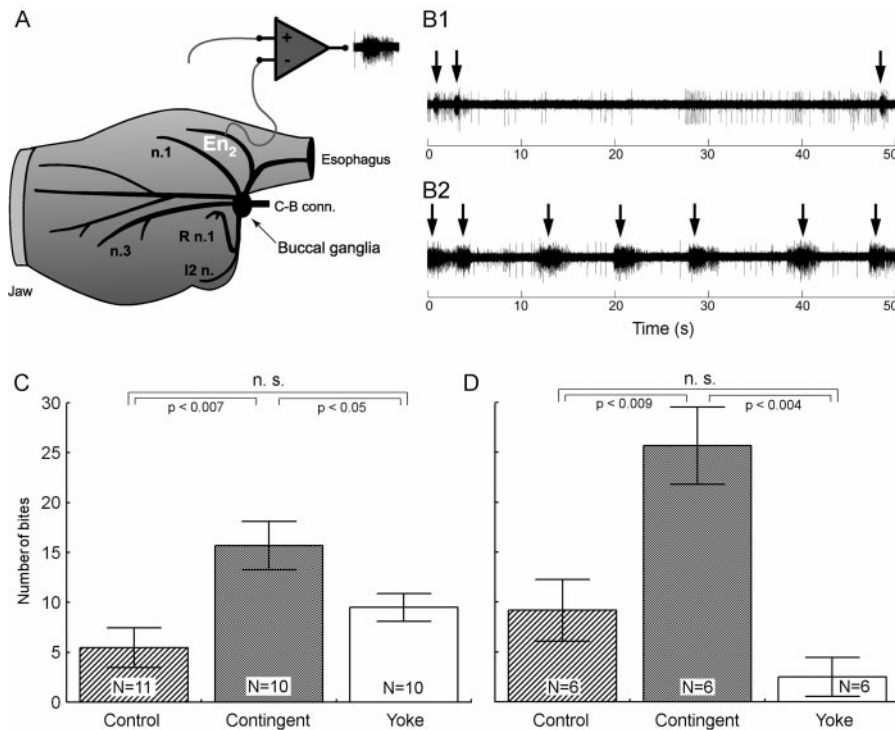
(En<sub>2</sub>) (Fig. 1A) is both necessary and sufficient for effective reinforcement during in vivo classical conditioning and in vitro analogs of classical and operant conditioning (15–18, 20–23). Presumably, En<sub>2</sub> conveys information about the presence of food during ingestive behavior. Consequently, we investigated the role of En<sub>2</sub> in the reinforcement pathway by recording from it in freely behaving *Aplysia* via chronically implanted extracellular hook-electrodes (24) (see supplemental methods) (Fig. 1A). Little nerve activity was observed during spontaneous biting in the absence of food (Fig. 1, B1), whereas bouts (duration: ~3 s) of high-frequency (~30 Hz) activity in En<sub>2</sub> were recorded during the ingestion of food (Fig. 1, B2). Specifically, this activity was observed in conjunction with ingestion movements of the odontophore/radula (a tongue-like organ). Electrical stimulation of En<sub>2</sub> might thus be used to substitute for food reinforcement in an operant conditioning paradigm. Therefore, in vivo stimulation of En<sub>2</sub> at approximately the frequency and duration as observed during feeding was

made contingent upon each spontaneous bite in freely behaving animals (see supplemental methods). Such a preparation is unique among studies of learning in invertebrates and analogous to commonly used self-stimulation procedures in rats (e.g., 13).

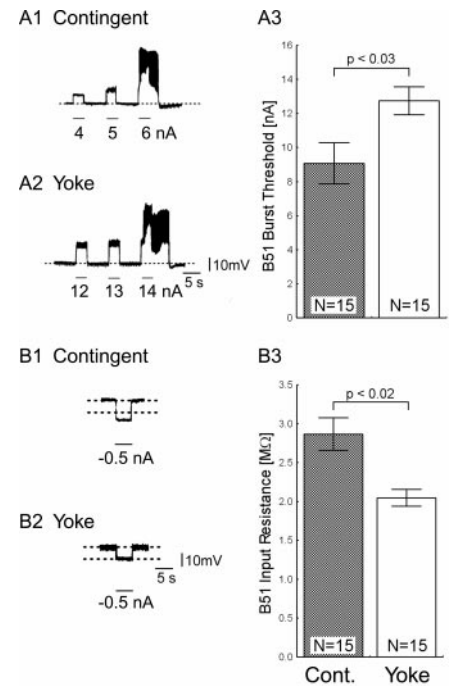
One day after implanting the electrodes, animals were assigned to one of three groups: (i) a control group without any stimulation, (ii) a contingent reinforcement group for which each bite during training was followed by En<sub>2</sub> stimulation, or (iii) a yoked control group that received the same sequence of stimulations as the contingent group, but the sequence was uncorrelated with their behavior (25). Animals that had been contingently reinforced showed significantly more spontaneous bites during a 5-min test period than did both control groups, regardless of whether they were tested immediately after training (Fig. 1C) or 24 hours later (Fig. 1D). These results indicate that during 10 min of contingent stimulation, the animals acquired an operant memory that lasted for at least 24 hours.

We next sought to identify changes in the

nervous system that were associated with the behavioral modification. The neural activity that underlies the radula movements during feeding is generated by the buccal CPG. This neural network consists of sensory, inter-, and motor neurons that continue to produce buccal motor patterns (BMPs), even when the ganglia are removed from the animal (15). In the intact animal, ingestion-like BMPs correspond to radula movements transporting food through the buccal mass into the foregut, as opposed to rejection-like BMPs that correspond to radula movements that remove inedible objects from the foregut (24). Buccal neuron B51 is pivotal



**Fig. 1.** In vivo recordings and behavioral results. (A) Schematic representation of electrode placement. (B1) Activity in En<sub>2</sub> during spontaneous bites in the absence of food. Depicted are three bites (arrows). (B2) Activity in En<sub>2</sub> during biting and swallowing behavior in the presence of food. Seven bite-swallows are shown (arrows). (C and D) Behavioral results. (C) Spontaneous bite rate in the final unreinforced test phase immediately after training. There was a significant difference among the three groups (Kruskal-Wallis ANOVA,  $H_2 = 9.678$ ,  $p < 0.008$ ). A post-hoc analysis revealed that the number of bites in the contingently reinforced group was significantly higher than both control and yoked groups (Mann-Whitney  $U$  tests,  $U = 16.5$ ,  $p < 0.007$ , and  $U = 24.0$ ,  $p < 0.05$ , respectively). The two control groups did not differ significantly (Mann-Whitney  $U$  test,  $U = 29.0$ ,  $p = 0.07$ ). (D) Spontaneous bite rate in the unreinforced test phase 24 hours after the beginning of the experiment. There was a significant difference among the three groups (Kruskal-Wallis ANOVA,  $H_2 = 11.9$ ,  $p < 0.003$ ). The number of bites taken by the contingent reinforcement group was higher than the two control groups (Mann-Whitney  $U$  tests,  $U = 1.5$ ,  $p < 0.009$ , control; and  $U = 0.0$ ,  $p < 0.004$ , yoke). The two control were not significantly different (Mann-Whitney  $U$  test,  $U = 9.5$ ,  $p = 0.17$ ). In this and subsequent illustrations, bar graphs display means  $\pm$  S.E.M.



**Fig. 2.** Changes in burst threshold and input resistance in B51 after operant training. (A) Burst threshold. (A1) and (A2) Intracellular recordings from B51 cells from a matched pair of contingently reinforced and yoked control animals. Depolarizing current pulses were injected into each B51 until the cell generated a plateau potential. In this example, a 6-nA current pulse was sufficient to generate a plateau potential in B51 from a contingently reinforced animal (A1), whereas 14 nA were required to generate a plateau potential in B51 from the corresponding yoked-control animal (A2). (A3) Summary data. B51 cells from the contingent reinforcement group required significantly less current to elicit the plateau potential (Mann-Whitney  $U$  test,  $U = 59.5$ ,  $p < 0.03$ ). (B) Input resistance. (B1) and (B2) Intracellular recordings from B51 cells from both contingently reinforced and yoked control animals. Hyperpolarizing current pulses were injected into B51 and the cells' input resistance was measured. In this example, the membrane potential of B51 from a contingently trained animal (B1) deflected more in response to the current pulse than the potential of B51 from a yoked control animal (B2). (B3) Summary data. B51 input resistance was significantly increased in contingently reinforced animals (Mann-Whitney  $U$  test,  $U = 37.0$ ,  $p < 0.002$ ).

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for the selection of BMPs. Specifically, B51 exhibits a characteristic, sustained, all-or-nothing level of activity (plateau potential) during ingestion-like BMPs. Moreover, B51 can gate transitions between BMPs. Direct depolarization of B51 leads to the production of ingestion-like BMPs, whereas hyperpolarization inhibits ingestion-like BMPs (18). We thus examined whether the observed increase in number of bites was associated with an increase in excitability of B51.

To test the hypothesis that B51 was a site of memory storage for operant conditioning, another set of animals was conditioned (26). Immediately after the last training period, the animals were anaesthetized and dissected, and the buccal ganglia were prepared for intracellular recording (see supplemental methods). Resting membrane potential, input resistance, and burst threshold were measured in B51. Burst threshold was defined as the amount of depolarizing current needed to elicit a plateau potential [see also (16, 18)]. Cells from the contingent group exhibited a significant decrease in burst threshold (Fig. 2A) and a significant increase in input resistance (Fig. 2B), as compared to cells from the yoked control. The resting membrane potential did not differ among the groups (27). The decrease in burst threshold and increased input resistance both increase the probability of B51 becoming active and thus increase the probability that a BMP will become ingestion-like. Our

data validate an in vitro analog of operant conditioning in isolated buccal ganglia (16) and extend the research to include operant conditioning in freely moving *Aplysia*.

Although the expression of intrinsic changes in the membrane properties of B51 was associated with operant conditioning, the maintenance of these changes could be due to extrinsic factors such as a tonic change in modulatory input to B51. If so, the locus of the associative neuronal mechanism may be upstream of B51. Moreover, as B51 is active during ingestion-like BMPs, the changes in B51 could be the effect of repeated activation, rather than a cause of operantly conditioned animals taking more bites than do the yoked control animals. To solve this question, we isolated the neuron in primary cell culture and developed a single-cell analog of the operant procedure. B51 neurons were removed from naïve *Aplysia* and cultured (see supplemental methods). Dopamine mediates reinforcement in an in vitro analog of operant conditioning (17), and  $En_2$  is rich in dopamine-containing processes (28). Therefore, reinforcement was mimicked by a brief (6 s) iontophoretic “puff” of dopamine onto the neuron. Because B51 exhibits a plateau potential during each ingestion-like BMP, this reinforcement was made contingent upon a plateau potential elicited by injection of a brief depolarizing current pulse. Contingent reinforcement of such B51 activity in the ganglion with  $En_2$  stimulation is sufficient

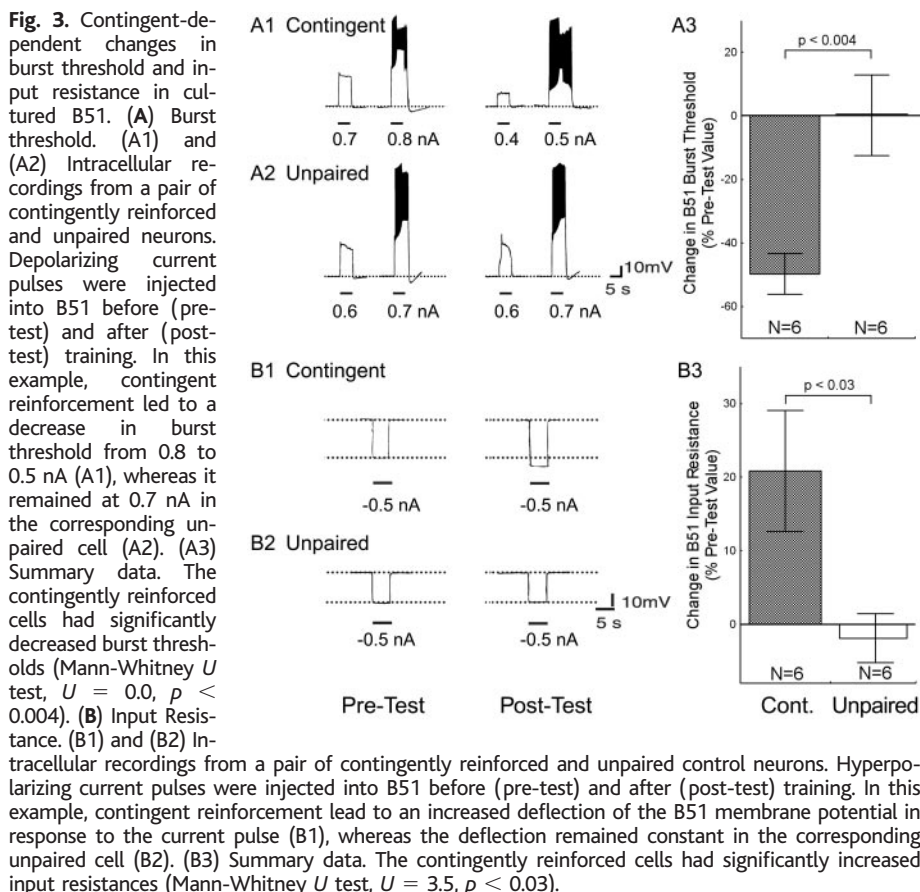
for in vitro operant conditioning (18). Two experimental groups were examined. Building on the experience with in vitro operant conditioning (18), we administered seven supra-threshold current pulses in a 10-min period to a contingent reinforcement group. Dopamine was iontophoresed immediately after cessation of the plateau potential. An unpaired group received the same number of depolarizations and puffs of dopamine, but dopamine iontophoresis was delayed by 40 s after the plateau potential. Contingent application of dopamine produced a significant decrease in burst threshold (Fig. 3A) and a significant increase in input resistance (Fig. 3B). Apparently, processes intrinsic to B51 are responsible for the induction and maintenance of the biophysical changes associated with operant reward learning.

The combination of rewarding a simple behavior with physiologically realistic, in vivo stimulation uncovered neuron B51 as one site where operant behavior and reward converge (see supplemental discussion). The results presented here suggest that intrinsic cell-wide plasticity contributes to operant reward learning. Such cell-wide plasticity is also associated with operant conditioning in insects (10). Although B51 is a key element in the neural circuit for feeding, the quantitative contribution of the changes in B51 to the expression of the behavioral changes needs to be elucidated. Given the number of neurons in the feeding CPG (19), it is likely that B51 will not be the only site of plasticity during operant conditioning (nor will cell-wide plasticity likely be the only mechanism). However, the persistent involvement of contingent-dependent cell-wide plasticity in B51 in different levels of successively reduced preparations suggests an important role for this mechanism.

Research on *Aplysia* has provided key insights into mechanisms of aversive conditioning that are evolutionary conserved. The utility of this model system for learning and memory has now been extended to dopamine-mediated reward learning on the behavioral, network, and cellular level. Our study expands a growing body of literature that shows that dopamine is an evolutionary conserved transmitter used in reward systems. Future research on *Aplysia* will likely provide insights into the subcellular effects of dopamine reward, an area currently under intense investigation in vertebrates (8, 13).

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# Anterior Cingulate: Single Neuronal Signals Related to Degree of Reward Expectancy

Munetaka Shidara<sup>1\*</sup> and Barry J. Richmond<sup>2</sup>

As monkeys perform schedules containing several trials with a visual cue indicating reward proximity, their error rates decrease as the number of remaining trials decreases, suggesting that their motivation and/or reward expectancy increases as the reward approaches. About one-third of single neurons recorded in the anterior cingulate cortex of monkeys during these reward schedules had responses that progressively changed strength with reward expectancy, an effect that disappeared when the cue was random. Alterations of this progression could be the basis for the changes from normal that are reported in anterior cingulate population activity for obsessive-compulsive disorder and drug abuse, conditions characterized by disturbances in reward expectancy.

During normal activity, we continually compare our current status against our expectation for reaching a goal, with expectation increasing over the course of the activity. That implies that there are neural signals underlying this increasing expectation.

Over the past several years, we have used visually cued multitrial reward schedules in monkeys. In this task monkeys change their error rates according to reward expectancy (1–4). To obtain a reward, monkeys must successfully complete a set (or schedule) of visual color-discrimination trials (Fig. 1A) [(2); see (5) for details of experimental procedures]. In the schedule task, the monkey has to complete between one and four color-discrimination trials successfully to obtain the reward (Fig. 1B). An unsuccessful trial is not explicitly punished, but the monkey only progresses to the next stage of a schedule when a trial is completed successfully. A second set of visual stimuli used as cues indicate progress of the schedule. The cues become brighter as the schedule progresses (cued condition). The only information available about the schedule and trial is provided by the cue. As in all of the previous studies making use of this task (5), the monkeys here made progressively fewer errors as the rewarded trial approached, with the fewest errors occurring in the rewarded trials (Fig. 2A), showing that the cue is actually being used by the monkey to regulate its behavior. When we randomized the cues with respect to the schedule so that the cues were no longer related to the schedule (random condition) (5), the monkey's error rate was always low, regardless of cue brightness (Fig. 2B). Thus, there is a substantial behavioral dif-

ference between knowing for certain what will happen in each successfully completed trial (cued condition) versus knowing the overall reward rate without knowing the outcome of each trial for certain (random condition).

For neurons in ventral striatum (2) and perirhinal cortex (4), responses occurred in specific trials of the reward schedules, with the response strengths being similar in all trials showing responses. The trials in which responses occurred appeared idiosyncratic. Thus, although the populations of neurons in either ventral striatum and perirhinal cortex could be used to decode progress through reward schedules, no single neuron carried a signal that varied directly with schedule progress or reward expectancy.

We hypothesized that within the brain's reward system, there should be a signal related to the degree of reward expectancy. For several reasons, the anterior cingulate cortex (6–10) seemed a promising site for such a signal. It appears to have a role in performance monitoring and error detection, conflict monitoring, and response selection, all of which depend on assessing reward proximity or likelihood (11–18). Several neuronal recording studies have shown associations between sensory stimuli and the expectation of various outcomes, such as reward, or pain (19–24). Finally, in several imaging studies of patients with disturbances in motivation and reward expectation, such as obsessive-compulsive disorder and drug abuse, the anterior cingulate has shown increased activation when compared with anterior cingulate in normal subjects (25–38).

We recorded from 106 single neurons in area 24c of anterior cingulate cortex [ventral bank of anterior cingulate sulcus, a part of rostral cingulate motor area (39), confirmed by magnetic resonance imaging (40)] of monkeys performing the cued multitrial reward schedule task. A substantial number of neurons (94/106) showed selective responses during the reward schedule task. For 69 neurons, activity was idiosyncratic

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26. In the conditioning experiment conducted to search for correlates of the operant memory in B51, an additional 5-min training period replaced the last test, to minimize extinction and ensure a high level of conditioning. Because unstimulated and yoked control groups did not differ significantly in the previous experiment, only two groups were used: contingent reinforcement and yoked control. Comparisons of the number of bites taken during the last 5-min training period assessed the success of the operant conditioning procedure. Confirming the previous results, contingently reinforced animals took significantly more bites in the last training period than did animals in the yoked control group: Mean contingent, 13.5; mean yoke, 8.4; Mann-Whitney  $U$  test,  $U = 62.0$ ,  $p < 0.04$ .
27. Mean contingent,  $-65.7$  mV,  $N = 13$ ; mean yoke,  $-65.3$  mV,  $N = 12$ ; Mann-Whitney  $U$  test,  $U = 77.0$ ,  $p < 0.96$ .
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## Supporting Online Material

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Materials and Methods  
SOM Text  
Fig. S1 and S2  
References and Notes  
Movies S1 and S2

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<sup>1</sup>Neuroscience Research Institute, National Institute of Advanced Industrial Science and Technology, 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan. <sup>2</sup>Laboratory of Neuropsychology, National Institute of Mental Health, Bethesda, MD 20892, USA.

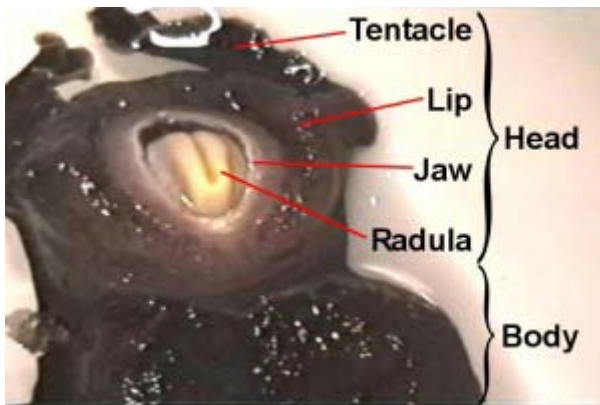
\*To whom correspondence should be addressed. E-mail: m.shidara@aist.go.jp

## Operant reward learning in *Aplysia*: Neuronal correlates and mechanisms

Björn Brembs, Fred D. Lorenzetti, Fredy D. Reyes, Douglas A. Baxter,  
John H. Byrne

### Supplementary Material

**Supplemental Video.** *Aplysia biting behavior.* The consummatory phase of *Aplysia* feeding behavior (biting) occurs in an all-or-nothing fashion and is unambiguously quantifiable (1, 2). It consists of four phases: jaw opening, odontophore/radula protraction in the open state, odontophore/radula retraction in the closed state and jaw closure. Biting occurs spontaneously as well as reflexively, in nature as well as in the laboratory. If food is present, it leads to the ingestion of food through the buccal cavity. In this 8 s video sequence, the animal has positioned itself under the water surface (as they often do). Its tentacles (anterior) are at the top of the screen. The sequence contains the opening of the jaws, followed by the protraction of the radula (cream colored tongue-like organ) in the open state, the closure of the radula, the retraction of the radula in the closed state and the closing of the jaws.



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**Supplemental Methods 1.** *Surgical procedures and in vivo recordings.* *Aplysia californica* (100-200 g) were obtained from Alacrity Marine Biological Specimens (Redondo Beach, CA) and Marinus (Long Beach, CA). They were kept individually in rectangular perforated plastic cages floating in aerated artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH) at a temperature of 12-15°C. Animals were fed ~1 g of dried laver, 3 times a week. To help ensure that all animals were in a similar motivational state, experimental animals were food deprived 3-5 days before surgery.

Extracellular electrodes were prepared from 25.4 µm insulated stainless steel wire (304 TRI-ML, California Fine Wire Co., Grover Beach, CA) by scraping the insulation from 2 mm at one end of a piece (~30 cm) of wire and from about 15 mm at the other end. Two such wires were used, one for recording/stimulating, the other for reference. The two long de-insulated ends were soldered to gold contacts, whereas one of the short de-insulated ends was fashioned into a small hook for placement on the nerve (for stimulation/recording). The other wire served as the reference electrode and its tip was left straight.

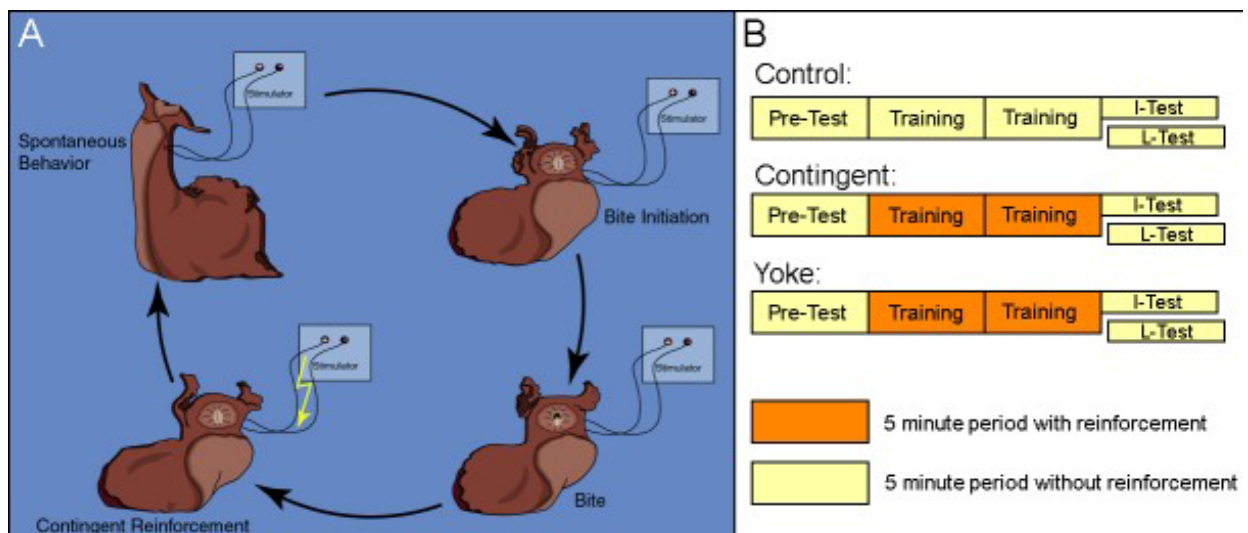
Just prior to surgery, the animals were fed a single bite of seaweed, to probe the motivational state, overall feeding behavior and health of the animals. Animals that did not feed were discarded. Each animal was then anaesthetized by injecting isotonic MgCl<sub>2</sub> (360 mM) solution (30% body weight) into the hemolymph and transferred onto a block of ice (made of

seawater), covered with artificial seawater. The animal was positioned with its left body side up and two hooks shaped from hypodermic needles (30 G1/2; Becton Dickinson, Franklin Lakes, NJ) were placed in the skin ~3 cm apart ~1.5 cm ventral of the eye and in parallel with the anterior-posterior axis of the animal. The hooks were fastened by threads on either end of the tank and tightened so as to lift the fold of skin between them above the water surface. A small (1-1.5 cm) incision was made between the hooks alongside the fold and with the eye being at the height of the anterior third of the incision. During the surgery, the thread suspended hooks were used to keep the wound above the water level to prevent leakage of hemolymph, or seawater from entering the animal. The incision was kept open using a second pair of hooks arranged perpendicularly to the first pair. A moveable support was used to lift the buccal mass and expose the buccal ganglia and their nerves. Additional manipulators were then used to place the hook-electrode around the anterior branch of the esophageal nerve ( $En_2$ ), close to the anterior/posterior branch point. The electrode was secured and insulated by a drop of superglue (Loctite Quick Gel, part # SGG-2B, Loctite, Rocky Hill, CT). After inserting the reference electrode to float free in the hemocoel, the animal was closed using 4-6 stitches of 4-0 black braided silk suture (Ethicon, Somerville, NJ), with the electrodes exiting the animal at the posterior end of the incision. The entire procedure lasted between 45-60 minutes for each animal. After the surgery, the animals were placed into individual rectangular perforated plastic cages and left to recover overnight at 12-15° C. Electrode signals were amplified using a differential AC amplifier (model 1700; A-M Systems, Everett, WA), filtered by a 100 Hz low cut-off filter and a 1 kHz high cut-off filter. One day after surgery, extracellular activity in the anterior branch of the esophageal nerve was recorded while the animal was observed in a round glass bowl (radius: 80 mm, depth: 70 mm) placed on a mirror. During the observation period, the animal was stimulated to bite and swallow with pieces of seaweed. The animals were not restrained and the length of the wires permitted a full range of motion in the bowl. Animals used for *in vivo* recording were not used in the conditioning studies.

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**Supplemental Methods 2. Operant reward learning.** The occurrence of spontaneous bites is dependent on a number of variables. While it can be observed in freshly caught specimens, it is comparatively rare. To successfully conduct experiments, certain measures need to be taken to increase the frequency at which this behavior occurs. To help ensure that all animals were in a similar motivational state, experimental animals were food deprived 3-5 days before surgery. One day after implanting the stimulating electrodes, the animals were fed a single bite of seaweed 30 minutes before the experiment to motivate the animal to search for food. Seaweed extract was prepared by incubating a 10 cm x 20 cm piece of seaweed in 300 ml of artificial seawater for 30 minutes. Pilot studies found seaweed extract to increase the overall probability of biting behavior to occur. Just prior to the experiment, 50 ml of the supernatant were added to 400 ml of fresh artificial seawater. The animal was then transferred into a round glass bowl (radius: 80 mm, depth: 70 mm) containing these 450 ml of diluted seaweed extract and the bowl placed on a mirror to be able to better observe the animal. The experiment was performed in a climate chamber at 15° C and 60% rel. humidity. Unrestrained in the bowl, the animal moved around freely and engaged in spontaneous behaviors (A). Throughout the experiment, the animal was observed and all bites recorded. A bite (see supplemental video) was defined as opening of the jaws and protraction of the radula. Before the start of the experiment, animals were assigned to one of three groups (B): i) a control group that did not receive any stimulation, ii) a contingent reinforcement group which

received  $\text{En}_2$  stimulation whenever the jaws closed after a bite during training, or iii) a yoked control group that received the same sequence of stimulations as the contingent group, but the stimulation occurred uncorrelated with their behavior. Except for the different reinforcement schedules, all animals in all three groups were treated identically. Application of reinforcement was the only difference between training and test. In the early phase of the study, the animals were assigned randomly to each group. As the study progressed, the animals were assigned to each group so as to balance pre-test bite rate between groups. Experimental sessions consisted of four consecutive five-minute periods. In each period, the number of bites was recorded. The final test period was either immediately after training (I-Test) or 24 h after the beginning of the experiment (L-Test). A Grass S48D stimulator (Grass Instruments, Quincy, MA) generated 10 ms pulses for extracellular nerve stimulation (30 Hz, 3 s). Pilot studies determined that a suitable intensity of the stimulation was 8 V. At this voltage, usually no behavioral response could be observed. Occasionally, an animal (mostly yoked controls) would show a jaw opening without radula protraction or a rejection-like behavior (i.e., the radula appeared to be closed during protraction) to the first few stimulations only. Such responses were never observed spontaneously. If they met the definition of a bite (i.e., the radula was protracted), they were scored as bites irrespective of the subjective impression of the observer. Animals that were tested after 24 h spent the time between training and test in individual rectangular perforated plastic cages in aerated artificial seawater at 12-15° C. On the next day, the animals were placed back into the glass bowl with seaweed extract, but without being fed before the test. After the experiments, all animals were sacrificed and electrode placement verified. Only animals that produced  $0 < n < 31$  bites in the pre-test were used. Several experimenters independently replicated this experiment and all their data were pooled.



**Supplemental Methods 3. Biophysical correlates of the operant memory in B51.** Animals from a second behavioral study were anesthetized by injecting a volume of isotonic  $\text{MgCl}_2$  equivalent to 50% of the animal's weight. Buccal ganglia were removed and pinned on a Sylgard-coated Petri dish containing artificial seawater (ASW). The composition of the ASW was: 450 mM NaCl, 10 mM KCl, 30 mM  $\text{MgCl}_2(6\text{H}_2\text{O})$ , 20 mM  $\text{MgSO}_4$ , 10 mM  $\text{CaCl}_2(2\text{H}_2\text{O})$ , 10 mM HEPES, with pH adjusted to 7.4. The ganglion ipsilateral to the

esophageal nerve stimulation was desheathed on the rostral side. Desheathing was performed in the presence of high divalent cation ASW solution, which contained concentrations of  $\text{CaCl}_2$  and  $\text{MgCl}_2$  that were three times the normal level. Osmolarity was maintained by correspondingly decreasing the concentration of  $\text{NaCl}$ . After desheathing, the medium was changed to normal ASW.

Neuron B51 was identified based on its relative size and position in the ganglia. The identity of the cell was confirmed by its pattern of electrical activity during a buccal motor program, membrane properties, and its characteristic plateau potential as described in (3,4). Recording temperature was  $15^\circ \text{C}$ .

Conventional two-electrode current-clamp techniques were used for intracellular recordings (Axoclamp-2A, Axon Instruments, Burlingame, CA). Fine-tipped glass microelectrodes (resistance 10-15 M $\Omega$ ) were filled with 2 M potassium acetate. The cell was current-clamped at -60 mV for the duration of the experiment. Five minutes after impalement with the electrodes, the input resistance and the burst threshold were measured. If a spontaneous motor pattern occurred during testing, the test was halted and then resumed 1 minute after the cessation of the pattern. 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 activity in B51 that outlasted the current pulse. The burst threshold was tested by a series of successively higher amplitude depolarizing current pulses (in 1 nA increments) with a duration of 5 s. The series was spaced with 10 s between the end of one pulse and the start of another. In this way the minimum amount of current necessary to elicit a plateau potential could be determined. After these two properties were measured, the cell was released from current clamp and the resting membrane potential was determined. On average, membrane properties of B51 were recorded 100 minutes after the last training period. The experimenter performing the intracellular measurements was unaware of the experimental history of the animals.

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**Supplemental Methods 4.** *B51 cell culture and electrophysiology.* Culturing procedures followed those described in (5-8). Buccal ganglia from adult *Aplysia* were incubated in 1% protease type IX (Sigma, St. Louis, MO) at room temperature for 24 hours and then desheathed. In pilot studies, B51 neurons in the buccal ganglia were first identified by the electrophysiological methods described previously and then dye-labelled with Fast Green (Sigma). B51 neurons were removed from the ganglia by microelectrodes with fine tips and plated on poly-L-lysine coated glass slides in 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. It was found that neurite morphology coupled with the size and the relative position of the cell in the ganglia was sufficient to identify B51. Thus, these criteria were adopted as the means of identification for all the neurons used in this report.

The electrophysiological methods used to record from cultured neurons were largely the same as those used to record from neurons in the ganglia. Due to the high input resistance in cultured cells, the cells were current clamped to -80 mV. Five minutes after impalement, input resistance and burst threshold were determined. Input resistance was tested by injecting a hyperpolarizing current pulse of 0.5 nA for 5 s and burst threshold was tested in 0.1 nA increments. The cells were then divided into a contingent reinforcement and an unpaired 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 in a ten-minute training period. The cells in the contingent reinforcement group received a 6 s iontophoretic pulse of dopamine immediately after the cessation of the plateau potential, whereas iontophoresis was delayed by 40 s in the unpaired group. Dopamine was iontophored through a fine-tipped glass microelectrode (resistance 10-15 MOhms). 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. After training, the membrane properties were measured again and compared to the pre-test levels.

Recordings were performed at room temperature.

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**Supplemental Discussion.** From postural adaptation to social interaction, operant conditioning is one of the essential processes leading to the generation and modulation of behavior. However, its analysis has been complicated because most learning situations inseparably comprise operant and classical components. Specifically, behaving organisms constantly receive a stream of sensory input that is both dependent and independent of their behavior. The classic debate as to whether one or two processes account for the operant/classical dichotomy reflects this entanglement (e.g., 9-14). Interrupting the operant feedback loop by restraining an animal can at least partly isolate classical conditioning from the operant components. Once isolated from spontaneous behavior, in a number of systems the stimuli have been traced into the nervous system to find the point of convergence where the classical association is formed. Until now, the convergence of reinforcement and the operant behavior has remained elusive, however.

Similar to the successful isolation of the stimuli from spontaneous behavior in classical conditioning, the development of the present procedure is a critical step towards operant conditioning without any external stimuli other than the reward being contingent upon the behavior (i.e., 'pure' operant conditioning, 14). So far, it can not be ruled out that the animal can perceive the sound and vibrations associated with recording the sequence of stimulations and activating the stimulator. However, given the nature of the sensory organs in *Aplysia*, this appears highly unlikely. Thus, in practical terms, the bite occurs spontaneously and except for the reinforcement, all external stimuli are either constant or independent of the biting behavior. The nature of the reinforcement also rules out classical contamination by predictive 'internal' stimuli generated by other types of reinforcement. Whereas other reinforcers like food or water need consummatory behavior (preceding the reward) to be effective, stimulation of En<sub>2</sub> is not preceded by any other behavior or stimuli (external or internal) other than the rewarded operant behavior. It cannot be ruled out that internal stimuli are generated by the innervation of the buccal musculature and the salivary gland by En<sub>2</sub>. However, such stimuli would be sensed after the onset of the reward and can therefore not acquire any predictive properties other than that they are not followed by reinforcement.

Contextual cues are always present during the experiment. Indeed, without contextual cues, the association would most likely have extinguished in the home tank before the 24 h test. However, these cues were constant throughout the experiment and thus are not predictive as to when exactly the reinforcement will occur (such as a classical conditioned stimulus would). Therefore, it is safe to contend that any contextual cues act as 'occasion setters' to indicate whenever the behavior – reinforcer relation is true and do not directly enter into the primary operant association.

With the development of in vivo and in vitro classical and operant procedures in *Aplysia*, the first detailed mechanistic comparison between operant and classical conditioning in the same preparation is within reach. Ultimately, the tools now available in *Aplysia* will allow for studies of operant-classical interactions (e.g., 14).

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