Implication of dopaminergic modulation in operant reward learning and the induction of compulsive-like feeding behavior in *Aplysia*

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Feeding in *Aplysia* provides an amenable model system for analyzing the neuronal substrates of motivated behavior and its adaptability by associative reward learning and neuromodulation. Among such learning processes, appetitive operant conditioning that leads to a compulsive-like expression of feeding actions is known to be associated with changes in the membrane properties and electrical coupling of essential action-initiating B63 neurons in the buccal central pattern generator (CPG). Moreover, the food-reward signal for this learning is conveyed in the esophageal nerve (En), an input nerve rich in dopamine-containing fibers. Here, to investigate whether dopamine (DA) is involved in this learning-induced plasticity, we used an in vitro analog of operant conditioning in which electrical stimulation of En substituted the contingent reinforcement of biting movements in vivo. Our data indicate that contingent En stimulation does, indeed, replicate the operant learning-induced changes in CPG output and the underlying membrane and synaptic properties of B63. Significantly, moreover, this network and cellular plasticity was blocked when the input nerve was stimulated in the presence of the DA receptor antagonist cis-flupenthixol. These results therefore suggest that En-derived dopaminergic modulation of CPG circuitry contributes to the operant reward-dependent emergence of a compulsive-like expression of *Aplysia*’s feeding behavior.

[Supplemental material is available for this article.]
Results

Monosynaptic dopaminergic connectivity of En.2 afferents with radula pattern-initiating neurons

In a first step, the synaptic relationship of En inputs with the B63 neurons and their pattern-initiating partners (B65/B30) of the buccal CPG network was assessed by stimulating En.2 in isolated buccal ganglia (B.g.) while simultaneously recording these cells and monitoring radula motor output with peripheral nerve recordings (Fig. 1A). In control artificial sea water (ASW), when the CPG was otherwise quiescent, an En.2 stimulation (8 V, 0.3-msec pulses at 10 Hz for 5 sec) at approximately the frequency and duration of spontaneous nerve activity observed during actual feeding (Brembs et al. 2002) elicited a rapid-onset, long-lasting depolarization in all three types of pattern-initiating neuron (Fig. 1B; Supplemental Fig. 1A). The concurrent depolarization and resultant burst firing was associated with an activation of the buccal CPG, which produced 1–3 cycles of fictive radula movement recorded in the motor nerves. To test whether this En.2-induced excitation was mediated by monosynaptic influences from input fibers and/or resulted from polysynaptic connectivity, the same experiment was conducted in ASW containing elevated divalent ion concentrations, which is known to decrease excitability and block polysynaptic pathways in the Aplysia nervous system (Byrne et al. 1978). Under this saline condition, the same electrical stimulation of En.2 was still able to elicit a fast onset and sustained (lasting several tens of seconds) 5–8-mV depolarization of the pattern-initiating cell (Fig. 1C; Supplemental Fig. 1B), but these cells no longer fired action potentials and radula motor patterns ceased to occur. This blockade of CPG activation was reversed by reexposing the B.g. to control ASW (Fig. 1D). These results therefore indicated that at least some of the En.2 influences are mediated by direct excitatory connections with the pattern-initiating neurons, whereas polysynaptic pathways appear to be responsible for the actual triggering of motor pattern genesis.

Histochemical studies have previously shown that En.2 afferent fibers contain DA and its synthesizing enzyme, tyrosine hydroxylase (Kabotyanski et al. 1998; Martínez-Rubio et al. 2009). Moreover, antagonists of dopamine receptors were found to block the postsynaptic effects of electrically stimulating En.2 (Nargeot et al. 1999c; Reyes et al. 2005). We therefore determined whether the monosynaptic and sustained depolarizing responses of the B63/B65/B30 neurons to En.2 stimulation are suppressed by flupenthixol, a DA antagonist that in Aplysia has been found to act through the selective blockade of D1-like receptors (Barbas et al. 2006). In the presence of flupenthixol added to high divalent ASW, the amplitudes of the pattern-initiating cell depolarizations evoked by En.2 stimulation were strongly reduced or abolished (Fig. 2A,B; Supplemental Fig. 1C), but were reversed after washout with saline in which the antagonist was absent (Fig. 2C).
Moreover, as seen in the corresponding dose-inhibition relationship for B63 (Fig. 2D), the amplitude reduction increased with increasing antagonist concentrations, with the effective concentration required to decrease the control response by one-half (IC50) being ~10^{-3} M, a value similar to that previously reported to block cloned D1-like receptors in *Aplysia* (Barbas et al. 2006) and insects (Mustard et al. 2005).

The attenuating effect of flupenthixol was specific to the depolarizing responses to brief En.2 stimulation since radula motor pattern genesis induced by tonic stimulation (2 Hz, 8 V) of the sensory n.2,3 nerve (Nargeot et al. 1997) was not prevented by antagonist exposure (Fig. 3A–C). Rather, with increasing flupenthixol concentrations, the frequency of buccal motor patterns elicited by n.2,3 stimulation progressively increased, with the dose–excitation relationship of the antagonist’s influence on pattern expression indicating an EC50 value of ~10^{-5} M (Fig. 3D). However, this facilitating effect was statistically significant only for concentrations >10^{-5} M ($\chi^2 = 12.646, P < 0.025$; 5.10^{-5} M flupenthixol vs. ASW, $Q = 3.5, P < 0.05$; for all other concentrations of flupenthixol vs. ASW, $Q < 2.1$).

Together, these results indicated that afferent processes in En.2, in contrast to the n.2,3 sensory pathway, make direct dopaminergic connections with the pattern-initiating B63/B65/B30 neurons, producing their long-lasting depolarization and a triggering of radula motor pattern expression that is mediated by an accompanying polysynaptic pathway.

### Contingent-dependent DA release mediates plasticity in radula motor pattern genesis

We next explored whether a contingent electrical stimulation of the dopaminergic En.2 pathway in isolated B.g. preparations is able to replicate the input nerve’s food reward-induced activation in vivo and thereby increase the frequency and regularity of radula motor pattern generation as found after behavioral operant conditioning (Nargeot et al. 2009). For this, in vitro preparations were assigned to one of three groups—Non-contingent, Contingent, and Contingent + Flupenthixol—that were subjected to pre-training, training, and post-training experimental protocols (Fig. 4A). In the first 10 min of the pre-training period, spontaneous cycles of radula motor output were recorded to ensure that all preparations were in a similar initial functional state. During the subsequent 30-min training period, a tonic (2 Hz, 8 V) inciting stimulation of n.2,3 was used to elicit radula motor pattern genesis while the bilateral En.2 nerves were briefly stimulated either in strict association with each motor pattern emission, at the end of the protraction phase (Contingent group), or in a regularly repeating sequence that was uncorrelated with the timing of motor pattern occurrences (Non-contingent group) (Fig. 4B). For the latter, the same number of En.2 stimuli was delivered as during the corresponding training periods for Contingent preparations. The training protocols were conducted either in control saline for the Contingent and Non-contingent groups, or in the presence of ASW containing flupenthixol for the Contingent + Flupenthixol group. The DA antagonist was used at 10^{-5} M (i.e., its IC50; see Fig. 2D) in order to significantly reduce the monosynaptic dopaminergic influence of En.2 stimulation on the pattern-initiating neurons, but without significantly modifying the frequency of radula motor pattern emissions compared to those of the Contingent group. The mean number of contingent En.2 stimuli delivered in the Contingent + Flupenthixol group training was not significantly different from that applied during the Contingent group training ($25.8 \pm 10.8$ and $21.4 \pm 11.1$, respectively, $U = 200.5, P = 0.209$). After the training period, all preparations were bathed for 45 min in control ASW (without flupenthixol) and then in the ultimate post-training test period recordings were made to determine any changes both in n.2,3-induced motor pattern expression and in the cellular properties of the two B63 neurons. Any data collected in this post-training test period that exceeded the 4-h retention period of operant learning in vivo (Nargeot et al. 2007) were excluded from subsequent analysis.

Before training (Pre-test) the frequency of motor patterns generated by isolated B.g. was similar in the three groups of preparation (Non-contingent, 0.56 ± 0.17 patterns/min; Contingent, 0.92 ± 0.27; Contingent + Flupenthixol, 0.93 ± 0.25; $H = 0.618$). In contrast, after training (Post-test), the frequency of output patterns and B63 burst occurrences were different between the three groups ($H = 6.772, P < 0.05$) (Fig. 4C–F), with the rate of motor patterns in the contingent group being significantly higher than those in either the Non-contingent ($q = 3.320, P < 0.05$) or Contingent + Flupenthixol groups ($q = 4.515, P < 0.005$). This finding therefore indicated that the contingent stimulation of En.2 was sufficient to produce a long-lasting modification in the frequency of radula motor pattern genesis and associated B63 bursting, which was similar to that produced by the contingent action/reward association of behavioral operant conditioning (Fig. 4C,D; see Nargeot et al. 2007, 2009). Moreover, this plasticity depended specifically on DA release because the same protocol of contingent En.2 stimulation in the presence of flupenthixol

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**Figure 3.** Flupenthixol does not prevent n.2,3-elicited motor pattern genesis. (A) Simultaneous extracellular recordings of radula motor nerves (upper traces) and an intrasomatic recording of a B63 neuron during radula motor activity elicited by tonic (2 Hz, 8 V) stimulation of bilateral n.2,3 in ASW. (B) The additional presence of flupenthixol did not impair, but rather slightly increased, the frequency of radula motor patterns elicited by inciting n.2,3 stimulation in the same preparation as in A. (C) Same preparation after return to control saline conditions. (D) Dose–excitation curve of flupenthixol’s effect on the frequency of n.2,3-elicited radula motor patterns. The antagonist’s half maximal effect (EC50) on the pattern frequency was attained at ~10^{-5} M.
of En.2 no longer led to stable rhythmic pattern expression in preparations exposed to flupenthixol, the contingent stimulation of En.2 being significantly lower (indicating higher regularity) compared to both the Contingent + Flupenthixol and Non-contingent groups ($q = 4.17, P < 0.005; q = 0.333, P < 0.05$, respectively). The latter two groups were not significantly different ($q = 0.798$).

Taken together, these data indicated that a contingent stimulation of En.2 input fibers in vitro is sufficient to induce both the long-lasting acceleration and regularization of radula motor pattern generation found in vivo after appetitive operant conditioning. Furthermore, the induction of this motor plasticity appears to be critically dependent on En.2 afferent dopamine release and monosynaptic DA receptor activation.

### Contingent-dependent DA release induces plasticity in B63 membrane properties

Since the increased rate and regularization of buccal pattern genesis and B63 bursting after in vivo operant conditioning is accompanied by specific alterations in the excitability and oscillatory properties of the pattern-initiating neurons (Nargeot et al. 2009), we assessed whether the contingent stimulation of En.2 in vitro was able to produce equivalent bioelectrical changes in these cells. The excitability of B63, which was defined as the amount of depolarizing current necessary to attain impulse threshold, was tested by injecting brief (1-sec) current pulses of increasing intensity into a recorded cell from a holding membrane potential of $-70 \text{ mV}$. B63 excitability was found to differ according to the training history of preparations ($H = 8.437, P < 0.025$) (Fig. 5A–C), with the spike threshold being significantly lower in B63s of the Contingent group than in either Non-contingent or Contingent + Flupenthixol ganglia ($q = 3.965, P < 0.01; q = 4.011, P < 0.025$) (Fig. 5D). No significant difference was evident between the latter two groups ($q = 1.998$). Thus, the contingent stimulation of esophageal dopaminergic fibers was able to induce a similar increase in B63 excitability as that found after behavioral operant learning.

We next examined the ability of the contingent En.2 activation to evoke long-lasting modifications in the oscillatory properties of the B63 neurons by injecting steady depolarizing current (+4 nA) into a B63 cell while its contratralateral partner was held silent by continuous hyperpolarization (Fig. 5E–G). Under this latter condition, activity of the buccal CPG was prevented and thus the

(10$^{-5}$ M) no longer caused an increase in motor pattern rate (Fig. 4E,F), which remained similar to that of non-contingent controls ($q = 0.435$).

In addition to inducing a cycle frequency increase, the contingent stimulation of En.2 reproduced the regularization of pattern occurrences and B63 bursting previously found after in vivo operant conditioning (Nargeot et al. 2007). Whereas non-contingent B.g. generated motor patterns at highly irregular and unpredictable intervals (Fig. 4C,G), contingent preparations typically produced stereotyped, rhythmically repeating patterns and B63 bursting (Fig. 4D,H). Again, this regularization of buccal CPG activity was dependent on DA receptor activation since in preparations exposed to flupenthixol, the contingent stimulation of En.2 no longer led to stable rhythmic pattern expression and bursting, but rather, this motor activity remained irregularly expressed as in non-contingent controls (Fig. 4E,F). This DA-dependent regularization of buccal CPG output was further confirmed by comparing the coefficients of variation (CV) of 100 successive inter-pattern intervals in the three groups of preparations (Fig. 4J). The CVs were significantly different among the three groups ($H = 6.406, P < 0.05$), being significantly lower (indicating higher regularity) compared to both the Contingent + Flupenthixol and Non-contingent groups ($q = 4.17, P < 0.005; q = 0.333, P < 0.05$, respectively). The latter two groups were not significantly different ($q = 0.798$).

**Figure 4.** Dopamine receptor blockade prevents radula motor output plasticity in an in vitro analog of operant conditioning. (A) Experimental protocol for associative learning in vitro. Each experiment consisted of four successive periods: a pre-training phase in which radula motor patterns were recorded for 10 min before the preparation was superfused for a further 20 min with either ASW alone or with ASW containing 10$^{-5}$ M flupenthixol; a training phase which differed according to the experimental groups (cf. B); after training, preparations were washed for 45 min in control ASW (i.e., without flupenthixol) before the properties of n.2,3-elicted radula motor output and associated bursting in the bilateral B63 neurons were tested. (B) In the training protocol, three groups of isolated B.g. preparations were used: in two Contingent groups (upper trace) exposed either to control ASW (Contingent) or to ASW containing 10$^{-5}$ M flupenthixol (Contingent + Flupenthixol), a phasic stimulation (10 Hz, 5 sec) of bilateral En.2 (arrowheads) was associated with each spontaneous emission of a radula motor pattern (vertical bars). In a third (Non-contingent) group (lower trace), En.2 stimulations (arrowheads) were applied at constant intervals and with the same number as for Contingent preparations, but independently of pattern occurrences (vertical bars). (C–E) Recordings of radula motor output and B63 activity elicited by tonic n.2,3 stimulation during the test period of three representative Non-contingent (Non-cont.) (C), Contingent (Cont.) (D), and Contingent + Flupenthixol (Cont. + Flu) (E) preparations. The frequency and regularity of radula motor patterns were increased in a B.g. after contingent En.2 stimulation in control ASW (Cont.) as compared to those in ganglia subjected to contingent stimulation in ASW + Flupenthixol (Cont. + Flu) or to non-contingent (Non-cont.) stimulation. (F) Comparison of the rate of pattern occurrences during the post-training test period in the three groups of preparations. The contingent-dependent increase in motor pattern frequency (cf. Cont. with Non-cont.) was blocked by Flupenthixol (Cont. + Flu). (G–J) Autocorrelation analyses of 100 successive radula motor patterns after in vitro training. In a Contingent B.g., the buccal motor patterns were rhythmically expressed as indicated by the significant data fit by a Gabor function (bold line). In contrast, in a contingently trained B.g. in the presence of flupenthixol and in a non-contingent preparation, radula patterns were randomly generated (histograms not significantly fitted by a Gabor function). (J) Comparison of mean coefficients of variation (CV) of inter-pattern intervals of the three preparation groups. The significantly lower mean CV in the contingent group indicated an increase in the regularity of motor pattern occurrences compared to those in the other two groups.
expression of cyclic bursting in the depolarized B63 cell is consistent with a current-induced activation of an underlying endogenous oscillatory mechanism (Nargeot et al. 2009). In accordance with their increased excitability seen above, the frequency of impulse bursts in such functionally isolated B63 neurons was significantly higher in the Contingent than in either the Non-contingent \( (q = 3.403, P < 0.05) \) or Contingent + Flupenthixol groups \( (q = 4.303, P < 0.005) \). Again the latter two groups were not significantly different \( (q = 0.736, H = 6.799, P < 0.05) \) (Fig. 5E–H), indicating that the antagonist prevented the associative rate increase.

The temporal distribution of B63 burst occurrences also differed among the three experimental groups in a manner consistent with En.2-derived dopaminergic neuromodulation. In non-contingent B.g., functionally isolated and continuously depolarized B63 neurons expressed bursting at highly irregular intervals (Fig. 5E,J) in correspondence with typical CPG network output of such control preparations (see Fig. 4C,G). In contrast, in contingent ganglia, B63 bursting in response to the same depolarizing current level was generated rhythmically with constant inter-burst intervals (Fig. 5E,J), as seen with the radula output patterns of the functionally intact buccal network (see Fig. 4D,H). Moreover, the regularization of endogenous B63 bursting was a specific consequence of En.2 DA release since the stimulus-induced plasticity was impaired in the presence of Flupenthixol and depolarization-activated bursting occurred indiscriminately as in non-contingent preparations (Fig. 5G,K). This En.2 DA-dependent regularization of the B63 oscillatory mechanism was further evident in statistical comparisons of the coefficients of variation in inter-burst intervals, which varied significantly between three groups of preparation \( (H = 6.669, P < 0.05; \text{Fig. 5L}) \). The CVs were lower in contingent ganglia than in either the Non-contingent or Contingent + Flupenthixol groups \( (q = 3.441, P < 0.05; q = 4.076, P < 0.01) \), whereas the latter two groups were not significantly different \( (q = 1.019) \).

These findings thus indicated that contingent DA release elicited by En.2 stimulation in vitro is able to selectively modify the biophysical properties of the pattern-initiating B63 neurons in an equivalent manner to the cellular changes found previously with in vivo operant conditioning.

Contingent-dependent DA release increases electrical coupling between the pattern-initiating neurons

In addition to changing the intrinsic properties of the B63/B65/B30 neurons, operant learning also increases the strength of their electrical coupling (Nargeot et al. 2009). To determine the involvement of En.2-mediated DA release in the synaptic plasticity, the coupling coefficients of the left and right B63 neurons were compared in the three groups of preparation. With both neurons of a recorded pair initially held at a membrane potential of \(-70\) mV, the injection of a brief (2-sec) hyperpolarizing current pulse \((10\) nA\) into a pre-junctional B63 was typically found to evoke a greater hyperpolarizing deflection in the contralateral (post-junctional) B63 of contingent preparations than in that of non-contingent preparations (Fig. 6A,B lower traces). In contrast, in the presence of flupenthixol, the post-junctional B63 response to contingent En.2 stimulation remained similar to that observed in non-contingent preparations (Fig. 6A,C lower traces), again indicating that the associative increase was mediated by DA. This DA-dependent coupling increase is further evident in the summary data of Figure 6D, where B63–B63 coupling coefficients were significantly higher in contingent B.g. than in either the non-contingent or contingent + flupenthixol ganglia \( (q = 3.552, P < 0.025; q = 3.863, P < 0.025) \), which themselves were not significantly different \( (q = 2.205, H = 7.599, P < 0.025) \). A similar increase in electrical coupling resulting from contingent En.2 stimulation, which was blocked by flupenthixol, was also found.

Figure 5. Flupenthixol blocks the plasticity in B63 membrane properties induced by in vitro operant conditioning. (A–C) Intracellular recordings of B63 neurons in Non-contingent (A), Contingent (B), and Contingent + Flupenthixol (C) B.g., during 1-sec depolarizing current injections of increasing intensity into cells initially held at \(-70\) mV. Less current was required to reach spike threshold in the B63 cell of the Cont. preparation, indicating an increased excitability. (D) Group comparisons of B63 spike thresholds. The mean threshold value was significantly lower in Cont. B.g. than in either Non-cont. or Cont. + Flu. ganglia. The latter two groups were not significantly different. \((\text{E–G})\) Oscillatory bursting properties of functionally isolated B63 neurons \((\text{lower traces in each case})\) in response to constant depolarizing current \((\pm 4\) nA\) injection while the contralateral B63 cell \((\text{B63c, upper traces})\) was simultaneously held hyperpolarized \((\pm 5\) nA\) to prevent activation of the buccal CPG. Isolated B63 cells in the Non-cont. \((\text{E})\) and Cont. + Flu. \((\text{G})\) preparations generated slow, irregularly repeating spike bursts when continuously depolarized, whereas in the Cont. B.g. \((\text{F})\), depolarized-induced B63 bursts occurred at a higher frequency and with regular interburst intervals. \((\text{H})\) Group comparison of burst rates in functionally isolated B63 neurons. The frequency of B63 bursting increased significantly in the Cont. group as compared to the Non-cont. or Cont. + Flu. groups, which themselves were not significantly different. \((\text{I–K})\) Autocorrelation analyses of 2000 successive action potentials in functionally isolated B63 neurons. Impulses were randomly distributed in time in the Non-cont. \((\text{I})\) and Cont. + Flu. B.g. \((\text{K})\), as indicated by the flat histograms not significantly fitted by a Gabor function, but were organized in rhythmic bursts in a Cont. preparation \((\text{J})\) as indicated by a significant Gabor function fit (bold line). \((\text{L})\) Group comparison of the coefficients of variation \((CV)\) of inter-burst intervals in functionally isolated B63 neurons. The mean CV was significantly lower in the Cont. group than in Non-cont. and Cont. + Flu. preparations. The latter two groups were not significantly different.
Flupenthixol blocks the increase in B63–B63 electrical coupling induced by in vitro operant conditioning. (A–C) Simultaneous recordings of the left and right B63 neurons during constant hyperpolarizing current pulse injection (−10 nA for 2 sec, lower trace) in one of the cell pair (B63 trace) in different Non-contingent (A), Contingent (B), and Contingent + Flupenthixol (C) preparations. The current injection revealed a concomitant increase in membrane input resistance and electrical coupling with the contralateral partner (lower dashed lines in the B63 and B63c recordings, respectively) in the Cont. B.g. compared to either the Non-cont. or Cont. + Flu. preparations. Note that in each case the two neurons were initially held at −70 mV (upper dashed lines in B63 and B63c). (D, E) Group comparisons of B63 coupling coefficient (D) and input resistance (E). Both parameters were significantly higher in the Contingent group than in either the Non-contingent or Contingent + Flupenthixol groups. The latter two groups were not significantly different.

Discussion

Using an in vitro analog of operant conditioning of feeding behavior in *Aplysia*, we report that a contingent electrical stimulation of bilateral esophageal nerve branches is able to reproduce the rewarding effect of food seeking that results from operant reward learning in vivo (Nargeot et al. 2007, 2009). Specifically, direct En.2 stimulation increased both the frequency and regularity of radula motor pattern expression and produced a long-lasting enhancement in electrical coupling between these essential pattern-generating neurons. Importantly, however, the induction of these network and cellular changes was blocked by an *Aplysia* D1-like receptor antagonist, flupenthixol, thereby indicating a fundamental role for dopamine in mediating the operant plasticity (Barbas et al. 2006).

Several earlier studies have provided substantial evidence that esophageal input nerve fibers mediate food-reward processes in *Aplysia*: (1) food stimuli that contributed to the reinforcing process in different forms of both operant and classical conditioning were found to elicit transient esophageal nerve activity (Schwarz and Susswein 1986; Lechner et al. 2000; Brems et al. 2002); (2) such appetitive associative learning was impaired by lesions to theseafferent pathways; (3) in vitro analogs of this associative-learning phasic En.2 electrical stimulation to mimic food signals conveyed in vivo was found to reproduce various facets of the motor circuit- and/or cell-wide plasticity resulting from learning (Nargeot et al. 1999a,b; Brems et al. 2002, 2004; Reyes et al. 2005; Mozzachiodi et al. 2008). In addition, there is considerable data indicating that the esophageal nerve terminals release DA: (1) histofluorescence analyses showed these bilateral nerves to be rich in DA-containing fibers and En.2 was also found to be immuno-reactive to tyrosine hydroxylase (Kabotyaniski et al. 1998; Martinez-Rubio et al. 2009); (2) in vitro exposure to a DA antagonist, methylerygonovine, blocked the monosynaptic actions of esophageal inputs on identified neurons (e.g., B51; see below) of the buccal CPG circuit (Nargeot et al. 1999c) and transient iontophoretic application of DA to such neurons in culture reproduced their membrane responses to esophageal nerve stimulation in situ (Brems et al. 2002; Lorenzetti et al. 2008).

The pivotal contribution of DA to a rewarding function in associative learning has become increasingly evident in both invertebrates and vertebrates (Schultz 1997; Brems et al. 2002; Everitt and Robbins 2005; Reyes et al. 2005; Kemenes et al. 2011; Burke et al. 2012). Our present findings corroborate this general principle by indicating that buccal motor network and cellular plasticity, which is blocked by flupenthixol, is specifically induced by the contingent stimulation of En.2 with motor pattern expression to reproduce the reward/action association of operant learning. Although our data point strongly to a mediating role for DA in the contingent reinforcement process, further experiments are required to determine whether stimulation of En.2, which accesses the wider buccal CPG network, might also lead to indirect cellular and circuit changes via non-dopaminergic transmitter release. Moreover, DA can contribute to the autonomous process of motor pattern genesis. Such an additional effect of DA was indicated by the observation that high doses of flupenthixol increased spontaneous buccal motor pattern genesis and CPG responsiveness to tonic stimulation of the peripheral nerve 2,3. Whether this apparent inhibitory influence of DA on buccal CPG function also contributes to the operant learning-induced changes remains unknown. Nevertheless, it is noteworthy that this latter plasticity was selectively blocked by exposure to low doses of flupenthixol during the training period, impairing the rewarding effect of esophageal nerve stimulation but without affecting the autonomous process of motor pattern genesis. Therefore, without excluding the possibility of an additional DA-dependent modulation of buccal network operation, our data provide strong evidence that at least the neuronal plasticity arising from the rewarding effects of contingent En.2 stimulation is mediated by DA.
Operant conditioning of *Aplysia* feeding was previously found to modify the animal’s decision-making capability for the autonomous selection and initiation of radula feeding-related actions, leading to a long-lasting increase in the frequency and regularity of ingestive (biting) movement occurrences (Nargeot and Simmers 2012). In the present study, the in vitro analog of this operant conditioning used to explore the role of contingent-dependent DA release in the reinforcement pathway was similar to that initially developed to analyze the contribution of rewarding esophageal nerve input to selecting the type of radula motor pattern produced (Nargeot et al. 1997; Mozzachiodi et al. 2008). In this analog of learning the contingent stimulation of En.2 (10 Hz, 6 sec, 8–9 V), which was delivered at the end of the retrac-tion phase of each spontaneous ingestive pattern, was found to selectively favor the continued expression of the rewarded pattern. Moreover, this plasticity was associated with a DA-dependent enhancement of plateauing properties in buccal network neuron B51, which, in turn, during the retraction phase of pattern genesis modifies the decision process for the type of radula output expressed, biasing motor pattern selection toward ingestive (i.e., biting) rather than egestive pattern genesis (Nargeot et al. 1999a,b,c; Brembs et al. 2002; Mozzachiodi et al. 2008). Although an increased rate of radula pattern genesis was also observed in this analog of operant conditioning, the underlying cellular mechanisms and the ability of contingent En.2 stimulation and DA release to transform motor pattern production from sporadic to stereotyped rhythogenesis, as addressed in the present study, had not been previously investigated. In the present study, the decision process for when (buccal pattern initiation) rather than how (pattern selection) to act was examined. The in vitro experiments were therefore designed so that bilateral En.2 stimulation (10 Hz, 5 sec, 8 V) was delivered after the initiation of each motor pattern, i.e., after each protraction phase and activity in B63, to reproduce the in vivo concomitancy of delivery of reward at the end of protraction (Brembs et al. 2002; Nargeot et al. 2007). This paradigm does not allow correlating the En.2 stimulation with expression of the ingestion pattern alone, since the nature of the pattern can only be identified once the pattern has fully terminated (i.e., by the amount of overlap of closure activity with the retraction phase [Nargeot et al. 1997]). Thus, the paradigm of in vitro En.2 stimulation reproduced only one aspect of in vivo operant learning—the contingency of the reward with the autonomous process of radula motor pattern initiation—but it was unable to reproduce the strict contingency with the process of ingestive pattern selection. The autonomous initiation of ingestive and egestive patterns is accomplished predominantly by the same CPG neurons (e.g., B63 [Nargeot et al. 2012]). Consequently, the in vitro stimulation paradigm reinforces the expression of both patterns, reproducing the expected association between the rewarding stimulus and spontaneous bursting in these CPG cells, but potentially resulting in more frequent action/reward associations than with the ingestion-specific paradigm used in vivo. Nevertheless, the in vitro and in vivo paradigms both led to more frequent and regular motor pattern occurrences, suggesting an equivalent underlying plasticity involving the same pattern-initiating neurons. Our results indicate that the contingent activation of En.2 modifies motor pattern genesis in association with a DA-mediated increase in excitability, regularization of oscillatory properties, and enhancement in the electrical coupling of the buccal neurons B63. Thus, the contingent-dependent release of DA from the afferent pathway in vitro does, indeed, replicate the network and cellular plasticity previously found associated with the learning-induced expression of compulsive-like food-seeking behavior in vivo (Nargeot et al. 2009).

It is well known that endogenous DA release or exogenous DA application can modulate neuronal membrane properties. In *Aplysia*, the amine has been found to modify the excitability, bursting, and plateau-generating properties, and post-inhibitory rebound and oscillatory properties of neurons belonging to the buccal CPG network (Lewis et al. 1984; Nargeot et al. 1999a,b; Kabotyanski et al. 2000; Brembs et al. 2002; Serrano and Miller 2006). Moreover, these modulatory actions vary according to the target cell type and whether exposure to the amine is continuous or intermittent. For example, tonic DA application decreases the excitability of buccal circuit neurons B4/5, B34, B64 (Kabotyanski et al. 2000), enhances post-inhibitory rebound in B8 (Kabotyanski et al. 2000), and increases the bursting and plateauing properties of B67 (Serrano and Miller 2006; Serrano et al. 2007). Alternatively, phasic DA release induced by contingent reward reinforcement in vivo or contingent En.2 stimulation in vitro augments both the intrinsic excitability and plateauing capability of neuron B51 (Nargeot et al. 1999a; Brembs et al. 2002; Lorenzetti et al. 2008). Here, we report evidence that a similar contingently evoked DA release from En.2 fiber terminals in isolated B.g. increases the excitability and stabilizes the intrinsic oscillatory properties of the buccal pattern-initiating B63 neurons, and, as a result, is able to reproduce essential features of the cellular plasticity resulting from behavioral associative learning (Nargeot et al. 2009). DA has been previously found capable of activating endogenous oscillatory mechanisms in previously silent neurons or increasing the frequency of ongoing oscillatory activity (Flamm and Harris-Warrick 1986b; Kadiri et al. 2011). Our data extend these findings by showing that the amine can also regularize the otherwise erratic expression of neuronal oscillatory activity, thereby leading to stereotyped rhythmic bursting (see also Serrano and Miller 2006). The ability of DA to exert differential cell-specific effects on intrinsic membrane properties is attributable to several factors, including variability in DA receptor function (see MISSALE ET AL. 1998; MUSTARD ET AL. 2005) as well as differences in both the arrays of modulated conductances (HARRIS-WARRICK AND FLAMM 1987; LLEDO ET AL. 1992; BARNES ET AL. 1994; SCHIFFMAN ET AL. 1995; SURMEIER ET AL. 1995; KLOOPENBURG ET AL. 1999; JOHNSON ET AL. 2003; BÁLLO ET AL. 2010) and the nature of, and interactions between, the underlying second messenger cascades (LORENZETTI ET AL. 2008). Consequently, the amine can dynamically specify functional network variants according to specific behavioral demands (Flamm et al. 1986a,b; Nargeot et al. 1999b).

In addition to altering intrinsic membrane properties, DA is also known to modulate neuronal connectivity (see SURMEIER ET AL. 2011), including chemical and electrical synapses in vertebrates and invertebrates (Laser and Dowling 1985; Pereda et al. 1992; JOHNSON ET AL. 1993; JOHNSON AND HARRIS-WARRICK 1997; KUMAR AND FABER 1999; KABOTYANSKI ET AL. 2000; see also BLOOMFIELD AND VÖLGYI 2009). In *Aplysia*’s buccal network, tonic application of exogenous DA is capable of increasing or decreasing the strength of chemical synapses depending on the connected cell pair (Kabotyanski et al. 2000). Here, we provide evidence that a phasic (contingent) release of endogenous DA from esophageal nerve terminals enhances the strength of the electrical coupling within and between the bilateral B63 neurons and their pattern-initiating cell partners in an equivalent manner to their coupling plasticity resulting from behavioral operant learning (NARGEOT ET AL. 2009). However, it remains to be determined whether this modulation resides solely with an overall increase in the input resistance of non-junctional membrane in these neurons, as indicated by our current data, or, additionally, involves a direct DA-mediated increase in their junctional resistance, as found elsewhere for gap–junctional coupling (Yang ET AL. 1990; HARSANYI AND MANGEL 1992; JOHNSON ET AL. 1993; RIBELAYAGA ET AL. 2008; HU ET AL. 2010). In other central pattern-generating networks, dopaminergic modulation of network electrical coupling can modify the
frequency of motor pattern genesis or switch neuronal activity from irregular to regular and coordinated rhythmic bursting (Ayalì and Harris-Warrick 1999; Varona et al. 2001; Venance et al. 2005). In *Aplysia*, the DA-dependent increase in electrical coupling among the buccal pattern-initiating neurons is similarly associated with changes in the frequency and regularity of their bursting activity and radula motor pattern expression. However, whether these long-lasting changes are causally linked to the coupling increase or that the latter is a secondary consequence of the DA-mediated alterations in cellular properties remains to be established.

**Materials and Methods**

**Animals**

Experiments were performed on adult *Aplysia californica* (80% of animals) and *Aplysia fasciata* (20%), depending on seasonal availability, and were purchased from the University of Miami (FL) or caught locally in the Bay of Arcachon (France), respectively. All experimental protocols were repeated using both species and, consistent with a previous combined study on these two animals, no interspecies differences in electrophysiological findings were obtained (Katzoff et al. 2002; Nargeot et al. 2009). However, for consistency among the figures in this report, all illustrated electrical recordings were obtained from *A. californica*. The animal housing and feeding conditions were identical to those described previously (e.g., Nargeot et al. 2007).

**Saline and pharmacology**

Animals were first anesthetized with a hemolymphatic injection of a solution of MgCl2 (360 mM) buffered with HEPES (10 mM) and adjusted to pH 7.5. The bilateral buccal ganglia (B.G.) were dissected out and placed in a Petri dish under artificial sea water (ASW) composed of (mM): NaCl 450, KCl 10, MgCl2 (6H2O) 30, MgSO4 20, CaCl2 (2H2O) 10, HEPES 10, with the pH adjusted to 7.5 and maintained at 15°C by a Peltier cooling device. A high divalent bathing solution, which was used to reduce neuronal excitability, was composed of ASW containing (mM) CaCl2 30 and MgCl2 182, with the osmolarity of the solution being adjusted by an appropriate reduction in NaCl. Solutions of cis-(Z)-flupenthixol dihydrochloride (flupenthixol, Sigma-Aldrich) were prepared in control Saline and for the DA treatment. Solutions of the latter were prepared by the intracellular injection of sustained depolarizing current (+4 nA) with the cell at its resting membrane potential. The latter was not significantly different among the three experimental groups of preparations (Non-contingent, −66.1 ± 3.5 mV; Contingent, −68.8 ± 2.9 mV; Contingent + Flupenthixol, −65.0 ± 4.9 mV; H = 0.561).

**Electrophysiology**

Extracellular recordings and stimulations were made using wire pin electrodes insulated from the bath by petroleum jelly (Vaseline). Bipolar electrodes were used for tonic stimulation (8 V, 3-msec pulses at 2 Hz) of the bilateral 2.3 nerves (n.2,3) and phasic stimulation (8 V, 0.3-msec pulses at 10 Hz in 5-sec trains) of the anterior branches of the bilateral esophageal nerves (En.2 [Nargeot et al. 2007]) using a Grass S88 stimulator (Astro-Medical/Grass Instruments). In all experimental groups, measurements of neuronal activity started 10 min after the onset of inciting stimulation of the n.2,3 (Nargeot et al. 1997). In the contingent stimulans paradigm, stimulation of En.2 started at the end of the protraction phase of each radula motor pattern cycle. Intracellular recordings of identified cells were made with capillary glass electrodes filled with 2 M KCl/CO2 (tip resistance 20–30 MΩ). Individual cells were identified according to their known synaptic connections and the phase positions of their action potential bursts within each radula motor pattern (see Nargeot et al. 2009). Cell excitability, input resistance, and electrical coupling were measured with two intrasomatic electrodes per neuron, one for injecting current and the other for recording the resultant pre- and post-junctional voltage deflections. Measurements of these properties were made with cells held at an initial resting membrane potential of −70 mV. Input resistance was calculated from the maximal membrane potential deflection evoked by −10-nA injected current. Neuronal excitability was measured as the minimum amount of depolarizing current (1-sec pulses by steps of +0.5 nA from 0 nA) necessary to elicit an action potential. Electrical coupling between a cell pair was quantified by their coupling coefficient defined as the ratio of the post-junctional voltage response to a pre-junctional voltage change elicited by a 2-sec, −10-nA pulse in the pre-junctional neuron. The oscillatory membrane properties of B63 neurons were tested by the intracellular injection of sustained depolarizing current (+4 nA) with the cell at its resting membrane potential. The latter was not significantly different among the three experimental groups of preparations (Non-contingent, −66.1 ± 3.5 mV; Contingent, −68.8 ± 2.9 mV; Contingent + Flupenthixol, −65.0 ± 4.9 mV; H = 0.561).

**Data analysis**

Autocorrelation histograms of radula motor pattern genesis or of impulse bursts in individual neurons were made from 100 consecutive patterns or 2000 action potentials, respectively. Successive motor patterns or spike bursts were considered to be rhythmically elicited if they occurred within 0.67 TSD, where TSD is the SD of the period in the corresponding autocorrelation histogram could be fitted, with statistical significance, by a sinusoidal Gabor function (Nargeot et al. 2007). Data in dose–response curves were fitted by a standard Hill equation:

\[
y = y_0 + \frac{a}{1 + \left(\frac{X}{EC_{50}}\right)^n}
\]

In all cases, calculation of the equation’s parameters and the statistical significance of fits were performed using Sigmaplot software (SystatCA). Comparisons of two and more than two independent data groups were made using the Mann–Whitney Rank Sum Test (U) and Kruskal–Wallis test (H), respectively. Comparisons between more than two independent data groups were made using the Friedman repeated measures ANOVA on ranks (\(r^2\)). Post-hoc pair-wise multiple comparisons and multiple comparisons vs. the corresponding control group were made using the Newman–Keuls multiple range test (q) and Dunn’s test (Q), respectively. Differences were considered to be statistically significant when \(P < 0.05\). The numbers of preparations (n) used in each experimental treatment are provided in the group data analyses of the corresponding figure.

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**References**


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Schiffmann SN, Lledo PM, Vincent JD. 1995. Dopamine D1 receptor modulates the voltage-gated sodium current in rat striatal neurons through a protein kinase A. *J Physiol (Lond)* **483:** 95–107.


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