Activation of High and Low Affinity Dopamine Receptors Generates a Closed Loop that Maintains a Conductance Ratio and its Activity Correlate

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Activation of high and low affinity dopamine receptors generates a closed loop that maintains a conductance ratio and its activity correlate

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INTRODUCTION

Neuromodulators alter network output and have the potential to destabilize a circuit. The mechanisms maintaining stability in the face of neuromodulation are not well described. Using the pyloric network in the crustacean stomatogastric nervous system, we show that dopamine (DA) does not simply alter circuit output, but activates a closed loop in which DA-induced alterations in circuit output consequently drive a change in an ionic conductance to preserve a conductance ratio and its activity correlate. DA acted at low affinity type 1 receptors (D1Rs) to induce an immediate modulatory decrease in the transient potassium current (Ih) of a pyloric neuron. This, in turn, advanced the activity phase of that component neuron, which disrupted its network function and thereby destabilized the circuit. DA simultaneously acted at high affinity D1Rs on the same neuron to confer activity-dependence upon the hyperpolarization activated current (Ih) such that the DA-induced changes in activity subsequently reduced Ih. This DA-enabled, activity-dependent, intrinsic plasticity exactly compensated for the modulatory decrease in Ih to restore the I/Ih ratio and neuronal activity phase, thereby closing an open loop created by the modulator. Activation of closed loops to preserve conductance ratios may represent a fundamental operating principle neuromodulatory systems use to ensure stability in their target networks.

Keywords: activity-dependent intrinsic plasticity, metaplasticity, metamodulation, HCN channel, stomatogastric, pyloric network
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**FIGURE 1** | Phase recovery in the pyloric network. (A) In situ preparation: the stomatogastric nervous system (STNS) is dissected and pinned in a dish. The commissural ganglia (CoGs) contain DA neurons that project to the STG (black) and loculi, which are the source of neurohormonal DA (purple). The well surrounding the STG (blue rectangle) is continuously superfused with saline (in/out arrows). There are ∼30 neurons in the STG. The pyloric network comprises 14 STG neurons; two are drawn: pyloric dilator (PD, red), lateral pyloric (LP, blue). Network neurons interact locally within the STG and can project axons to striated muscles surrounding the foregut. The diagram shows that PD and LP neurons project their axons through identified nerves to innervate muscles (rectangles). (B) Spontaneous pyloric network output from one experiment during a 1 h 5 μM DA application: one set of traces comprises two intra-cellular recordings (top) and two extra-cellular recordings (bottom) from the in situ preparation diagrammed in (A). The three sets of traces represent recordings from the indicated time points, in minutes, directly before and after the start of DA application. Red and blue dashed lines reveal how cycle period and LP-on delay change with time. The two red lines demarcate one cycle. Cycle period (a) is defined as the time between the last spike in one PD burst and the last spike in the subsequent PD burst. Note that for each time point the last spike in the first PD burst is aligned with the first red line; however, the last spike in the second PD burst is not aligned with the second red line except at t = 0. This is because 5 μM DA produces a sustained average 10% reduction in cycle period. Thus, for t = 10 and 60 min, the spike in the second PD burst occurs prior to the second red line. Within the indicated cycle, a blue line aligns with the first spike in LP at t = 0. The time between the last spike in PD and the first spike in LP (LP-on delay) and LP-on phase is b/a. Note that for the t = 10 min cycle, the first spike in LP occurs well before the blue line. This is because DA produces a sustained average 20% LP-on phase advance. LP-on phase recovery can be seen in the cycle at t = 60 min because the first LP spike is again aligned with the blue line. Measures of pyloric output parameters can be obtained from either intra- or extra-cellular traces, and LP burst duration is indicated by (c) on the extracellular traces; scale bars: 20 mV and 500 ms. (C) The pyloric circuit: the diagram represents pyloric neuron interactions within the STG. Open circles represent the six cell types, numbers indicate more than one cell within a cell type: anterior burster (AB), inferior cardiac (IC), ventricular dilator (VD); filled circles, inhibitory chemical synapses; resistors and diodes, electrical coupling; red, pacemaker kernel and its output connections. (D) Phase recovery: the preparation shown in (A) was superfused with one of the two indicated treatments for 1 h and LP-on phase was measured every 10 min throughout the experiment (n ≥ 6/treatment). Average fold-changes in LP on-phase are plotted for each group; yellow asterisks, significantly different from t = 0, data taken from Rodgers et al. (2011a). Note that phase recovery in 5 μM DA was blocked by Cs.
We suggest that a mechanism to maintain the \( I_h: I_A \) ratio may also prevail during DA modulation of pyloric neurons (Rodgers et al., 2011a). There is a single LP follower neuron in the pyloric network, and it contributes to cycle frequency regulation (Weaver and Hooper, 2003). The timing of the LP activity phase is critical for this function (Johnson et al., 2011). LP expresses D1Rs but not D2Rs (Zhang et al., 2010), and DA-induced changes in burst duration and cycle frequency are maintained (Rodgers et al., 2011a); thus, a compensatory mechanism operates to restore neuronal activity phase during neuromodulation. Here we investigate this mechanism and show that LP phase recovery involves a DA- and activity-dependent (DAD) decrease in \( I_h \) that compensates for the modulatory decrease in LP \( I_h \) to restore the LP \( I_h: I_A \) ratio and LP activity phase.

**MATERIALS AND METHODS**

**ANIMALS AND DRUGS**

California spiny lobsters, *Panulirus interruptus*, were purchased from Catalina Offshore Products (San Diego, CA, USA) and Marimi Scientific (Long Beach, CA, USA) and housed at 16–18°C in saltwater aquariums at Georgia State University (Atlanta, GA, USA). Animals of both sexes were used in these experiments. TTX was obtained from TCI (Tokyo, Japan) and 3 M KCl connected to Axoclamp 2B or 900A amplifiers (Molecular Devices, Foster City, CA, USA). Extracellular recordings were analyzed using DataView v6.3.2 (Heitler, 2009) to determine cycle period, spike frequency, burst duration, LP-on/off delays, and LP activity phase as previously described (Rodgers et al., 2011a). Reported values for all parameters represent a 10 cycle average.

Experiments in TTX blocked action potential firing and slow voltage oscillations in STG neurons. Under these conditions, the resting membrane potential of most pyloric neurons is between \(-52 \) and \(-62 \) mV. Pyloric neuron input/output curves suggest that graded transmitter release will be minimal to non-existent at these voltages (Johnson et al., 1991, 1995). DA \((100 \mu M)\) can shift the curves (Johnson and Harris-Warrick, 1990), but a 10-fold lower concentration has a minimal effect on the strength of graded release (Kvarta et al., 2012). Pyloric neurons can oscillate in TTX if bathed in 100 \( \mu M \) DA, but we do not observe pyloric oscillations in TTX at \(<5 \) \( \mu M \) DA. a

**TWO-ELECTRODE VOLTAGE CLAMP (TEVC)**

For TEVC of LP \( I_h \), the LP neuron was impaled with two micropipettes \((8–10 \) MΩ connected to Axoclamp 2B or 900A amplifiers (Molecular Devices, Foster City, CA, USA). The well surrounding the STG was superfused with P. saline containing 100 nM TTX for \( \geq 5 \) min. LP was clamped to \(-50 \) mV holding potential using pClamp software. \( I_h \) was elicited using a series of 4 s hyperpolarizing voltage steps, from \(-60 \) to \(-120 \) mV in 10 mV increments with 6 s between steps. Steady-state peak currents were measured by fitting the current trace back to the beginning of the hyperpolarizing voltage step using a single exponential equation. In some experiments small oscillations interrupted the current trace at \( t = 0 \) (e.g., Figure 2) and prevented curve fitting. In those experiments, peak \( I_h \) was measured by subtracting the initial fast leak current from the slowly developing peak of \( I_h \) at the end of each negative voltage step. Currents were converted to conductance using \((G = I/V_{\text{peak}}) = V_{\text{holding potential}} - V_{\text{peak}})\) and fitted to a first-order Boltzmann equation.

\[ V_{\text{mem}} - V_{\text{h}} = -35 \text{ mV (Kiehn and Harris-Warrick, 1992) \}.} \]

For TEVC measurement of peak \( I_h \), the command potential was stepped from \(-50 \) to \(-90 \) mV for 200 ms to remove resting inactivation. The deinactivating prepulse was immediately followed by an activation pulse to \(-60 \) mV for 480 ms to ensure that channels were maximally activated and observed changes could not be due to alterations in voltage dependence (Zhang et al., 2010). To subtract the leak current the hyperpolarizing prepulse was omitted and instead the prepulse was set to \(-40 \) mV to remove \( I_h \) activation from the \(-30 \) mV holding potential. For recordings to measure the LP \( I_h: I_A \) ratio in 5 \( \mu M \) DA, the saline also contained 20 \( \mu M \) TEA and 1 \( \mu M \) PTX to block DA-induced modulatory changes in other conductances that could interfere with measures of peak currents. Recording voltage steps to mimic slow wave oscillations and action potentials were constructed with pClamp software. When currents were not being measured, and recording steps were not being implemented, LP was held at its initial resting membrane potential in TTX (on average, \(-59 \) mV).
**FIGURE 2** | DA-enables activity-dependent alterations in LP $I_h$. (A) The protocols used to measure DA- and/or activity-induced changes in LP $I_h$ are diagrammed in the top two panels. Asterisks indicate points where TEVC measures of LP $I_h$ were made. Bottom panels show typical LP $I_h$ recordings at $t = 0$ and $t = 10$ min for each of the four indicated treatment groups; scale bars: 500 ms and 5 nA. Note that distal compartments of LP neurons are not completely space clamped and oscillatory activity at $t = 0$ was observed in all treatment groups in ∼20% of the experiments due to the short exposure to TTX (example seen in TTX group); nevertheless, $I_h$ could be measured from the traces. (B,C) Plots of the fold-changes in LP $I_h G_{\text{max}}$ in each treatment group at $t = 10$ min. Each symbol represents one experiment; solid lines indicate the means; *$p < 0.05$, t-tests. (D) Typical LP $I_h$ recordings for additional experiments in 5 nM DA. (E) Plots of the fold-changes in LP $I_h G_{\text{max}}$ in each treatment group in 5 nM DA at $t = 10$ min. Each symbol represents one experiment; solid lines represent means *$p < 0.05$, t-tests.

**DYNAMIC CLAMP**

We used the dynamic clamp to introduce an artificial injection current ($I_{\text{inj}}$) specified to counteract the metaplastic (DA modulation of activity dependent (AD) intrinsic plasticity) change in $I_h$ in LP neurons during ongoing rhythmic pyloric activity following bath application of 5 μM DA (Sharp et al., 1993a,b; Prinz et al., 2004a). The membrane potential of the LP soma was amplified, fed into a PCI-6052E DAQ board (National Instruments, Austin, TX, USA), and digitized at 20 kHz. The dynamic clamp program was written in the C programming language and designed to use the real time Linux dynamic controller (Dorval et al., 2001). This dynamic clamp software calculated the $I_{\text{inj}}$ that would be active at the measured membrane potential ($V_m$) given a set of model parameters as follows:

$$I_{\text{inj}} = G_{\text{max}} m(V_m - E_{\text{rev}}),$$

where $m$ changed according to $\frac{dm}{dt} = (m_\infty - m)/\tau_m$, computed numerically using the first-order forward Euler method, and $m_\infty$ was given by $m_\infty = 1/(1 + \exp((V_m - V_1/2)/V_{\text{slope}}))$. $E_{\text{rev}}$ was set to −35 mV (Kiehn and Harris-Warrick, 1992). Values for $I_h$, $V_m$, $V_1/2$, and $V_{\text{slope}}$ were determined from a Boltzmann fit as described above. The predicted metaplastic change in LP $I_h G_{\text{max}}$ was determined using the activity-dependence curve in Figure 3 and the measured change in LP burst duration after a 10 min application of 5 μM DA. The predicted metaplastic change in $I_h$ conductance was subtracted with the dynamic clamp, which calculated and continuously injected current according to...

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the above model, where $G_{\text{max}}$ = measured LP $I_h$, $G_{\text{max}} \times$ predicted meta plastic change of LP $I_h$, $G_{\text{max}}$. Intracellular and extracellular recordings of LP activity throughout the experiment were obtained using a separate computer equipped with Axoscope and Clampex 9.2 software (Axon Instruments).

STATISTICAL ANALYSIS

Data were checked for normal distribution and analyzed using parametric statistical tests with Prism software package v5.01 (GraphPad, La Jolla, CA, USA). Significance was set at $p < 0.05$ in all cases. Individual samples that were more than 2 standard deviations from the mean were excluded from the analyses after determining the mean. This eliminated two experiments. ANOVAs are followed by post hoc tests that make all possible comparisons between columns (Tukey’s) or that compare all columns to a single column, usually $t = 0$ (Dunnett’s). Means are followed by standard errors.

RESULTS

THE EXPERIMENTAL MODEL

The pyloric circuit is located in the crustacean STG (Figure 1A), and it produces a rhythmic motor output in vivo. Each pyloric cell type displays oscillatory activity in membrane potential with a burst of spikes riding on the depolarized plateau (Figure 1B). The circuit comprises six oscillatory cell types coupled by fast inhibitory synapses and/or gap junctions (Figure 1C). The pacemaker kernel (anterior burster (AB) + 2 PIR neurons) rhythmically inhibits the four follower neuron cell types, which then display different rates of PIR. The different rates of PIR are due, in part, to differences in the expression of $I_h$ in each follower neuron (Baro et al., 1997, 2000). $I_h$ delays pyloric neuron PIR (Tierney and Harris-Warrick, 1992): the hyperpolarizing phase of $I_h$ removes resting inactivation from the $K_v4$ channels mediating membrane potential oscillation.

In the presence of $5 \mu M$ TTX, the pyloric network displayed normal slow wave activity but no SP activity (TTX). TTX was also used to block SP activity. In previous experiments we clearly demonstrated that phase recovery in the presence of $5 \mu M$ DA could be blocked by bath application of CoCI to reduce $I_h$ (Figure 1D).

DA- AND ACTIVITY-DEPENDENT (DAD) REGULATION OF LP $I_h$ IN $5 \mu M$ DA

We first tested the idea that DA conferred activity-dependence upon LP $I_h$ by measuring $I_h$ in LP neurons that showed different activity patterns. In these experiments, LP neurons have one of two activity patterns: either LP activity is completely blocked (TTX, 1 h), or DA increased pyloric network cycle frequency by reducing the inherent period of the pacemaker AB neuron (Harris-Warrick et al., 1992; Rodgers et al., 2011a). DA application also reduced LP burst duration and advanced LP firing phase. The traces indicate that by 60 min in DA, network cycle frequency was still increased and LP burst duration was still decreased, but LP-on phase was restored. In previous experiments we clearly demonstrated that phase recovery was AD: if the experiment shown in Figure 1B was repeated with continuous injection of a depolarizing bias current into LP to block the DA-induced decrease in LP burst duration, then the LP phase advance occurred, but phase recovery did not (Rodgers et al., 2011a). We also showed that phase recovery in the presence of $5 \mu M$ DA could be blocked by bath application of CoCI to reduce $I_h$ (Figure 1D).
The results were consistent with the hypothesis; in the presence membrane potential in TTX (nadir of the LP oscillation at step and holding potentials corresponded to the average peak and in membrane potential (Johnson et al., 2003; Kadiri et al., 2011). Micromolar DA can regulate calcium dynamics during oscillations significantly different between treatment groups ($p < 0.0285$). In the absence of DA the fold-change in LP $I_h \text{G}_{\max}$ was not significantly different between treatment groups (Figure 2C, $t$-test, $p = 0.236$) and there was no significant change in LP $I_h \text{G}_{\max}$ by $t = 10$ min relative to $t = 0$ in preparations where slow wave activity was mimicked (paired $t$-test, $p = 0.1166$) or activity was completely blocked (Wilcoxon matched pairs signed rank test, $p = 0.2969$). We previously demonstrated that 5 nM DA acting at high affinity LP D1Rs permitted a decrease in LP burst duration to produce an increase in LP $I_h \text{G}_{\max}$ that persisted well beyond DA washout (Rodgers et al., 2011a). This suggested that perhaps high affinity D1Rs receptors might also mediate the more rapid DAD regulation of LP $I_h \text{G}_{\max}$ observed in Figure 2B. To test this hypothesis, we repeated the experiments diagrammed in Figure 2A, but applied 5 nM rather than 50 nM DA (Figure 2D). The results were consistent with the hypothesis, in the presence of 5 nM DA, the fold-change in LP $I_h \text{G}_{\max}$ at $t = 10$ min varied according to activity (Figure 2E, $t$-test, $p = 0.0321$). Interestingly, LP $I_h \text{G}_{\max}$ did not change over time in 5 nM DA preparations where slow wave activity was mimicked (paired $t$-test, $p = 0.5996$); however, a complete block of activity produced a clear trend toward an increase in LP $I_h \text{G}_{\max}$ (paired $t$-test, $p = 0.0596$), and the magnitude of the increase was similar to that observed in 5 nM DA (compare Figures 2B vs. 2E). The difference in the TTX + OSC treatment groups in 5 nM DA (no change in $\text{G}_{\max}$) vs. 50 nM DA (decrease in $\text{G}_{\max}$) may be due to the fact that micromolar DA can regulate calcium dynamics during oscillations in membrane potential (Johnson et al., 2003; Kadiri et al., 2011).

For all treatment groups the voltages of half activation changed by 5±3 mV on average, and LP $I_h$ voltage dependence is not considered further here. In sum, ≥5 nM DA permitted activity to differentially regulate LP $I_h \text{G}_{\max}$ but, neither 5 nM DA alone nor changes in activity alone significantly altered LP $I_h \text{G}_{\max}$, i.e., DA did not modulate LP $I_h$, but conferred activity-dependence upon LP $I_h$.

**DAD REGULATION OF LP $I_h$ IS NECESSARY FOR PHASE RECOVERY**

Our previous study suggested that LP phase recovery during sustained DA modulation was triggered by a change in LP burst duration (Rodgers et al., 2011a). In order to understand if and how DAD regulation of LP $I_h$ restored the timing of the LP activity phase in 5 μM DA, it was necessary to determine how LP $I_h$ varied according to changes in LP burst duration. An LP $I_h$ activity-dependence curve for changes in burst duration was constructed by repeating the previous experiments in 5 μM DA for the TTX + OSC treatment group, except that the length of the depolarizing step varied across experiments to mimic a change in burst duration (Figure 3A). A plot of the fold-change in LP $I_h \text{G}_{\max}$ vs. percent change in LP burst duration at $t = 10$ min was best-fitted with a Boltzmann sigmoidal equation. DA (5 μM) produced an average 30% decrease in LP burst duration (Rodgers et al., 2011a), and so, according to the activity-dependence curve, LP $I_h \text{G}_{\max}$ should be reduced by ∼6% in 5 μM DA during on-going activity (Figure 3B, dashed line). This decrease in LP $I_h$ is consistent with our hypothesis that DAD regulation of LP $I_h$ compensates for the DA-induced modulatory decrease in LP $I_h$ to restore the $I_h/I_h$ ratio and the timing of LP activity phase. In order to determine if DAD regulation of LP $I_h$ was necessary for phase restoration, we used the activity-dependence curve in conjunction with dynamic clamp experiments to abrogate DAD regulation of LP $I_h$ (Figure 4).

The experimental preparation was as shown in Figure 1A. After dissection and cell identification the STG was superfused with TTX for 5 min; LP $I_h$ was measured with TEVC and values for $\text{G}_{\max}$, $V_{1/2}$ and $V_{\text{dip}}$ were subsequently incorporated into the dynamic clamp model for $I_h$ (see Section "Materials and Methods"). TTX was immediately washed out with saline for 90 min. LP burst duration was measured at the end of the wash followed by application of 5 μM DA from $t = 0$–0 min. The predicted fold-change in LP $I_h \text{G}_{\max}$ due to DAD regulation was determined using the activity-dependence curve in Figure 3.

**FIGURE 4 | DAD regulation of LP $I_h$ is necessary for phase recovery in 5 μM DA.** Plots of fold-changes in LP $I_h$ phase over time for dynamic clamp (solid line) and control (dashed line) experiments indicate that introduction of a dynamic clamp current to abrogate DAD regulation of LP $I_h$ prevents phase recovery; that is, individual experiments with dynamic clamp (in 5 μM) thick line, average for experiments with dynamic clamp; dashed line, control experiment that was exactly the same as the dynamic clamp experiments except that the dynamic clamp was turned off during the 1 h superfusion with 5 μM DA. Repeated measures ANOVA with Dunnett’s post hoc tests that compared all time points to $t = 0$ showed that average LP$I_h$ phase did not recover in experimental preparations, $F(6,4) = 16.04$, $p = 0.0001$.* $p < 0.05$. Note that phase did recover in the control experiment.
and the measured change in LP burst duration from $t = 0$ to $t = 10$ min, and was subsequently incorporated into the dynamic clamp model for $I_{h}$ (see section "Materials and Methods"). From $t = 10$ to 60 min, dynamic clamp was used to remove the predicted DAD regulation of LP $I_{h}$, i.e., to add back, in the form of dynamic clamp current, the same amount of $I_{h}$ that was predicted to have been lost because of DAD regulation. LP-on phase was subsequently measured every 10 min from $t = 6$–60 min.

Plots of the fold-change in LP-on phase over the course of the experiment demonstrated that 5 μM DA-induced the usual phase advance, but removing DAD regulation of LP $I_{h}$ prevented LP-on phase recovery (compare Figures 4 vs. 1D). It also prevented LP-off phase recovery (repeated measures ANOVA, $F(6,4) = 3.119$, $p = 0.0210$). However, it should be noted that the recovery of LP-off phase may be complicated by the PY cell activity phase. The PY-LP synapse contributes to the timing of LP-off phase, especially when pyramidal cell activity phase. The $I_{h}$ ratio was subsequently measured every 10 min from $t = 6$–60 min. The $I_{h}$ ratio was not measured, LP received a recurring step. Plots of the average peak $I_{h}$ ratio by decreasing LP $I_{h}$, by repeating the experiments to measure $I_{h}$ ratio (Gunay and Prinz, 2010), thus spike activity and DA-induced changes in slow wave activity might have opposing effects on steady-state Ca$^{2+}$, and spike activity could delay the compensatory decrease in LP $I_{h}$ by slowing the rate of change of steady-state Ca$^{2+}$. To investigate this idea, we repeated experiments to measure the LP $I_{h}$ ratio using a recurring step that mimicked not only slow wave activity, but also spike activity.

During normal LP activity, spikes passively spread to the soma and neuropil from a distal spike initiation zone (siz). We mimicked spike activity generated at the siz with depolarizing current injections into the soma. We reasoned that LP HCN channels, which are located in the neuropil (Cooritz et al., 2011), will experience a similar depolarization regardless of whether the spikes initiate at the soma or siz, because the two structures are roughly equidistant from the neuropil. This logic rests on the untested assumption that the electrotonic properties and protein composition of the entire primary neurite membrane between soma and spike initiation zone are homogeneous and that electronic potentials spread with similar efficiency in both directions. We also made untested assumptions about LP spike amplitude and duration. Peak voltage (+40 mV) and duration (2 ms) of PD spikes have been directly measured from intra-axonal recordings (Ballo et al., 2012). We assumed LP and PD spikes would be similar and used these values here.

Previous work suggested that activity-dependent regulation can be coded by the pattern of spike activity and not simply the total amount of depolarization (Gorbunova and Spitzer, 2002). We performed two series of experiments to determine if spike activity influenced the LP $I_{h}$ ratio either by the total amount of depolarization produced or by the pattern of depolarization. The total amount of depolarization was mimicked with a step to +40 mV whose duration equaled the average number of spikes per burst multiplied by 2 ms. Patterned spike activity was mimicked by 2 ms depolarizations to +40 mV separated by the average interpulse
In cycle frequency completely abolished the effect of DA-induced mimicked a 30% decrease in LP burst duration and a 10% increase short depolarization on top of the usual recurrent voltage step that change in LP peak 

average number of spikes per burst does not change significantly 

there was no reduction in LP 

repeated measures ANOVA: 

ratio significantly decreased under these conditions 

was superimposed upon the recurrent voltage step that mimicked the DA-induced 30% decrease in LP burst duration and no change in cycle frequency. The ratio significantly decreased with time; repeated measures ANOVA with Dunnett’s post hoc tests that compare all time points to t = 0: F(3,6) = 7.322, p = 0.0032. (B) Plots of the fold-changes in peak LP, _i_ and _l_, (mean ± SEM) from the same experiments as in (A). Repeated measures ANOVA with Dunnett’s post hoc tests that compare all time points to _t_ = 0 indicate that only LP _l_ was significantly decreased (LP _l_, F(3,6) = 10.68, p < 0.0025; LP _l_, F(3,6) = 12.18, p = 0.0014; interaction, F(3,28) = 0.7789; time, F(3,28) = 6.83, p = 0.0014; interaction, F(3,28) = 0.33, p = 0.8065). 

We next asked if we could delay, but not abolish the compensatory decrease in LP _l_ _G_ _max_ by better mimicking the spike pattern (Figure 6B). To do this, we included an ISI in between each 2 ms depolarization to +40 mV that was equal to the average ISI at _t_ = −10 min multiplied by 0.66, because a 1 h 5 μM DA application reduced the mean ISI to 66% of its initial value [repeated measures ANOVA: F(6,8) = 4.002, p = 0.0065, data not shown]. Including patterned spike activity in the recurrent voltage step delayed the compensatory reduction in LP _l_ _G_ _max_ (Figure 6F). By 10 min in 5 μM DA, the compensatory reduction in LP peak _l_ _G_ _max_ was elicited with protocol B, it was not large enough to compensate for the decrease in LP _l_ _G_ _max_ even by 2 h (Figure 6F). This is because the patterned spike activity also unexpectedly regulated LP _l_ _G_ _max_, the reduction in peak LP _l_ _G_ _max_ was significantly larger for protocols that did (Figure 6F) vs. did not (Figure 6D) mimic spike activity along with DA-induced changes in slow wave activity (two-way ANOVA: treatment, F(1,28) = 0.08, p = 0.7789; time, F(3,28) = 6.83, p = 0.0014; interaction, F(3,28) = 0.33, p = 0.8065).

In the first set of experiments a depolarizing step to +40 mV was superimposed upon the recurrent voltage step that mimicked LP slow wave activity in 5 μM DA (Figure 6A). The duration of the step to +40 mV corresponded to the average number of spikes per burst at _t_ = −10 min multiplied by 2 ms. Note that the average number of spikes per burst does not change significantly during a 1 h 5 μM DA application [repeated measures ANOVA, F(6,8) = 0.8920, p = 0.5083; data not shown]. Surprisingly, this short depolarization on top of the usual recurrent voltage step that mimicked a 30% decrease in LP burst duration and a 10% increase in cycle frequency completely abolished the effect of DA-induced changes in slow wave activity upon LP peak _l_. The LP _l_ _G_ _max_ ratio significantly decreased under these conditions (Figure 6C: repeated measures ANOVA: F(3,3) = 6.114, p = 0.0149) because, there was no reduction in LP _l_ (Figure 6D, mean ± SEM fold-change in LP peak _l_ at 10 min = 1.008 ± 0.010). The insignificant change in LP _l_ _G_ _max_ throughout the 1 h 5 μM DA application could not compensate for the significant decrease in LP _l_ (Figure 6D; repeated measures ANOVA: _l_ _G_ _max_, F(3,4) = 1.001, p = 0.9078; _l_, F(3,3) = 5.251, p = 0.0228). Note that the change in LP _l_ was not significantly different between experiments that did (Figure 6D) vs. did not (Figure 6D) mimic spike activity along with DA-induced changes in slow wave activity (two-way ANOVA: treatment, F(1,28) = 0.08, p = 0.7789; time, F(3,28) = 6.83, p = 0.0014; interaction, F(3,28) = 0.33, p = 0.8065).

interval (ISI), and the number of depolarizations was equal to the average number of spikes per burst.

![Figure 5](https://example.com/figure5.png)
DISCUSSION

The principal finding of our study is that 5 μM DA simultaneously generates flexibility and stability in a rhythmically active neural network by activating a closed loop (Figure 7). DA acts at both low and high affinity D1Rs to alter activity and enable AD intrinsic plasticity, respectively. The feedback loop re-established a conductance ratio that was modified by DA, and thereby restored a neuronal phase relationship during a sustained increase in cycle frequency. The generation of closed loops via modulator-enabled AD intrinsic plasticity may represent a fundamental organizing principle used by modulatory systems to preserve conductance ratios and their associated activity correlates, while at the same time altering other aspects of circuit output.

DA SIMULTANEOUSLY GENERATES FLEXIBILITY AND STABILITY BY ACTIVATING HIGH AND LOW AFFINITY D1Rs

Like most systems, DA transmission takes two forms in the stomatogastric nervous system, tonic, and phasic. DA neurons in the commissural ganglia project to the STG and release DA into open synapses, DA then diffuses to its sites of action before re-uptake (Oginsky et al., 2010). To the best of our knowledge, DA levels have not been measured in the STG, but in other systems that use volume transmission, DA is tonically present at μM levels (range: 0.1–100 nM) and can transiently increase to ~μM levels (range: 0.1–100 μM) near the release sites of bursting DA neurons (Zoli et al., 1998; Schultz, 2007; Fuxe et al., 2010). In addition, the STG is located in a blood vessel and is bathed by neurohemofugal DA (Sullivan et al., 1977; Marder and Bucher, 2007). Generally...
speaking, high affinity receptors respond to ~nM DA (tonic) and low affinity receptors respond to ~μM DA (phasic). We have previously shown that LP possesses both high and low affinity D1Rs that mediate different effects on $I_{A}$. High affinity receptors were activated by a tonic 1 h application of 0.5 nM but not 0.05 nM DA and produced a persistent (i.e., non-reversible) increase in LP $I_{A}$ through a translation-dependent mechanism (Rodgers et al., 2011b, in press). On the other hand, low affinity D1Rs responded to bath application of ~μM DA and immediately and reversibly decreased LP $I_{A}$ by altering its biophysical properties (Zhang et al., 2010). In this study we showed that high affinity D1Rs do not simply act through slow mechanisms (hours) to produce persistent changes in ionic currents, but can also rapidly (seconds to minutes) confer activity-dependence upon an ionic conductance to generate a feedback loop.

Concomitant stimulation of both low and high affinity LP D1Rs altered pyloric network activity (Rodgers et al., 2011a); therefore, DA acts at low affinity receptors to modulate circuit output. At least three key aspects of pyloric network output are modulated by DA (Rodgers et al., 2011a): on average, cycle frequency is increased by ~10%, LP burst duration is decreased by ~30%, and LP firing phase is advanced by ~20%. The LP phase advance is largely due to a DA-induced reduction in LP $I_{A}$ (Harris-Warrick et al., 1995; Zhang et al., 2010). These alterations in network output disrupt LP network function (Johnson et al., 2011). Normally, LP acts through the LP-PD synapse to slow increasing cycle frequencies (Nadim et al., 1999; Weaver and Hooper, 2003; Mamija and Nadim, 2004, 2005; Johnson et al., 2011). The timing of LP activity phase is critical for this function because, LP inhibition has different effects according to when it occurs during the pacemaker oscillation, and a phase advance can even increase cycle frequency (Thirumalai et al., 2006; Johnson et al., 2011). This creates a potential for spiraling changes in network output that would destabilize the system. However, besides eliciting these alterations in network activity, DA acts at high affinity D1Rs to permit AD regulation of LP $I_{A}$. This allows the DA-induced changes in cycle frequency and LP burst duration to subsequently elicit a reduction in LP $I_{A}$ that exactly compensates for the modulatory decrease in LP $I_{A}$ to restore the timing of LP activity phase. Restoring LP firing phase re-establishes LP network function which is to slow increasing cycle frequency (Johnson et al., 2011). This could limit the DA-induced increase in cycle frequency driven by DA actions on the pacemaker and stabilize circuit performance at the increased network cycle frequency, decreased LP burst duration, and potentially altered LP input-output gain (Burdakov, 2005). It should also restore the initial phasing of rhythmic pyloric muscle contractions, but at an increased cycle frequency. Interestingly, burst duration and on/off-delays scale with cycle period in the natural population throughout development and over a wide range of temperatures (Bucher et al., 2005; Goaillard et al., 2009; Tang et al., 2010). Thus, the closed loop uncovered here may be part of a more extensive control system that synchronizes these network characteristics over multiple time scales and through multiple mechanisms.

**DOPAMINERGIC TONE MIGHT MAINTAIN THE $I_{A}/I_{h}$ RATIO DURING NON-DOPAMINERGIC PERTURBATIONS TO ACTIVITY**

Landmark studies from the Masder group demonstrated equivalent neuronal and network firing patterns can arise from different sets of intrinsic and synaptic conductances (Godowsch et al., 1999a,b; Prinz et al., 2004b; Schulz et al., 2006, 2007). This work led to the idea that conductances co-vary over time in order to maintain a particular activity feature, an idea that was supported by existing ion channel overexpression studies (MacLean and Selverston, 2005). These findings were unexpected and caused the Selverston group to ask: can the output of a network made up of disparate components be robust to perturbation (Szasz and Selverston, 2006)? Within a population, peak PD $I_{A}$ and PD $I_{h}$ vary by >3-fold across individuals; but, all individuals maintain the same PD $I_{A}/I_{h}$ ratio (Temporal et al., 2012). Selverston’s group reasoned that if PD $I_{h}$ were blocked with 4-AP in multiple preparations, and the variable amounts of PD $I_{h}$ in each preparation would be revealed in distinct PD activity patterns (Szasz and Selverston, 2006; Nowotny et al., 2007). To their surprise, all blocked neurons produced similar activity patterns, suggesting either that the pyloric network is not made up of disparate components in each individual or that rapid compensatory mechanisms must exist to maintain activity. Our findings suggest the latter may be true: modulator-enabled, AD feedback loops could have produced compensatory changes in $I_{A}$ that maintained activity in the Selverston group’s experiments. Indeed, modulatory inputs were intact in the latter studies (Szasz and Selverston, 2006), and 4-AP significantly alters pyloric cycle period and neuronal burst

**FIGURE 7 | DA (5 μM) activates a closed loop.** DA (5 μM) acts at high affinity D1Rs to confer activity-dependence upon LP $I_{A}$ (DAD regulation, coral). In addition, 5 μM DA acts at low affinity D1Rs to modulate LP $I_{h}$ and circuit output (DA modulation, green). Note that the D1R high affinity (coral) and low affinity (green) effects each provide an arm of a closed loop. DA (5 μM) initially increases network cycle frequency, decreases LP burst duration and advances LP activity phase. The latter is due to a decrease in LP $I_{A}$. The phase advance not only prevents LP network function, which is to act as a brake on increasing cycle frequencies, but may even drive further increases in cycle frequency. DAD regulation permits these DA-induced changes in activity to subsequently produce a compensatory decrease in LP $I_{A}$, $G_{h}$. This restores the LP $I_{h}/I_{A}$ conductance ratio and the timing of LP activity phase at the increased cycle frequency and decreased burst duration. This will stabilize circuit output by limiting further increases in cycle frequency.
Both intrinsic and synaptic mechanisms can operate over different time scales to maintain pyloric neuron phase relationships when cycle frequency varies. Synaptic depression rapidly promotes phase maintenance by proportionately delaying neuronal firing as synapses increasingly recover from depression with longer cycle periods (Nadim et al., 1999; 2003; Manor et al., 2003). DA can modulate synaptic dynamics to promote phase maintenance: 10 μM DA decreased the time constants of short-term depression and its recovery at the PD–LP graded synapse, thus contributing to phase maintenance with changing network frequency (Kvarta et al., 2012). It is also worth noting that PY inhibition onto LP plays an important role in determining LP off-phase and this impact can modulate synaptic dynamics to promote phase maintenance with changing network frequency (Kvarta et al., 2011). Fast intrinsic conductances, including Ih, can act in conjunction with synaptic mechanisms to promote phase maintenance in pyloric neurons (Rose et al., 2004; Greenberg and Manor, 2005; Rabbah and Nadim, 2005). Slower processes can also play a role in pyloric neuron phase maintenance. In a combined physiological and computational study on the spiny lobster, Hooper et al. (2009) demonstrated that a conductance with slow activating and inactivating kinetics (seconds to minutes) could explain adjustment of PIR and phase maintenance in PY neurons in the presence of altered cycle period. Gaillard et al. (2010) showed the crab LP neuron possessed a similar mechanism. Neither of these studies identified the slow conductance. Ih was considered, but blocking Ih did not terminate the mechanism. The authors suggested the conductance could be an unidentified slow potassium or calcium conductance, deactivation of a fast sodium current, a pump current or a combination of opposing currents with fast kinetics. Our research extends these previous findings by revealing the existence of a DA-enabled mechanism(s) for phase maintenance that involves preserving the Ih–IP3 ratio. DAD regulation of LP Ih may contribute to phase maintenance in other rhythmically active systems where phase relationships are maintained amidst changes in cycle frequency (Diacrepo et al., 1997; Jacobson et al., 2009).


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Dopamine activates a closed loop


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