Specific Plasticity Loci and Their Synergism Mediate Operant Conditioning

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Despite numerous studies examining the mechanisms of operant conditioning (OC), the diversity of OC plasticity loci and their synergism have not been examined sufficiently. In the well-characterized feeding neural circuit of Aplysia, in vivo and in vitro appetitive OC increases neuronal excitability and electrical coupling among several neurons leading to an increase in expression of ingestive behavior. Here, we used the in vitro analog of OC to investigate whether OC reduces the excitability of a neuron, B4, whose inhibitory connections decrease expression of ingestive behavior. We found OC decreased the excitability of B4. This change appeared intrinsic to B4 because it could be replicated with an analog of OC in isolated cultures of B4 neurons. In addition to changes in B4 excitability, OC decreased the strength of B4’s inhibitory connection to a key decision-making neuron, B51. The OC-induced changes were specific without affecting the excitability of another neuron critical for feeding behavior, B8, or the B4-to-B8 inhibitory connection. A conductance-based circuit model indicated that reducing the B4-to-B51 synapse, or increasing B51 excitability, mediated the OC phenotype more effectively than did decreasing B4 excitability. We combined these modifications to examine whether they could act synergistically. Combinations including B51 synergistically enhanced feeding. Taken together, these results suggest modifications of diverse loci work synergistically to mediate OC and that some neurons are well suited to work synergistically with plasticity in other loci.

Key words: Aplysia; feeding behavior; operant learning; plasticity

Significance Statement

The ways in which synergism of diverse plasticity loci mediate the change in motor patterns in operant conditioning (OC) are poorly understood. Here, we found that OC was in part mediated by decreasing the intrinsic excitability of a critical neuron of Aplysia feeding behavior, and specifically reducing the strength of one of its inhibitory connections that targets a key decision-making neuron. A conductance-based computational model indicated that the known plasticity loci showed a surprising level of synergism to mediate the behavioral changes associated with OC. These results highlight the importance of understanding the diversity, specificity and synergy among different types of plasticity that encode memory. Also, because OC in Aplysia is mediated by dopamine (DA), the present study provides insights into specific and synergistic mechanisms of DA-mediated reinforcement of behaviors.

Introduction

Appetitive operant conditioning (OC) increases expression of specific behaviors because of the association of the behavior with rewards (Skinner, 1981; Thorndike, 1911). This form of learning is ubiquitous (for review, see Martin-Soelch et al., 2007; Nargeot and Puygrenier, 2019). Neuronal mechanisms of appetitive OC include changes in synaptic strength and intrinsic excitability of individual neurons (for review, see Mozzachiodi and Byrne, 2010; Cox and Witten, 2019; Nargeot and Puygrenier, 2019). Learning likely involves diverse plasticity mechanisms that work synergistically (Gao et al., 2012). However, the diversity of plasticity loci, and how they might act synergistically to mediate OC, remains relatively unexplored.

To address this issue, we exploited the technical advantages of Aplysia feeding behavior, which is mediated by a central pattern generator (CPG) located primarily in the buccal ganglia (Church and Lloyd, 1994; Elliott and Susswein, 2002; Cropper et al., 2004) and can be modified by appetitive OC (Brembs et al., 2004; Baxter and Byrne, 2006; Nargeot and Simmers, 2011). The CPG...
generates at least two mutually exclusive behaviors, egestion and ingestion. During OC, ingestion behavior is reinforced by contingent presentation of food (in vivo) or stimulation of dopaminergic afferents (in vitro; Nargeot et al., 1997, 2007; Brembs et al., 2002). Previously identified neuronal correlates of OC include increases in the excitability and electrical coupling of neurons that initiate the behavior (Nargeot et al., 2009; Sieling et al., 2014; Costa et al., 2020), and increased excitability and input resistance of a plateau generating neuron, B51, in selected inactivation of ingestion (Nargeot et al., 1999a,b; Lorenzetti et al., 2006, 2008; Mozzachiodi et al., 2008).

Surprisingly, to date all of the correlations of OC in the Aplysia feeding system are associated with either increases in intrinsic neuronal excitability or electrical coupling. These results raise several important questions. First, does OC decrease excitability of neurons? Second, does OC modify chemical synaptic transmission? Third, are modifications of some neurons or synapses more effective than others? Fourth, in what ways would such a diverse array of neuronal plasticity act synergistically to alter behavior? To address these issues, we examined whether OC produces complementary modifications in both the excitability and strength of inhibitory connections of B4, a decision-making neuron that suppresses ingestion, in part, by its inhibitory synaptic connections to B8 and B51 (Plummer and Kirk, 1990; Kabotyanski et al., 1998; Sasaki et al., 2009; Dacks et al., 2012).

We then investigated the behavioral consequences of any observed modifications by including these changes in a conductance-based model of the CPG and examining whether they synergistically enhanced feeding.

Materials and Methods

Three distinct but complementary approaches were used in this study: (1) an in vitro analog of OC in isolated preparations of buccal ganglia dissected from naive animals; (2) a single-cell analog of OC in cell culture with neurons isolated from buccal ganglia of naive animals; and (3) a conductance-based model of the CPG to assess the relative contributions of the changes in excitability and synaptic strength to the generation of BMPs.

Animals

Aplysia californica (30–70 g) were obtained from the National Resource for Aplysia (University of Miami, FL), Alacrity Marine Biological Specimens (Redondo Beach, CA), and Marinus (Westchester, CA). Animals were housed individually in perforated plastic cages in aerated seawater tanks at a temperature of 15°C and were fed ~1 g of dried seaweed three times per week.

Classification of buccal motor patterns (BMPs)

Feeding behavior can be broadly classified into behaviors that lead to the ingestion or rejection of food. Both ingestion and rejection consist of two phases that involve an outward (protraction) and inward movement (retraction) of the radula, a grooved tongue-like structure. Radula closure was estimated automatically by a custom-built spike detection algorithm analyzing recordings of R n.1 (Fig. 1A), a nerve that contains radula closing motor neurons, or B8 in preparations that included intracellular recordings from B8 (Fig. 2B), an important radula closing neuron that projects through R n.1 (Morton and Chiel, 1993). The spike detection consisted of a simple threshold that was manually set for each nerve recording, to be above the smallest R n.1 spike during protraction (likely noise) but lower than spikes (likely motor) occurring outside of BMP activity. Occasionally, B8 fires spontaneously outside of motor patterns producing large unit activity. Therefore, large unit R n.1 spikes (likely B8) occurring outside of BMP activity were ignored when setting the threshold. Patterns with >50% of the total duration of R n.1 activity occurring during retraction were classified as an ingestive motor pattern, iBMP (Nargeot et al., 1997, 1999a,b,c; Brembs et al., 2002; Mozzachiodi et al., 2008) and for simplicity, all other patterns were classified as rejection BMPs (rBMPs). For each preparation, BMP classification was conducted by an individual blind to the stimulus protocol used (OC vs control).

In vitro analog of OC

Animals were food-deprived for 1–3 d before the experiment and fed a piece of seaweed 30 min before dissection, at which time animals were anesthetized by isotonic MgCl2 with a volume equal to half the animal’s body weight (Brembs et al., 2004). The protocol for the in vitro analog of OC has been described previously (Nargeot et al., 1999a). Briefly, the buccal ganglia were isolated and transferred to the recording chamber containing artificial seawater with a high concentration of divalent cation (HiDi-ASW) composed of the following: 330 mM NaCl, 10 mM KCl, 30 mM CaCl2, 90 mM MgCl2, 20 mM MgSO4, and 10 mM HEPES (pH was adjusted to 7.5 with NOH; Nargeot et al., 1997; Fig. 1B1,B2). HiDi-ASW was used to reduce neuronal activity during dissection. The high osmolarity was desalted on the rostral side. Immediately after desalting, the chamber solution was changed to normal ASW composed of the following: 450 mM NaCl, 10 mM KCl, 10 mM CaCl2, 30 mM MgCl2, 20 mM MgSO4, and 10 mM HEPES (pH was adjusted to 7.5 with NOH; Nargeot et al., 1997). The preparations were at rest 20 min before the recording (Fig. 1B3) and maintained at 13–15°C by means of a Peltier cooling device for the duration of the experiment.

First, pre-test measurements of input resistance (Rin), excitability, and synaptic strengths were made following a 5 min rest period after the beginning of intracellular recordings (see below). Then, sustained rhythmic BMP activity was elicited by continuous low-frequency stimulation of contralateral nerve n.2,3 (0.5 ms, 2 Hz; Nargeot et al., 1997). Stimulation of En.2 (0.5 ms, 10 Hz), which contains dopaminergic afferents to the buccal ganglia, served as reinforcement during training (Fig. 1B2,B4; Nargeot et al., 1997, 1999a; c; Kabotyanski et al., 1998). The intensity (2–4 V) of nerve stimulation of n.2,3 was adjusted so 10 consecutive stimuli elicited one-for-one spikes in B4. The intensity (3–8 V) of En.2 stimulation was adjusted so that (1) one-for-one EPSPs were elicited in B4 by three consecutive stimuli at 1 Hz; and (2) 1–4 BMPs were generated with En.2 stimulation of 10 Hz for 6 s. Experiments consisted of a 10 min pre-test period, a 10-min training period, and a 10-min post-training period (Fig. 1B3). The pre-training period was initiated 5 min after the first occurrence of a BMP by tonic n.2,3 stimulation. During the post-training period, which immediately followed the training period, n.2,3 stimulation was paused briefly and post-test Rin, excitability, and synaptic strength were measured. Each experiment included two groups: a contingent reinforcement group that received En.2 stimulation immediately following the expression of iBMPs and a yoke control group that received En.2 stimulation that was uncorrelated to pattern expression (Fig. 1B4). The experiments were done sequentially with a yoke experiment following each contingent experiment. The sequence of En.2 stimulation of each contingent preparation served as the template for the sequence of En.2 stimulation for its corresponding yoke preparation.

Cell identification

Neurons were identified by their relative size, location, and physiological characteristics. For example, B51 was identified based on its small size, apposition to B15, and characteristic plateau potential (Plummer and
Kirk, 1990). B8 was identified by its lateral position, large size, and one-for-one spikes in the radula nerve (Nargeot et al., 1997). B4 was identified by its medial position, large size, and inhibitory inputs to B8 (Rosen et al., 2000). B51 (Plummer and Kirk, 1990).

Testing B4, B8, and B51 properties in ganglia

Intracellular recordings were performed using conventional fine-tipped glass microelectrodes filled with 2 M potassium acetate (resistance 7–15 MΩ), with signals amplified by an Axoclamp-2B and digitized with a Digidata1322a (Molecular Devices). Intracellular recording signals were acquired at 5 kHz and filtered using a 300-Hz low-pass filter. Simultaneous recordings were made from the presynaptic (i.e., B4) and postsynaptic neurons (B8 or B51). B4 and B8 were recorded with dual electrodes whereas B51 was recorded with a single electrode. Experiments on the B4-to-B51 connection were performed in a different set of preparations than those on the B4-to-B8 connection. Rin was measured in B4 and B8 by injecting a 5-s, 5-nA hyperpolarizing current pulse. The excitability was measured as the number of spikes elicited during injecting a 5-s duration depolarizing current pulse. B4 and B8 differ in their baseline excitability, so the intensity of depolarizing current was 4 nA for B4 and 2 nA for B8. Measurements in which less than three spikes were elicited for measurement of excitability during pre-test were excluded in the analysis for both the contingent and its yoke pair. Intrinsic properties were measured at resting membrane potential in the absence of n.2,3 stimulation. For measurements of IPSPs, B8 was held at −100 mV and B51 at −70 mV. The membrane potential was held at −100 mV to increase the ionic driving force and thereby increase the signal-to-noise ratio and obtain a more accurate measurement of the B4-to-B8 IPSP. The reversal potential of the B4-to-B8 IPSP was about −70 mV. The B4-to-B51 IPSP was measured with a holding potential of about −100 mV because its reversal potential was about −100 mV. A rest period of at least 4 min preceded the measurements of IPSPs before and after training. The effects of OC on excitability, Rin, Vrest (resting membrane potential), and IPSP were assessed by the percent change in value between post-test and pre-test \[\frac{(Post-Pre)}{Pre} \times 100\].

Cell culture

Culturing procedures for B4 followed those described in Brembs et al. (2002) and Lorenzet et al. (2008). Briefly, ganglia from adult Aplysia were treated with Dispase II (10 units/ml; neutral protease, Grade II; Roche) at 35°C for −3 h and then desheathed. Fine-tipped glass
microelectrodes were used to remove individual B4 cells from the ganglia. Each cell was plated on poly-L-lysine (0.75 mg/ml)-coated Petri dishes with culture medium containing 50% hemolymph, 50% isotonic L15 (Invitrogen). L15 was made of 350 mM NaCl, 25 mM MgSO4, 11.4 mM CaCl2, 29 mM MgCl2, 10 mM KCl, streptomycin sulfate (0.10 mg/ml), penicillin-G (0.06 mg/ml), dextrose (6 mg/ml) and 15 mM HEPES. The pH of the culture medium was adjusted to 7.5. Cells were allowed to grow for 4–5 d. Before recording, the culture medium was exchanged for a solution containing 50% ASW and 50% isotonic L15.

**Single-cell analog for B4**

The procedures for the single-cell analog were similar to those established previously (Brembs et al., 2002; Lorenzetti et al., 2008) in which excitability and Rm were measured immediately before and after training. Rm was measured by injecting a 5-s duration -1-nA hyperpolarizing current pulse. Neuron B4 in culture had a higher Rm and lower firing threshold (Rm = 6.3 ± 0.6 MΩ; threshold = 0.6 ± 0.1 nA, N = 10) than cells in ganglia (Rm = 3.3 ± 0.1 MΩ; threshold = 3.0 ± 0.2 nA, N = 10), which is presumably due, at least in part, to the absence of the electrical coupling in the isolated neurons. Excitability of cultured B4 was measured as the number of spikes elicited by a 5-s duration 1.5-nA depolarizing current pulses. The membrane potential was held at −70 mV, which was slightly more negative than the average resting potential (−63 ± 0.7 mV) of B4 in ganglia. Dopamine (DA; Sigma-Aldrich) was iontophoresed through a fine-tipped glass microelectrode (resistance 10–15 MΩ; Fig. 3C). The concentration of DA in the electrode was 200 mM with an equal concentration of ascorbic acid to reduce oxidation of DA. A retaining current of −3 nA in the DA electrode was used during the course of the experiment. The current was transiently stepped to +1 nA (6-s duration) to eject DA.

**DA antagonist**

Sulpiride (Tocris) DA antagonist was used because previous studies showed that 1 mM sulpiride blocked several dopaminergic connections in *Aplysia* including input to B4 (Díaz-Ríos and Miller, 2005). In experiments using the isolated buccal ganglion, the chamber was perfused with a HiDi-ASW solution (see *in vitro* analog of OC) to minimize activation of polysynaptic pathways. En.2 was stimulated with a single shock (0.5-ms duration) adjusted to yield a response in B4 several mV in amplitude. En.2 was then retested after being perfused in HiDi-ASW containing sulpiride, and tested again after being perfused with HiDi-ASW to wash out the DA antagonist. Perfusion consisted of 5 min with a 6 ml/min perfusion rate (6× bath volume).

**Statistical analyses**

Data are represented as mean ± SEM. Statistical significance was set at p < 0.05 and all comparisons were two-tailed. For parametric data, unpaired t tests were used for comparisons between two independent groups and paired t tests were used for comparisons between dependent samples. For nonparametric data, samples were analyzed with Wilcoxon signed-rank tests. For comparison among three dependent groups, one-way ANOVA was performed with Tukey’s post hoc test when data showed a parametric distribution. Shapiro–Wilk test was used to confirm the normal distribution of the mean samples. Statistical analyses were performed using Sigmaplot 12.0 (Systat Software).

**Computational model**

This study used a conductance-based model representing a subset of CPG neurons sufficient to generate BMP-like activity elicited in isolated ganglia. The model is a modified version of that in Costa et al. (2020), implemented with the Simulator for Neural Networks and Action Potentials (SNNAP, version 8.1; Baxter and Byrne, 2007). The methods used to develop the CPG model have been described previously (Ziv et al., 1994; Cataldo et al., 2006; Baxter and Byrne, 2007; Costa et al., 2020).
Briefly, the CPG model includes sub-models of the cerebrobuccal interneuron CBI-2, six protraction neurons (B20, B31, B35, B63, B65, and B40), three retraction neurons (B4, B51, and B64), and a postretraction neuron (B52). Neurons B31, B51, and B64 were modeled with two compartments (soma and axon), whereas the other neurons were modeled with a single compartment. The entire model has 49 excitatory, 32 inhibitory, and 22 electrical coupling synapses. Each synaptic connection was based on empirical data, with one exception. Because the mechanism of the transition between protraction and retraction is still under investigation, we included a fictive B63-to-B64 synapse, as used in Costa et al. (2020), to mediate the transition between these two phases. Conductance-based models of voltage-gated ion channels were added to each compartment, including only the necessary channels sufficient to simulate the unique firing properties of each neuron documented in the literature. Noise was a random variation in all conductances with an amplitude chosen to give a variety of patterns; 30 min were simulated, which generated 56–118 BMPs depending on the parameter values of the OC plasticity loci. For experiments examining synergism 30 min simulations were repeated six times. The simulations were concatenated and 100 BMPs were randomly sampled without replacement. This process was repeated 1000 times to obtain a distribution of the iBMP rates. The mean and SD were calculated as estimates of the width and central tendency of the distribution.

Results

Contingent reinforcement of iBMPs reduced the excitability of B4 but not B8

To evaluate the mechanisms of OC, this study used a previously developed in vitro protocol in which tonic stimulation of n.2,3 elicited a mix of ingestion-like and rejection-like BMPs (iBMPs and rBMPs; Fig. 1A). As previously described (Nargeot et al., 1997), stimulation of En.2 was contingent on the expression of iBMPs during training (Fig. 1B). Figure 1C illustrates an example recording of BMP patterns expressed during pre-test and post-test periods from two preparations, one in the contingent reinforcement group (Fig. 1C1), and one in the noncontingent group (Fig. 1C2). Training led to a 17.8 ± 10.9% [mean ± SEM; ((Post-Pre)/Pre)*100] change in iBMP rate for preparations that received contingent reinforcement, compared with a −47 ± 8.4% change in iBMPs in a yoke
group (Fig. 1D). There was a statistical difference between groups (Wilcoxon signed-rank test, \( W = 105, p < 0.001, n = 14 \) in each group). The number of pre-test iBMPs was not statistically different between yoke and contingent groups (contingent, \( 7.7 \pm 0.8 \); yoke, \( 7.1 \pm 0.9 \); paired \( t \) test, \( t_{(13)} = 0.430, p = 0.674, n = 14 \) in each group). The decrease of iBMP frequency in the yoke group is consistent with observations in previous studies (Nargeot et al., 1997). The decrease in iBMPs in the yoke group could be because of a time-dependent reduction in effectiveness of Bn2,3 stimulation. Consistent with this idea, the yoke group also had a reduction in total number of patterns (pre, \( 9.8 \pm 1.3 \); post, \( 5.8 \pm 1.3 \); paired \( t \) test, \( t_{(13)} = 3.655, p = 0.003 \)).

Previous studies found increased excitability of neurons B30, B63, B65, and B51 (Fig. 2A; Nargeot et al., 1999a,b, 2009; Lorentzetti et al., 2006, 2008; Mozzachiodi et al., 2008). To determine whether OC leads to a complementary decrease in excitability of a neuron that suppresses iBMPs, the intrinsic properties of B4 were measured (Fig. 2). A recording of B4 during an iBMP is shown in Figure 2B. Excitability was measured as the number of spikes during injection of an intracellular depolarizing current pulse (Materials and Methods). Contingent reinforcement led to a decrease in the excitability of B4 (\(-72.5 \pm 10.9\%\) that was significantly different from the yoke control group (\(-15.5 \pm 16.0\%\); paired \( t \) test, \( t_{(9)} = 3.330, p = 0.009, n = 10 \) in each group; Fig. 2C). The excitability at pre-test was not statistically different between the groups (contingent, \( 25.0 \pm 4.0\%\); yoke, \( 22.5 \pm 3.1\%\); paired \( t \) test, \( t_{(9)} = -0.517, p = 0.618, n = 10 \) in each group). The decrease in excitability was not because of changes in \( R_{\text{in}} \) (contingent, \(-27.1 \pm 2.0\%\); yoke, \(-27.4 \pm 2.1\%\); paired \( t \) test, \( t_{(7)} = 0.090, p = 0.931, n = 8 \) in each group) or \( V_{\text{rest}} \) (contingent, \( 2.0 \pm 4.0\%\); yoke, \( 2.9 \pm 2.4\%\); paired \( t \) test, \( t_{(9)} = -0.182, p = 0.859, n = 10 \) in each group).

We next investigated the excitability of closure motor neuron B8 (Fig. 2A). B8 is a potential site of OC plasticity because an increase in its excitability could increase iBMP expression (Fig. 2A). In contrast to the changes in B4 excitability, no significant difference was observed in B8 excitability (Fig. 2D) between contingent (\( 0.7 \pm 6.8\%\) and yoke groups (\( 10.2 \pm 11.9\%\); paired \( t \) test, \( t_{(6)} = 0.871, p = 0.417, n = 7 \) in each group). There was no statistical difference in \( R_{\text{in}} \) (contingent, \(-2.0 \pm 2.8\%\); yoke, \(-9.7 \pm 6.8\%\); Wilcoxon signed-rank test, \( W = 50, p = 0.625, n = 5 \) each group) or \( V_{\text{rest}} \) (contingent, \( 2.7 \pm 2.5\%\); yoke, \( 3.9 \pm 3.3\%\); paired \( t \) test, \( t_{(5)} = 0.50, p = 0.584, n = 7 \) each group). These results indicate that OC reduces excitability of a specific subset of neurons (i.e., B4 but not B8).

A single-cell analog of OC reduced B4 excitability

We hypothesized the OC-induced decrease in B4 excitability is intrinsic to B4 and that the OC-induced changes could be replicated by pairing activity in B4 with exogenous application of DA. This hypothesis is supported by evidence that DA is released by stimulation of nerve En.2 (Kabotyanski et al., 1998; Nargeot et al., 1999c) and neuron B4 is enveloped by tyrosine hydroxylase labeled fibers (Kabotyanski et al., 1998). As a first step, we extended previous studies of Nargeot et al. (1999c) implicating a role for DA in En.2-mediated responses in B4. B4 responses during En.2 stimulation were compared before and after application of a DA receptor antagonist, sulpiride (1 mM; Díaz-Ríos and Miller, 2005; Fig. 3A). The single-pulse stimulation of En.2 elicited depolarizing responses in B4 and these responses were partially blocked by application of sulpiride (control, \( 2.7 \pm 0.3 \text{ mV} \); sulpiride, \( 1.5 \pm 0.3 \text{ mV} \)) and recovered to control levels after washing out (\( 2.8 \pm 0.5 \text{ mV} \)). One-way RM ANOVA revealed an overall effect of treatment (\( F_{(2,18)} = 6.797, p = 0.006, n = 10 \)). The amplitude of En.2-elicited responses in B4 in the presence of sulpiride was statistically different from both control measures and washout (before, \( q = 4.266, p = 0.010 \); washed, \( q = 4.730, p = 0.019, n = 10 \)). The lack of a complete block of the En.2-mediated responses could be because of the presence of nondopaminergic afferents in En.2, or that the DA released from the afferents engages DA-receptor subtypes in B4 not affected by sulpiride. Despite the lack of a total block, these data indicate that B4 receives DA input from En.2, a finding that is consistent with previous studies (Kabotyanski et al., 1998; Nargeot et al., 1999c).

We next examined whether the OC-induced changes were intrinsic to B4 using a single cell analog of OC where DA is iontophoretically applied to B4 neurons that were isolated and grown separately in culture. We first confirmed that isolated cultured B4 neurons expressed DA receptors. Similar to En.2 stimulation in ganglia, iontophoretic application of DA near the axon hillock region produced depolarizing responses (Fig. 3B). Two groups of B4 neurons were used, which differed in the delay between B4 stimulation and DA iontophoresis (Fig. 3C). The contingent group received DA iontophoresis (6-s duration) immediately following suprathreshold depolarizing current injections (5 s) into B4, whereas the noncontingent control group received DA 35 s after B4 stimulation. \( R_{\text{in}} \) and excitability were measured immediately before and after conditioning (Fig. 3D1). The decrease in excitability of the contingent group (\(-62.7 \pm 11.3\%\)) was significantly greater than the noncontingent control group (\(-22.3 \pm 12.1\%; t_{(10)} = 2.39 i = 0.038, n = 7 \) and 5 in contingent and noncontingent group, respectively; Fig. 3D2). The pre-test excitability was not statistically different between the groups (contingent, \( 13.9 \pm 2.1\%\); yoke, \( 13.2 \pm 3.0\%\); paired \( t \) test, \( t_{(10)} = -0.191, p = 0.852, n = 7 \) and 5 in each group). No significant differences were observed in \( R_{\text{in}} \) between the contingent (\(-22.1 \pm 4.5\%\)) and the noncontingent control group (\(-12.9 \pm 3.7\%; t \) test, \( t_{(10)} = -1.476, p = 0.171, n = 7 \) and 5 in each group).

These data indicate that the single-cell analog of OC reduced B4 excitability, and similar to in vitro conditioning, this decrease appeared to be independent of a change in input resistance. Moreover, these results indicate that the contingent-dependent decrease in B4 excitability was intrinsic to B4 and mediated by DA. We next examined whether the strength of chemical synaptic connections is altered by OC.

An in vitro analog of OC reduced the B4-to-B51 IPSP but not the B4 to B8 IPSP

B4 makes a large number of synaptic connections in the buccal ganglion (Gardner, 1977; Gardner and Kandel, 1977; Fiore and Meunier, 1979; Susswein and Byrne, 1988; Kabotyanski et al., 1998; Sasaki et al., 2009; Dacks et al., 2012). Here, we focused on the B4-to-B51 and B4-to-B8 inhibitory synapses. These two connections could contribute to the changes in pattern generation associated with OC. A reduction in B4-to-B8 inhibitory synaptic strength would directly disinhibit B8, thereby favoring the expression of iBMPs. A reduction of the B4-to-B51 inhibitory connection could indirectly promote the expression of iBMPs by allowing increased activity in B51, and thus, increased activation of the B51-to-B8 excitatory connection (Fig. 4A).

A simultaneous recording of B4 and B51 during a BMP is shown in Figure 4B. The B4-to-B51 IPSPs from the contingent
Contingent training decreased the IPSP in B51.

**A** Simplified schematic of the feeding CPG. **B** A typical recording of B4 and B51 during an iBMP. Contingent training decreased the IPSP in B51. **C1** Representative intracellular recordings illustrating the measurement of the B4-to-B51 IPSP amplitude before (pre-test) and after (post-test) contingent (red-traces) and yoke control (blue-traces). Because the membrane potential was held at −100 mV, IPSPs were reversed in this panel. **C2** Summary data. **D1** Representative intracellular recordings illustrating the measurement of the B4-to-B8 IPSP amplitude before (pre-test) and after (post-test) contingent training (red-traces) and yoke control (blue-traces). Because the membrane potential was held at −100 mV, IPSPs were reversed in this panel. **D2** Summary data. **Fig. 4.** In vitro analog of OC reduced the B4-to-B51 synaptic connection but had no effect on the B4 to B8 synaptic connection. **Fig. 1** A conductance-based model of the CPG. We hypothesized that a decrease in the excitability of B4 and in the B4-to-B51 inhibitory connection would contribute to biasing the CPG toward generating iBMPs. It is difficult, however, to estimate the relative contributions of B4 changes compared with previously discovered OC-induced loci of plasticity, such as increased excitability and coupling of neurons active during protraction that initiate BMPs (i.e., B30, B63, B65, B30→B63, B63→B51, B63→B65; Nargeot et al., 2009; Sieling et al., 2014), and B51 that selects iBMPs (Nargeot et al., 1999a, b; Lorenzetti et al., 2006, 2008; Mozzachiodi et al., 2008). To address this issue, we used a conductance-based computational model of the CPG to examine the relative contributions of plasticity loci.

The CPG circuit model is illustrated in **Figure 5A**. Simulations of this model with all parameters set to control values generated activity resembling most of the features of physiological BMPs (compare **Fig. 5B** and **Figs. 2B, 4B**). Note the unique pattern of activation of each neuron during the simulated BMPs. The CPG also randomly switches between iBMPs and rBMPs (**Fig. 6**). The variability was built into the model by introducing noise to the conductances. Consistent with literature, some neurons are more active during iBMPs (e.g., B8 and B51), whereas others are more active during rBMPs (e.g., B20, B34). Thus, this model reflects the selective engagement of subcircuits specific for each behavior. Taken together, these results suggested the model recapitulates the salient features of fictive feeding.

**Contingent reinforcement increased** iBMP expression compared with yoke training (**Fig. 1**). Therefore, we first tested whether concurrently modifying the parameters for all the above plasticity loci (**Fig. 5A2**) increased iBMP expression. To model OC-induced increases in excitability for B51, B30, B63, and B65, we decreased the leakage conductance of each cell. To model OC-induced increases in electrical coupling, we increased the coupling conductance of the B30→B63 and B63→B65 electrical synapses. For simplicity, parameter changes involving the protraction loci B30, B63, B65, B30→B63, and B63→B65 were always imposed simultaneously and not examined in isolation. To model OC-induced changes in B4, we added a Ca²⁺-activated potassium (KCₐ) conductance to B4. The parameters for this conductance were taken from Baxter et al. (1999). This model reproduced the spike rate adaptation observed in B4 and increasing KCₐ maximum conductance above control values mimicked the indicated that plasticity is selectively induced among B4 chemical synaptic connections (e.g., plasticity at the B4-to-B51 connections, but not at the B4-to-B8 connection).

**A conductance-based model of the CPG**

We hypothesized that a decrease in the excitability of B4 and in the B4-to-B51 inhibitory connection would contribute to biasing the CPG toward generating iBMPs. It is difficult, however, to estimate the relative contributions of B4 changes compared with previously discovered OC-induced loci of plasticity, such as increased excitability and coupling of neurons active during protraction that initiate BMPs (i.e., B30, B63, B65, B30→B63, B63→B51, B63→B65; Nargeot et al., 2009; Sieling et al., 2014), and B51 that selects iBMPs (Nargeot et al., 1999a, b; Lorenzetti et al., 2006, 2008; Mozzachiodi et al., 2008). To address this issue, we used a conductance-based computational model of the CPG to examine the relative contributions of plasticity loci.

The CPG circuit model is illustrated in **Figure 5A**. Simulations of this model with all parameters set to control values generated activity resembling most of the features of physiological BMPs (compare **Fig. 5B** and **Figs. 2B, 4B**). Note the unique pattern of activation of each neuron during the simulated BMPs. The CPG also randomly switches between iBMPs and rBMPs (**Fig. 6**). The variability was built into the model by introducing noise to the conductances. Consistent with literature, some neurons are more active during iBMPs (e.g., B8 and B51), whereas others are more active during rBMPs (e.g., B20, B34). Thus, this model reflects the selective engagement of subcircuits specific for each behavior. Taken together, these results suggested the model recapitulates the salient features of fictive feeding.

**Contingent reinforcement increased** iBMP expression compared with yoke training (**Fig. 1**). Therefore, we first tested whether concurrently modifying the parameters for all the above plasticity loci (**Fig. 5A2**) increased iBMP expression. To model OC-induced increases in excitability for B51, B30, B63, and B65, we decreased the leakage conductance of each cell. To model OC-induced increases in electrical coupling, we increased the coupling conductance of the B30→B63 and B63→B65 electrical synapses. For simplicity, parameter changes involving the protraction loci B30, B63, B65, B30→B63, and B63→B65 were always imposed simultaneously and not examined in isolation. To model OC-induced changes in B4, we added a Ca²⁺-activated potassium (KCₐ) conductance to B4. The parameters for this conductance were taken from Baxter et al. (1999). This model reproduced the spike rate adaptation observed in B4 and increasing KCₐ maximum conductance above control values mimicked the
decrease in excitability (Fig. 5C). The decrease in the B4-to-B51 IPSP was mimicked by decreasing the maximal conductance for this connection. We set the above parameters to values matching yoke control ganglia (Y values). OC was simulated by adjusting parameters to new values (C values) determined in this study and the literature. Parameter variations were obtained using the equation $Y_A(C - Y_A)$, where $A$ is a value between 0 and 2, and abbreviated as $A_xOC$. Values of $A$ above 1 examined whether further modification above post-test OC values enhanced iBMP expression. When all parameters were set to yoke values (i.e., 0xOC) the simulated iBMP rate was 0.250 ± 0.022 iBMP/min (mean ± SEM, $N = 4$). Simulations of concurrent OC modifications at all loci increased the iBMP rate to 1.77 iBMP/min at 1xOC levels (Fig. 6A4). Further modification of all loci to 2xOC levels increased the iBMP rate to 2.73 iBMP/min (Fig. 6A6). These modifications also enhanced the rate of total BMPs, from 2.03 BMP/min at yoke parameter values to 3.33 BMP/min at 1xOC (data not shown). These simulations indicated that OC-induced plasticity increased total BMP expression and biased that activity toward the genesis of iBMPs. Moreover, these results suggested that more substantial modifications of these loci may further increase iBMP expression.

We next examined the ways in which alterations at individual plasticity loci change iBMP expression when the parameters for all other plasticity loci are set to yoke (i.e., 0xOC) values. Decreasing the strength of the B4-to-B51 connection alone increased the iBMP rate to 0.933 iBMP/min at 1xOC and to 1.57 iBMP/min at 1.5xOC (Fig. 6B1, B5). Decreasing B4 excitability alone led to a modest increase in iBMP rate to 0.400 iBMP/min at 1xOC and to 0.600 iBMP/min at 2xOC (Fig. 6B1, B4). Increasing B51 excitability alone led to the greatest increase in iBMP rate to 1.50 iBMP/min at 1xOC and 1.87 iBMP/min at 2xOC (Fig. 6B1, B6). In contrast, concurrently modifying the parameters for the protraction loci toward their OC values led to a decrease in iBMP rate to 0.033 iBMP/min at 1xOC and 0.000 iBMP/min at 2xOC (Fig. 6B1, B4). Increasing B51 excitability alone led to the greatest increase in iBMP rate to 1.50 iBMP/min at 1xOC and 1.87 iBMP/min at 2xOC (Fig. 6B1, B3). Importantly, modifying the protraction loci enhanced the rate of total BMPs, from 2.07 BMP/min at yoke parameter values to 3.53 BMP/min at 1xOC (data not shown). These results indicate that increase of B51 intrinsic excitability, reduction of B4-to-B51 chemical synaptic strength, and to a lesser extent reduction of B4 intrinsic excitability can each increase iBMP expression, and that OC modifications of the protraction loci increase total pattern production while actually decreasing iBMP rate.

Figure 5. Overview of the CPG model and the OC plasticity loci. A1, Diagram of the synaptic connections and the loci of OC plasticity (*) identified in this and previous studies. Neurons depicted with two circles have an axonal and soma compartment because these neurons exhibit plateau potentials. A2, Table illustrating the values of parameters simulating OC-induced changes as compared with yoke-control parameter values. Column 0 represents the post-test yoke-control values, column 1 represents the OC post-test values. Column 2 represents hypothetical 2xOC post-test values mimicking learning. B, Example of an iBMP (left) and rBMP (right) from a simulation where all parameters are set to yoke values. C1, Excitability of B4 model for yoke control (blue) and contingent (red) conditions compared with empirical data for yoke (dark-gray) and contingent (light-gray) measured during post-test during a 5-s, 4-nA pulse (see C3). Color scheme the same for C1–C4. C2, Excitability for all parameter values used to modify B4 excitability. Test stimuli same as C1, C2, C3. Comparison of the time course of activity (Hz) during 4-nA current injection for empirical and model data. The model results are the mean of five simulation repetitions. Apparent oscillations emerged from the average of the five responses because of similar interspike intervals of the responses. Solid lines indicate arithmetic mean. Gray fill indicates SEM. C4, Example simulation results showing the membrane potential trace during stimuli presented in C3. D, Comparison of the IPSP waveform for empirical and model data.
Given that changes of parameters at some loci toward their OC values increased iBMP rate, whereas changes at other loci decreased iBMP rate, we next examined what effect the plasticity loci of the current study (i.e., B4, B4-to-B51) had on iBMP expression when the parameters of the previously identified loci (i.e., increased excitability of B30, B51, B63, B65, and increased coupling of B30→B63 and B63→B65) are set to their 1xOC values. Setting the previously identified loci to their 1xOC values enhanced the effects of changes in the B4-to-B51 synaptic conductance and B4 excitability on rate of iBMPs (Fig. 6C). For example, for B4 excitability, the enhancement of iBMP rate was only 0.400 iBMP/min at 1xOC when all other parameters were set to 0xOC (Fig. 6B1,B4), but 1.70 iBMP/min at 1xOC when the B51 and protraction loci were set to their 1xOC values (Fig. 6C1,C3).

Synergism of the plasticity loci

The above results suggest potential synergistic effects between the different plasticity loci when modified combinatorially. To

Figure 6. Plasticity loci have differing levels of efficacy to promote iBMP expression. A1, iBMP rate for each independent simulation when the parameter values for all plasticity loci are set at varying levels relative to OC (Fig. 5A). Except where noted, 19 distinct parameter values were used to generate each line plotted in panels A1, B1, C1. The letters on the lines correspond to the simulations in A2–C6. A2–A6, Example traces from simulations in A1. B1, iBMP rate when only parameters for a single plasticity locus were modified at a time. For simplicity, all parameters modified by OC pertaining to protraction neurons (i.e., B30, B63, B65, B30→B63, B63→B65) were grouped into a single locus. The maximal conductance of the B4-to-B51 synapse was varied from 0.86 mS at Y to 0 mS at 1.5xOC and held at 0 mS for four additional simulations. C1, iBMP rate when protraction and B51 parameters were set to their 1xOC values and either the B4 or B4-to-B51 parameter was varied while the remaining parameter was held at yoke control value.
investigate this possibility further, we examined whether modifying a combination of parameters increased the iBMP rate to a greater extent than the arithmetic sum of iBMP rates when parameters were modified individually. For reference we first set the protraction loci alone to 0.625xOC, which resulted in an iBMP rate of 0.002 ± 0.020 iBMP/min (Fig. 7Ab). We kept the protraction loci at 0.625xOC for all remaining simulations and then, individually or in combination, set the other loci to 0.625xOC, a value unlikely to have a basement or ceiling effect. When B51 excitability was modified the iBMP rate increased to 0.224 ± 0.015 iBMP/min (Fig. 7Ab). However, B4 excitability (Fig. 7Ac) or B4-to-B51 modifications (Fig. 7Ad) did not show a meaningful increase in iBMP rate in this circumstance (0.002 ± 0.020 and 0.006 ± 0.034 iBMP/min, respectively), possibly because the decrease in iBMP rate caused by the protraction loci requires these sites to work synergistically to overcome the deficit. When B51 and B4 excitability were modified concurrently (Fig. 7Af) the iBMP rate increased to 0.926 ± 0.226 iBMP/min, which was much greater than the effects of modifying B4 or B51 alone, and greater than the sum of those effects, suggesting B51 and B4 excitability work synergistically. Synergism was also observed when B51 was modified concurrently with B4-to-B51 (1.46 ± 0.15 iBMP/min; Fig. 7Ag). However, the synergistic effect was marginal when B4 excitability and B4-to-B51 were modified concurrently (Fig. 7Ag), resulting in an iBMP rate of 0.066 ± 0.099 iBMP/min. We then compared the iBMP rate when all the loci were modified concurrently, with the effects of modifying only B51 and B4-to-B51 concurrently. Modifying all loci concurrently gave an iBMP rate of 1.47 ± 0.17 iBMP/min (Fig. 7Ah). This result indicates that B4 excitability has little added benefit on top of the B51 and B4-to-B51 synergism. Notably, B4 excitability has little added benefit on top of the B51 and B4-to-B51 synergism.

Finally, rather than setting the parameters at 0.625xOC, we examined the range of values for which these loci acted synergistically. We set the parameters to values ranging from 0.25xOC to 1.5xOC using the same combinations mentioned above. For nearly all values tested, modifying the B4 and B51 excitability concurrently resulted in a greater iBMP rate than did the sum of the individual modifications (Fig. 7B, orange trace). This effect was also seen when B51 and B4-to-B51 were modified concurrently (Fig. 7B, violet trace). However, modifying the B4 excitability and B4-to-B51 loci concurrently did not synergistically increase iBMP rate consistently compared with the sum of the individual modifications (Fig. 7B, green trace). In addition, modifying all of the parameters concurrently did not generate a greater iBMP rate as compared with concurrently modifying B51 and B4-to-B51 (Fig. 7B, brown trace). These results suggest that synergism only occurred in combinations including B51 excitability, and that this synergism was sufficient to produce the majority of the behavioral phenotype of OC.

In summary, these results indicated that the OC-induced decrease in the B4-to-B51 synaptic connection, and to a lesser extent the decrease in B4 intrinsic excitability, increased the pattern selection of the CPG toward iBMPs. Finally, these plasticity loci are capable of working synergistically to enhance iBMPs. However, this synergism was loci specific in that not all combinations were able to synergistically enhance iBMP rate.

Discussion

Although examples of neuronal correlates of OC have been found in many animal species (for review, see Martin-Soelch et al., 2007; Mozzachiodi and Byrne, 2010; Cox and Witten, 2019; Nargeot and Puygrenier, 2019), the full extent of changes and how these changes interact with each other has been challenging to investigate. The present study used *Aplysia* as a model system to extend understanding of OC by demonstrating: (1) OC decreased excitability of neuron B4; (2) OC decreased the strength of the chemical synaptic connection from B4 to B51; (3) OC-induced changes were site specific; (4) the relative contributions of these changes with a computational model; (5) potential synergism between the plasticity loci. These results provide important new insights into the neural mechanisms of behavior reinforcement during OC.
Reduced B4 excitability

Previous correlates of OC in *Aplysia* include increases in the excitability of neurons that initiate the behavior (Nargeot et al., 2009; Sieling et al., 2014; Costa et al., 2020), and increased excitability of a plateau generating neuron, B51, involved in selection of ingestion (Nargeot et al., 1999b; Brems et al., 2002; Lorenzetti et al., 2008). Here, we found OC decreased the excitability of B4. The B4 neuron has inhibitory connections to a variety of neurons, many of these neurons (e.g., B51) promote the expression of iBMPs. The results in this study indicate that reinforcement of motor patterns not only recruits neurons such as B51 into the motor pattern but also suppresses the activity of neurons that reduce expression of the reinforced motor patterns. This decrease in excitability appears to be intrinsic to B4 and not due, for example, to some change in a tonic modulatory circuit or in electrical coupling, because the excitability decrease can be mimicked by pairing B4 activity in an isolated neuron with application of DA (Fig. 3). Interestingly, pairing activity in B4 with DA leads to a decrease, whereas pairing activity in B51 with DA leads to increased excitability of B51 (Nargeot et al., 1999b; Brems et al., 2002; Lorenzetti et al., 2008). This difference in responses indicates that common signals (i.e., spike activity and DA) presented contiguously mediate opposite changes in B4 and B51. In mammalian systems, it is well known that DA modulation is mediated at least in part by differential expression of receptor subtypes, such as D1-like and D2-like receptors, which have different downstream mediators and lead to different cellular changes (Beaulieu and Gainetdinov, 2011; Liang et al., 2014). The increase in B51 excitability is mediated by a signaling pathway similar to D1-like receptor activation (Lorenzetti et al., 2008). It is possible that the decrease of B4 excitability was mediated by another receptor subtype, such as a D2-like receptor. We observed an increased spike frequency adaptation following OC (Fig. 5C), which may be because of an increase in $K_{Ca}$, a family of channels involved in spike frequency adaptation (Ha and Cheong, 2017) and implicated in many forms of learning (Kuiper et al., 2012; Typlt et al., 2013).

We observed a decrease in input resistance in both the contingent and yoke group in the *in vitro* and single-cell analog of B4 OC. This decrease in input resistance does not appear to be because of injury, given that the input resistance of B8 does not change. Also, previous studies showed an increase in B51 input resistance by contingent reinforcement of iBMPs, with no change in input resistance for the yoke or control groups (Nargeot et al., 1999a). The selective decrease in input resistance of B4 may reflect a nonassociative effect of En.2 stimulation.

Decrease in the chemical synaptic connection from B4 to B51 and its specificity

Previously identified neuronal correlates of OC include increases in the excitability and electrical coupling (Nargeot et al., 1999a, b, 2009; Lorenzetti et al., 2006, 2008; Mozzachiodi et al., 2008; Sieling et al., 2014; Costa et al., 2020). The present study identified the first example of an OC-induced change in the strength of a chemical synaptic connection in the *Aplysia* feeding CPG, indicating that both chemical synaptic, electrical synaptic (Sieling et al., 2014), and intrinsic changes (Nargeot et al., 1999a, b; Sieling et al., 2014) cooperate to mediate the change in motor patterns. A reduction of the B4-to-B51 inhibitory connection would indirectly favor the expression of iBMPs by allowing increased activity in B51 (disinhibition), and thus, increased activation of the B51-to-B8 excitatory connection (Fig. 4A).

Interestingly, the OC plasticity was target specific, decreasing the B4-to-B51 synapse but not the B4-to-B8 synaptic connection. Target specific homosynaptic depression was observed in the early studies of the feeding CPG by Gardner and Kandel (1977). Other examples of target specific plasticity have been observed in *Aplysia* (Trudeau and Castellucci, 1993; Martin et al., 1997) and in the hippocampus, where modulation of synaptic plasticity can be specific to neurons targeting either apical or distal dendrites of CA1 pyramidal neurons (for review, see Edelmann et al., 2017).

The mechanism underlying the decrease in the strength of the B4-to-B51 synapse is not known. This study indicates OC-induced plasticity occurs in the presynaptic neuron, B4, and Nargeot et al. (1999a) showed increased in excitability and input resistance in the postsynaptic neuron, B51, after the OC. It is possible the change in B4-to-B51 is mediated by either presynaptic or postsynaptic mechanisms. In the striatum, activation of D2-like receptor reduces acetylcholine release from local cholinergic neurons (Gorell and Czarnecki, 1986). The principal neurotransmitter of B4 is acetylcholine (Gardner and Kandel, 1977) and the D2-like receptor antagonist sulpiride blocks En.2 responses. A reasonable hypothesis is that release of acetylcholine from B4 is reduced by similar mechanisms as shown in the striatum.

We also did not examine the persistence of the B4-to-B51 or B4 excitability changes in the present study, but we expect that the changes would persist for at least 24 h because OC and at least one of the correlates (B51 excitability changes) persist for at least 24 h (Mozzachiodi et al., 2008).

Relative contributions and synergism between the loci of plasticity

It is well established that B4 biases the CPG away from expressing iBMPs (Sasaki et al., 2009). Therefore, we hypothesized that a reduction in excitability of B4 and its synaptic connection to B51 would bias the patterns toward iBMPs. We examined this possibility by using computer simulations of a conductance-based model derived from empirical biophysical data and cellular firing characteristics. Reduction of B4-to-B51 synaptic strength and to a lesser extent reduction of B4 excitability increased iBMP expression. It was surprising that the change in B4 excitability did not increase iBMP expression as potently as did B4-to-B51 or B51 excitability changes. This result suggested that changes in B4 excitability needed to be combined with changes at other loci to alter the motor pattern output.

Synergism between plasticity loci has been suggested to be important for learning (Gao et al., 2012). The potential for synergism was examined in the current study by computational modeling. The B4-to-B51 synaptic strength and B4 excitability worked synergistically with a previously identified loci of OC, B51 excitability. Combining decreased B4-to-B51 efficacy with increased B51 excitability was substantially more effective at producing the change in behavioral phenotype of OC. Not all combinations of parameter changes acted synergistically, suggesting that some information pathways may be more suited to cooperate synergistically and mediate changes in behavior. We hypothesize that the presence of synergism at some loci but not others can be explained by the intrinsic nature of the neurons. When modifications were combined in neurons with linear firing rate properties (i.e., B4 and B4-to-B51) then the changes did not work synergistically. However, when modifications at these loci were combined with modifications in a neuron with nonlinear
properties such as a plateau potential (i.e., B51) then the combination was seen to work synergistically. As new loci are identified, the relationship between nonlinear firing properties and synergism between loci can be further tested. This study suggests that synergism between plasticity loci plays an important role in understanding the neural mechanisms underlying changes in motor pattern activity in learning. Moreover, this study illustrates the need to consider how an entire network is being modified and the relationship between the different loci rather than considering each plasticity loci in isolation.

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