Activity and Neuromodulatory Input Contribute to the Recovery of Rhythmic Output After Decentralization in a Central Pattern Generator

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Central pattern generators (CPGs) are neuronal networks that control vitally important rhythmic behaviors including breathing, heartbeat, and digestion. Understanding how CPGs recover activity after their rhythmic activity is disrupted has important theoretical and practical implications. Previous experimental and modeling studies indicated that rhythm recovery after central neuromodulatory input loss (decentralization) could be based entirely on activity-dependent mechanisms, but recent evidence of long-term conductance regulation by neuromodulators suggests that neuromodulator-dependent mechanisms may also be involved. Here we examined the effects of altering activity and the neuromodulatory environment before decentralization of the pyloric CPG in Cancer borealis on the initial phase of rhythmic activity recovery after decentralization. We found that pretreatments altering the network activity through shifting the ionic balance or the membrane potential of pyloric pacemaker neurons reduced the delay of recovery initiation after decentralization, consistent with the recovery process being triggered already during the pretreatment period through an activity-dependent mechanism. However, we observed that pretreatment with neuromodulators GABA and proctolin, acting via metabotropic receptors, also affected the initial phase of the recovery of pyloric activity after decentralization. Their distinct effects appear to result from interactions of their metabotropic effects with their effects on neuronal activity. Thus we show that the initial phase of the recovery process can be accounted for by the existence of distinct activity- and neuromodulator-dependent pathways. We propose a computational model that includes activity- and neuromodulator-dependent mechanisms of the activity recovery process, which successfully explains the experimental observations and predicts the results of key biological experiments.

INTRODUCTION

Central pattern generators (CPGs) are neuronal networks that control rhythmic behaviors such as breathing, heartbeat, swimming, feeding, walking, and flying without requiring patterned sensory feedback (Marder and Bucher 2001). Studies in both vertebrates as well as invertebrates have shown that these networks remain functional even when they are isolated from the organism and receive no rhythmic neuronal input from the environment (Marder and Bucher 2001). The vital nature of many of the behaviors governed by CPGs suggests that evolutionary pressures may have led to the emergence of safeguard mechanisms that enable the recovery of the patterned activity after its disruption by trauma or disease. Indeed it has previously been shown that the pyloric network, located in the stomatogastric ganglion (STG) of decapod crustaceans, recovers its rhythmic activity after it is disrupted by the removal of all central neuromodulatory inputs to the network, a process we refer to as decentralization (Golowasch et al. 1999b; Khorkova and Golowasch 2007; Luther et al. 2003; Thoby-Brisson and Simmers 1998). The recovery of activity in the crab occurs within hours to days after decentralization and achieves levels and characteristics similar to those observed in control preparations (Golowasch et al. 1999b; Luther et al. 2003). Based on evidence that neurons of the pyloric network respond to changes in activity with changes in their intrinsic properties (Haedo and Golowasch 2006; Turngiano et al. 1994) and in their ionic currents (Golowasch et al. 1999a; Haedo and Golowasch 2006; Khorkova and Golowasch 2007), previous modeling work has shown that such recovery of activity can be based entirely on activity-dependent mechanisms (Golowasch et al. 1999b; Zhang and Golowasch 2007). However, new experimental evidence that shows that neuromodulators have long-term effects on ionic conductances in members of the pyloric CPG (that are different from their short-term effects) suggests that neuromodulators also play a role in the slow recovery process (Golowasch et al. 1999a; Haedo and Golowasch 2006; Khorkova and Golowasch 2007).

Here we examined the contributions of activity-dependent and neuromodulator input-dependent mechanisms on the process of recovery of the rhythmic activity of the crab pyloric network after decentralization. We have done this by studying the time course of network activity changes, namely the termination of rhythmic pyloric activity immediately following decentralization and the time course of the beginning of its subsequent recovery, in response to manipulations of the activity or neuromodulator environment prior to decentralization. Using a computer model of this process, we show that the recovery process can be explained by activity- and neuromodulator-dependent mechanisms acting via independent pathways. Finally, we used the model neuron to predict the results of biological experiments and experimentally confirm the prediction.

METHODS

Stomatogastric nervous system preparation

Jonah crabs, Cancer borealis, were obtained from local suppliers in Newark (NJ) and kept in seawater tanks at 10–13°C. Before dissec-

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tion, crabs were anesthetized by cooling in ice for 20–30 min. The stomatogastric nervous system, including the esophageal ganglion (OG), commissural ganglion (CoGs, which contain neuromodulator-releasing projection neurons), the stomatogastric ganglion (STG), their interconnecting nerves as well as the lateral ventricular (lvn), pyloric dilator (pdl), and pyloric constrictor (pym) motor nerves, was dissected and pinned down in silicone elastomer-lined Petri dishes (Sylgard 182, Fisher Scientific) in normal chilled *C. borealis* physiological saline. Normal saline had the following composition (in mM):440.0 NaCl, 11.0 KCl, 13.0 CaCl₂, 26.0 MgCl₂, 5.0 maleic acid, and 11.0 Trizma base; pH 7.4–7.5. The STG was desheathed before experiments began, even when neurons were not impaled. Neuronal cell types were identified as described before (Harris-Warrick 1992; Selverston et al. 1976).

Organotypic cultures of the stomatogastric nervous system (STNS) were recorded continuously for ≤2 days on an electrophysiology setup at 11–13°C in normal or modified saline (see following text) supplemented with 1 g/l dextrose, 35 μM penicillin and 50 μM streptomycin. To experimentally modify the activity of the pyloric network, we used several pharmacological methods: 1) low-Na⁺ saline, 2) GABA, 3) muscimol, 4) baclofen, and 5) proctolin bath applications or hyperpolarizing current injection into the pyloric dilator (PD) neurons. When low external Na⁺ solutions were used, we replaced the Na⁺ in the normal saline with equimolar amounts of N-methyl-D-glucamine (Acros Organics). GABA, muscimol, baclofen, and proctolin were dissolved immediately until an approximately stable steady state of very slow (<0.1 Hz) or no pyloric activity was obtained for 5–10 min. Proctolin was then bath-applied to the STG and motor nerves only.

Pyloric frequency was measured cycle by cycle using software developed in our laboratory (Datamaster) on a LabWindows platform, which determines bursts of action potentials from extracellular recordings by detecting threshold crossings by the action potentials. Frequency data for each of the 5 or 10 min of pyloric activity was averaged and plotted as one bar with variance indicated by a capped line whose height corresponds to the SD of this mean.

Statistical analysis was performed using nonpaired Student’s *t*-test on one-way ANOVAs with Tukey post hoc tests (SigmaStat 2.03, Aspire Software International, Leesburg, VA). All data are presented as averages ±SD.

**Mathematical model of a single pyloric neuron**

For simplicity and as a model of the pyloric network pacemaker AB neuron, a single two-compartment neuron was built. Although simplified compared with previous AB neuron models (Soto-Treviño et al. 2005), this model expressed endogenous oscillatory activity resembling that of the AB neuron (Figs. 5B and 7B, top traces). Because our goal was to reproduce and provide a mechanistic understanding of the essential aspects of the process of pyloric activity recovery after decentralization, rather than the details of AB neuron bursting activity, we have not attempted to perfectly capture all details of the AB neuron activity. Instead the ionic currents were modeled using a modified version of previous models of decentralization (Golowasch et al. 1999b; Zhang and Golowasch 2007)

\[
\frac{dV_{soma}}{dt} = I_{ext} - \left[ I_C + I_K + I_A + I_{Proc} + I_{GABA} + I_{leak,soma} \right] + G_{soma-axon}(V_{soma} - V_{axon})
\]

\[
\frac{dV_{axon}}{dt} = \left[ I_Na + I_Kd + I_{leak,axon} + G_{axon-axon}(V_{axon} - V_{soma}) \right]
\]

Each ionic current is described by Hodgkin-Huxley-type equations as described before (Zhang and Golowasch 2007), and identical parameter values were used in this study, including \(C_{soma}\) and \(C_{axon}\) except where indicated. The differential equations in the model were numerically solved with the software XPP using the default Runge-Kutta integration method (Ermentrout 2002).

Our previous modeling studies have shown that the regulation of an intracellular activity-dependent Ca²⁺ sequestration mechanism as well as slow activity-dependent K⁺ and Ca²⁺ conductance regulation are required to produce the intermittent on and off pattern of pyloric activity generation that characterizes the initial phase of the recovery process of the crab pyloric network following decentralization (Zhang and Golowasch 2007), an activity pattern we call “bouting” (Fig. 2, B–D). A similar activity-dependent feedback mechanism, using an intracellular Ca²⁺ sensor to regulate the maximal Ca²⁺ conductance, \(G_{Ca}\), and the transport rate of an internal calcium pump putatively localized on the endoplasmic reticulum (ER) membrane, \(R_{sump}\), was used. The intracellular cytoplasmatic Ca²⁺ concentration, \([Ca^{2+}]_{cyt}\), was treated with a first-order diffusion term responsible for clearing Ca²⁺ from the cytoplasm and an IP₃ and Ca²⁺-dependent Ca²⁺ channel responsible for extrusion of Ca²⁺ from the ER, exactly as described in Zhang and Golowasch (2007). This model is schematically illustrated in Fig. 1, which for simplicity depicts only the soma compartment and only a subset of the ionic currents included. The previous model (Zhang and Golowasch 2007) required three different activity sensors to generate the recovery process after decentralization. Here we found only one sensor to be necessary and sufficient to regulate both the Ca²⁺ pump rate (\(R_{sump}\)) and ionic conductances. Furthermore, we found that regulation of \(G_{Ca}\) is sufficient to reproduce the phenomenon and have thus simplified the original model further by excluding...
FIG. 1. Schematic diagram of intracellular activity- and neuromediator-dependent regulation. $G_{GABA}(V)$, $G_{Ca}(V)$, and $G_{pump}(V)$ are the voltage-dependent conductances of $I_{GABA}$, $I_{Ca}$, and $I_{pump}$, respectively. ER here represents the endoplasmic reticulum or any intracellular Ca$^{2+}$ store. IP$_3$RCa is the activated form of the IP$_3$-sensitive Ca$^{2+}$ receptor/channel on the ER membrane, and Ca pump on the ER membrane represents the intracellular Ca$^{2+}$ uptake process. The shaded arrow labeled Diff stands for intracellular Ca$^{2+}$ diffusion and passive buffering. $S_A$ is the activity-dependent Ca$^{2+}$ sensor. $S_A$ detects changes of [Ca$^{2+}]_{cyt}$, and in turn regulates both $G_{Ca}$, and Ca pump. $S_{NM}$ is the neuromodulator sensor that regulates $G_{Ca}$ and no other conductance. $S_{NM}$ is triggered directly by activation of neuromediator receptors, in particular type-B GABA receptors ($R_{GABA}$), and proctolin and other receptors ($R_{proc}$, ), putatively via G proteins or other activated signaling molecules downstream of the receptors. GABA also activates a voltage-dependent hyperpolarizing (or shunting) conductance via GABA$_A$-like receptors (not shown). Proctolin and other non-GABA neuromodulators activate the neuromodulator-sensitive conductance $G_{pump}(V)$.

activity dependence of regulation $G_{Ca}$. Other currents may certainly be regulated by activity in the biological pyloric network, but we have not included them here. We have therefore simplified the model of the $G_{Ca}$ dynamics to make it dependent on a single activity sensor, $S_A$

$$\tau_d G_{Ca}/dt = G_{Ca,min} + F_G(S_A) - G_{Ca}$$

$$\tau_d R_{pump}/dt = R_{pump,min} + F_R(S_A) - R_{pump}$$

$\tau_d$ is the time constant of Ca$^{2+}$ conductance regulation and $\tau_r$ is the time constant of ER pump regulation. $G_{Ca,min}$ and $R_{pump,min}$ are constants representing minimal values of $G_{Ca}$ and $R_{pump}$. The activity sensor, $S_A$, has a direct sigmoidal dependence on [Ca$^{2+}]_{cyt}$

$$S_A = \frac{S \cdot M_S}{1 + e^{M_S}}$$

$M_S$ represents the maximal value of $S_A$, and $M_S$ is the activation variable of $S_A$ and is a growing function of intracellular [Ca$^{2+}]_{cyt}$. $M_{thr,S}$ represents the value of [Ca$^{2+}]_{cyt}$ when $M_S = 0.5$.

The activity sensor in turn regulates both the maximal Ca$^{2+}$ conductance, $G_{Ca}$, and the maximal intracellular Ca$^{2+}$ pump activity, $R_{pump}$, via decreasing sigmoid functions of $S_A$

$$F_G(S_A) = \frac{\tilde{G}_{Ca,S}}{1 + e^{M_S_{Thr,G}}}$$

$$F_R(S_A) = \frac{\tilde{R}_{pump,S}}{1 + e^{M_S_{Thr,R}}}$$

$\tilde{G}_{Ca,S}$ and $\tilde{R}_{pump,S}$ are constants representing the maximal values of $G_{Ca}$ and $R_{pump}$ generated via activity-dependent (i.e., Ca$^{2+}$-dependent) regulation. $F_G(S_A)$ and $F_R(S_A)$ are both decreasing functions of $S_A$, and $S_{thr,G}$ represents the value of $S_A$ when $F_G(S_A) = G_{Ca,min}/2$, and $S_{thr,R}$ represents the value of $S_A$ when $F_R(S_A) = R_{pump,min}/2$. The parameters $r_d$ and $r_r$ represent the steepness of $F_G(S_A)$ and $F_R(S_A)$ regulation, respectively, as a function of $S_A$.

GABA activates two types of receptors: GABA$_A$-like, also activated by muscimol and evoking a current with a reversal potential near ~70 mV in PD neurons and a GABA$_B$-like receptor, also activated by $\beta$-guanidinopropionic acid and baclofen and evoking a current with a similar hyperpolarized reversal potential (Fig. 1) (Parnas et al. 1999; Swensen et al. 2000). To implement both GABAergic effects on pyloric activity in the preceding model, a GABA-activated current, $I_{GABA}$, was included in the somatic current balance equation [1]. $I_{GABA}$ is a voltage-independent current, but its conductance is dependent on GABA concentration, $G_{GABA}$, with a maximum conductance at saturation, $G_{GABA}$, and reversal potential, $E_{GABA}$ (Table 2) taken from Swensen et al. (2000)

$$I_{GABA} = G_{GABA}(V_{soma} - E_{GABA})$$

$G_{GABA} = \frac{G_{GABA}}{1 + e^{S_{NM}/G_{GABA}}}$

$[GABA]_{ext}$ represents the value of [GABA] when $G_{GABA} = G_{GABA}/2$.

To model the GABAergic metabotropic effects, we build a neuromodulator-dependent sensor $S_{NM}$ sensitive to the neuromodulator concentration [NM]. For simplicity, we assume this same sensor to depend on the levels of all metatropic neuromodulators known to be released by axon terminals. Thus $S_{NM}$ is sensitive to GABA (consequently also to GABA$_B$) receptor agonists and to proctolin and represents some intracellular signaling pathway likely activated by a G protein coupled to the neuromodulator-activated receptors (Parnas et al. 1999; Swensen et al. 2000). The introduction of a slow-acting neuromodulator-dependent sensor (or signaling pathway) is consistent with the recently discovered existence of long-term neuromodulator effects, including those of proctolin, on voltage-gated conductances in this system (Khorkova and Golowasch 2007). These effects are distinct from the acute (i.e., fast) activation of the neuromodulator-gated current $I_{pump}$ (Buchholz et al. 1992; Golowasch and Marder 1992; Swensen and Marder 2000) also included in this model. The sensor $S_{NM}$ activates with time constant $\tau_{NM}$ toward a steady-state value that depends on the neuromodulator concentration, [NM]. $[NM]_{thr}$ represents the [NM] needed to reach half the maximal $S_{NM}$ value

$$\tau_{NM} \frac{dS_{NM}}{dt} = (S_{NM} - S_{NM})$$

$$S_{NM} = \frac{1}{1 + e^{[NM]_{thr} - [NM]}}$$

and

$[GABA] = [GABA]_{endo} + [GABA]_{exo}$

$[Proc] = [Proc]_{endo} + [Proc]_{exo}$

$[NM] = [GABA] + [Proc]$
The concentration of neuromodulators other than GABA (proctolin in our experiments) is represented by \([\text{proc}]\) and activation of the conductances \(G_{\text{proc}}\) depends on voltage via \(m_{\text{proc}}(V)\) and on the neuromodulator concentration \([\text{proc}]\):

\[
m_{\text{proc}}(V) = \frac{1}{1 + e^{-\frac{V + 55}{5}}}
\]

\[
G_{\text{proc}} = \frac{\tilde{G}_{\text{proc}}}{1 + e^{-\frac{V - \tilde{v}_{\text{proc}}}{\nu_{\text{proc}}}}} \tag{7}
\]

\([\text{proc}]_{\text{thr}}\) represents the value of \([\text{proc}]\) when \(G_{\text{proc}} = \tilde{G}_{\text{proc}}/2. m_{\text{proc}}(V)\) is identical to what was used in Zhang and Golowasch (2007). In this model, all the time constants of the slow dynamical variables (\(\tau_p, \tau_e, \) and \(\tau_{\text{NM}}\)) have been scaled down \(\sim 10\)-fold from values that would produce a realistic time course of recovery solely for the purpose of speeding the computations. When using 10\(\times\) longer time constants, no qualitative differences from the results shown here are observed. As described before (Zhang and Golowasch 2007), the activity dependent sensor \(S_{\text{NM}}\) needs to be much faster than the other variables and is thus assumed to be instantaneous (see Eq. 3).

To introduce neuromodulator-dependence in the model, Eq. 2 was modified to include a dependence of \(G_{\text{Ca}}\) on a neuromodulator sensor \(S_{\text{NM}}\) via the function \(F_s(S_{\text{NM}})\):

\[
\tau_d \frac{d\tilde{G}_{\text{Ca}}}{dt} = \tilde{G}_{\text{Ca, min}} + F_s(S_\text{NM}) + \tilde{F}_s(S_{\text{NM}}) - \tilde{G}_{\text{Ca}}
\]

\[
F_s(S_{\text{NM}}) = \frac{\tilde{G}_{\text{Ca, NM}}}{1 + e^{-\frac{r_{\text{NM}} - S_{\text{NM}}}{\nu_{\text{NM}}}}} \tag{8}
\]

\(F_s(S_{\text{NM}})\) is an increasing sigmoid function of \(S_{\text{NM}}, \tilde{G}_{\text{Ca, NM}}\) is the maximal value of \(G_{\text{Ca}}\) that can be generated by neuromodulator-dependent regulation, \(S_{\text{thr, NM}}\) is the half-maximal value of \(S_{\text{NM}},\) and the parameter \(r_{\text{NM}}\) represents the sensitivity of \(F_s(S_{\text{NM}})\) regulation. Without exogenous neuromodulators, \(F_s(S_{\text{NM}})\) is much smaller than \(F_{\text{Ca}}(S_\text{NM}),\) and \(G_{\text{Ca}}\) is mainly determined by \(F_{\text{Ca}}(S_\text{NM}).\)

The values of all parameters are listed in Table 1.

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**Table 1. Parameter values of pacemaker model**

<table>
<thead>
<tr>
<th>Name</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\tilde{G}_{\text{Ca, min}})</td>
<td>0.12 (\mu)S</td>
</tr>
<tr>
<td>(R_{\text{pump, min}})</td>
<td>0.002 (\mu)M s MMs(^{-1})</td>
</tr>
<tr>
<td>(\tau_p)</td>
<td>300 s</td>
</tr>
<tr>
<td>(\tau_e)</td>
<td>4500 s</td>
</tr>
<tr>
<td>(S_{\text{thr}})</td>
<td>8</td>
</tr>
<tr>
<td>(M_{\text{Ca, S}})</td>
<td>0.9 (\mu)M</td>
</tr>
<tr>
<td>(\tilde{G}_{\text{Ca, S}})</td>
<td>0.039 (\mu)S</td>
</tr>
<tr>
<td>(R_{\text{pump, S}})</td>
<td>0.006 (\mu)M s MMs(^{-1})</td>
</tr>
<tr>
<td>(S_{\text{thr, G}})</td>
<td>0.17</td>
</tr>
<tr>
<td>(\tilde{S}_{\text{out, R}})</td>
<td>0.17</td>
</tr>
<tr>
<td>(\tilde{\tau}_g)</td>
<td>0.012</td>
</tr>
<tr>
<td>(\tau_g)</td>
<td>0.012</td>
</tr>
<tr>
<td>(E_{\text{GABA}})</td>
<td>(-66) mV</td>
</tr>
<tr>
<td>(G_{\text{Ca, max}})</td>
<td>0.5 (\mu)S</td>
</tr>
<tr>
<td>(\伽\text{GABA})_{\text{In}})</td>
<td>2</td>
</tr>
<tr>
<td>(\gamma\text{GABA}))</td>
<td>0.05</td>
</tr>
<tr>
<td>(S_{\text{NM}})</td>
<td>500 s</td>
</tr>
<tr>
<td>(\text{[NM]}_{\text{hr}})</td>
<td>3</td>
</tr>
<tr>
<td>(\tilde{G}_{\text{NM}})</td>
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</tr>
<tr>
<td>(\tilde{G}_{\text{Ca, NM}})</td>
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<tr>
<td>(S_{\text{thr, NM}})</td>
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<tr>
<td>(\tilde{S}_{\text{thr, NM}})</td>
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<tr>
<td>(G_{\text{proc, thr}})</td>
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</tr>
<tr>
<td>([\text{proc}]_{\text{hr}})</td>
<td>0.998</td>
</tr>
<tr>
<td>(r_{\text{proc, thr}})</td>
<td>0.005</td>
</tr>
</tbody>
</table>

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**RESULTS**

**Animal model**

The STNS of crustaceans, such as the crab *C. borealis*, controls the ingestion and movements involved in food processing in the foregut (Selverston et al. 1976). The movements of one of the forget chambers, the pylorus, are driven by the pyloric network, located in the STG. The pyloric rhythm is composed of alternating bursts of activity of several neuronal groups (see Fig. 2, B and C top). This bursting activity is the result of the interactions of reciprocal inhibitory synaptic inputs between the neurons in the circuit and the neuronal intrinsic properties, all driven by the pacemaker activity of the anterior burster neuron (AB) (Nusbaum and Beenakker 2002). This activity can be recorded both extracellularly from motor nerves (Fig. 2, B and C) as well as intracellularly. We have limited our recordings to extracellular nerve recordings and used intracellular impalements for current injection only.

Neuromodulatory input to the STG comes from the anterior OG and CoGs via a single input nerve, the stomatogastric nerve (stn, Fig. 2A). When the STG was isolated from the rest of the STNS by blocking action potential transmission along the stn, activity of the pyloric network decreased considerably or stopped completely (Fig. 2, B and C, 2nd trace) (see also Golowasch et al. 1999b; Luther et al. 2003; Thoby-Brisson and Simmers 1998; Zhang and Golowasch 2007). This is thought to be due to the cessation of neuromodulator release from axonal terminals of the stn (Marder and Bucher 2007) and the consequent deactivation of \(I_{\text{proc}}\). Over time, the rhythmic activity of the crab and lobster pyloric networks resumes and stabilizes (Golowasch et al. 1999b; Thoby-Brisson and Simmers 1998). In crabs, this occurs after a period characterized by the repeated turning on and off of the rhythm, called bursting (Luther et al. 2003; Zhang and Golowasch 2007). The third trace in Fig. 2, B and C, illustrates two examples of a burst of pyloric activity: in the first, a very brief bout is observed with three bursts of pyloric activity in a single bout (Fig. 2B); otherwise bouts are long-lasting and sometimes appear as an acceleration of a slow ongoing pyloric rhythm (Fig. 2C, cases 1 and 2). We defined a bout as an increase of the pyloric frequency for at least three pyloric cycles and by \(\geq40\%\) above the background frequency (see examples labeled boutting in Fig. 2, B and C). Case 2 in Fig. 2C shows an extreme example in which this acceleration is not easily distinguished by eye yet is clearly distinguished as a rapid increase in a frequency-versus-time graph followed by an exponential return to baseline (Fig. 2C, 5th trace). Figure 2D shows a bar graph summarizing the changes in pyloric rhythm frequency over several hours after decentralization (Fig. 2D, ↑). Figure 2D also illustrates the rate of pyloric activity deacceleration, i.e., rapid pyloric frequency decrease immediately after decentralization (Fig. 2D, inset) and the variability of the pyloric frequency during the boutting period (bouts in Fig. 2D, ↓).

In this study, we examined the hypothesis that the process of recovery of the pyloric network activity can be altered by changes in network activity and the neuromodulator environment preceding decentralization. We experimentally induced activity changes using GABA, GABA agonists, hyperpolarizing current injections into the pacemaker neurons and low external \(\text{Na}^+\) solutions. All experiments described in the following text followed the same protocol: after recording, the
normal pyloric network activity for ~30 min, a pharmacological agent or a manipulation of the membrane potential of identified pyloric neurons was applied for a defined period. The pharmacological agents were then washed out rapidly with normal saline (or the external current injection discontinued) until stable pyloric network activity was recovered (typically within 5–10 min). The STG was then decentralized by blocking action potential conduction along the input stn nerve.

We used two parameters to characterize the recovery process: the rate of rhythmic activity deceleration starting immediately after decentralization and the time to the first bout. We define the rate of activity deceleration as the time needed for the pyloric rhythm frequency to reach for the first time half of its maximum predecentralization value (t_{1/2}) at the time immediately before decentralization (Fig. 2D, inset). The time to the first bout (t_{bout}) of pyloric activity is the time from the moment of decentralization to the beginning of the first observed bout (Fig. 2D). We chose t_{bout} rather than the time to stable recovery as our landmark to estimate the rate of recovery because, although quite variable, t_{bout} is significantly shorter than the time to stable recovery of activity (Luther et al. 2003). Bouts are a clear sign of the impending recovery of pyloric activity after decentralization and are observed in every preparation examined (see also Luther et al. 2003). Thus even if other processes may be activated after bouting starts that are required for full and stable recovery of activity, bouting seems to be a

![Diagram](image-url)
hallmark of the overall process. We thus refer to $t_{bout}$ as a measure of the delay of the initiation of pyloric rhythm recovery. This choice thus maximizes the number of successful experiments. In control preparations, the average time to half-maximal pyloric rhythm frequency was $t_{1/2} = 0.25 \pm 0.26$ h, and the average time to first bout was $t_{bout} = 6.19 \pm 4.76$ h (Table 2, $n = 18$).

Activity suppression by hyperpolarization or low Na$^+$ advances recovery after decentralization

To determine how the activation of pyloric rhythm recovery is affected by a purely activity-dependent process, we first attempted to reduce or eliminate pyloric activity during several hours preceding decentralization. Reasoning that reduced pyloric activity before decentralization should upregulate any activity-dependent recovery mechanisms available to the neurons at that time, a shorter delay to the initiation of recovery would be expected if measured from the moment of decentralization.

The rhythmic activity of the pyloric network can be inhibited by either hyperpolarization of the PD neurons that are electrically coupled to the pacemaker AB neuron or by reducing the extracellular Na$^+$ concentration (Fig. 3). In preparations in which the PD neurons were hyperpolarized with current injection for ~5 h prior to decentralization, the average time to half-maximal pyloric rhythm frequency (i.e., of rhythm cessation) was not different from control values ($t_{1/2} = 0.21 \pm 0.18$ h, $n = 12$, Fig. 3A). A one-way ANOVA was used to compare control, hyperpolarized, and low-Na$^+$-treated (see following text) preparations, and all these effects were indistinguishable from each other ($P = 0.56$, Table 2). However, the average time to first bout in hyperpolarized preparations was significantly shorter than in control ($t_{bout} = 1.60 \pm 1.72$ h, $n = 12$, $P = 0.003$; post hoc Tukey test from 1-way ANOVA test; Table 2, Fig. 3A).

When preparations were incubated in 40–50% Na$^+$ saline for ~5 h prior to decentralization, results similar to those observed in preparations that were hyperpolarized before decentralization were obtained (Fig. 3B, Table 2). The time to half-maximal frequency was $0.33 \pm 0.35$ h ($n = 14$), which was not significantly different from control or from hyperpolarized PD preparations (overall 1-way ANOVA, $P = 0.56$; Fig. 3B, Table 2). However, the average time required to produce the first bout in these low-Na$^+$-treated preparations ($t_{bout} = 1.22 \pm 0.94$ h; $n = 14$) was significantly shorter than control (post hoc Tukey test $P < 0.001$; Table 2, Fig. 3B). The overall one-way ANOVA comparing these effects showed a significant difference between these treatments ($P = 0.0004$, Table 2).

Treatment with GABA before decentralization advances the recovery of activity but delays the rhythm deceleration after decentralization

Treatment of the nondecentralized pyloric network with 1 mM GABA stopped the pyloric rhythm completely and reversibly (Fig. 4A). For this reason, we initially intended to use GABA as a means of reducing pyloric network activity prior to decentralization. Examples of pyloric activity before GABA application, immediately after washout of GABA but before decentralization and at $t_{1/2}$ are shown in Fig. 4B. When preparations were pretreated with 1 mM GABA for 0 (control), 1, 5, and 12–18 h, the half-maximal time of pyloric rhythm deceleration showed a time-dependent increase ($t_{1/2} = 0.25 \pm 0.26$, 0.72 ± 0.85, 1.08 ± 1.05, and 2.27 ± 3.21 h, respectively) which was statistically significant ($P = 0.023$, 1-way ANOVA, Fig. 4A, Table 2). On the other hand, the time to first bout decreased when GABA was applied before decentralization for 0, 1, 5, or 12–18 h ($t_{bout} = 6.19 \pm 4.76$, 1.90 ± 0.84, 1.98 ± 1.30, and 3.91 ± 4.60 h, respectively). This dependence on GABA incubation time was statistically significant overall ($P = 0.015$, 1-way ANOVA; Table 2), but a post hoc Tukey test revealed that only preincubation of ≤5 h had significant effects on $t_{bout}$ ($P = 0.04$). We believe that this reveals a possible interplay between activity-dependent and neuromodulator-dependent mechanisms (see DISCUSSION). It is important to note that occasionally the activity never completely stopped after GABA pretreatment. Nevertheless, when this happened, bouts often appeared as transient frequency increases over the background frequency that could be easily distinguished from random frequency variation or transient artifacts by the criteria described before (see Fig. 2C, case 2).

If the recovery process was solely activity dependent, in GABA-pretreated preparations, only the advanced recovery time would be observed (i.e., a reduced $t_{bout}$), as in hyperpolarized or low-sodium saline-treated preparations with no ef-

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<tr>
<td>Control</td>
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<td>$t_{1/2}$, h</td>
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<td>Low-Na$^+$ saline</td>
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<td>Muscimol</td>
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<td>ANOVA $P = 0.56$</td>
<td>2.05 ± 1.60*</td>
<td>ANOVA $P = 0.0004$</td>
<td>$t_{1/2}$, h</td>
<td>0.18</td>
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<td>GABA overnight (12–18 h)</td>
<td>2.27 ± 3.21*</td>
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<td>3.91 ± 4.60</td>
<td>ANOVA $P = 0.015$</td>
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<td>GABA for 5 h</td>
<td>1.08 ± 1.05</td>
<td>ANOVA $P = 0.023$</td>
<td>1.98 ± 1.30*</td>
<td>ANOVA $P = 0.015$</td>
<td>$t_{1/2}$, h</td>
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<tr>
<td>GABA for 1 h</td>
<td>0.72 ± 0.85</td>
<td>ANOVA $P = 0.023$</td>
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<td>ANOVA $P = 0.015$</td>
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<td>Baclofen</td>
<td>5.89 ± 8.64**</td>
<td>ANOVA $P = 0.023$</td>
<td>6.74 ± 8.18</td>
<td>ANOVA $P = 0.015$</td>
<td>$t_{1/2}$, h</td>
<td>0.18</td>
<td>0.28</td>
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<tr>
<td>Proctolin</td>
<td>0.16 ± 0.14</td>
<td>ANOVA $P = 0.023$</td>
<td>2.34 ± 1.88*</td>
<td>ANOVA $P = 0.015$</td>
<td>$t_{1/2}$, h</td>
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<tr>
<td>Proctolin + Hyper-polarization</td>
<td>0.11 ± 0.11</td>
<td>ANOVA $P = 0.023$</td>
<td>0.22 ± 0.17*</td>
<td>ANOVA $P = 0.015$</td>
<td>$t_{1/2}$, h</td>
<td>0.18</td>
<td>0.28</td>
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Values represent averages ± SD. ANOVA was followed by post hoc Tukey tests, and the significance of the individual treatments obtained from post hoc tests is indicated (*if significantly different from control at $P < 0.05$ or ** if significantly different from control at $P < 0.01$).
effect on the rhythm deceleration rate (i.e., \( t_{1/2} \)). However, the experimental results also showed a significant reduction in the rate of pyloric activity deceleration after decentralization (i.e., an increase in \( t_{1/2} \)). Additionally, short-term (1 h) pretreatment with GABA only reduced \( t_{\text{bout}} \), whereas \( t_{1/2} \) was not significantly affected (Table 2). The difference in the kinetics of these two effects suggested to us the possibility that they may be mediated by two different mechanisms.

Because it is known that pyloric network neurons express at least two types of GABA receptors, an ionotropic GABA\(_A\)-type and a metabotropic GABA\(_B\)-type (Swensen et al. 2000), we examined whether the two observed effects could be mediated independently by the two different receptor types.

**Metabotropic GABA\(_B\) receptor activation only affects the kinetics of pyloric rhythm deceleration**

Pretreatment of the STG with the GABA\(_B\) receptor agonist baclofen (0.5 mM) for \( \sim 5 \) h before decentralization slightly (but not significantly) slowed the pyloric rhythmic activity from 1.15 ± 0.27 to 0.98 ± 0.24 Hz (\( P = 0.163, n = 6 \)), Student’s t-test, Fig. 4, C and D). Yet the time course of pyloric rhythm deceleration after decentralization in these preparations was significantly higher than control (\( t_{1/2} = 5.89 \pm 8.64 \text{ h} \), \( P = 0.004, n = 7 \), Student’s t-test; Fig. 4C, Table 2). On the other hand, the average time required to produce the first bout was not significantly different from control preparations (\( t_{\text{bout}} = 6.74 \pm 8.18 \text{ h} \); \( P = 0.58, n = 7 \), Student’s t-test; Table 2). Therefore the baclofen pretreatment resembles pretreatment with GABA in its effects on the time course of pyloric activity deceleration after decentralization but not on the delay of pyloric rhythm recovery initiation as indicated by the time to generate the first bout of pyloric activity (Fig. 4C, Table 2).

**Ionotropic GABA\(_A\) receptor agonist only reduces the time to first bout**

Pretreatment of the STG with the GABA\(_A\) receptor agonist muscimol (0.5 mM) for \( \sim 5 \) h before decentralization completely shut down pyloric activity (Fig. 4E). Decentralization after muscimol treatment resulted in the complete and rapid termination of rhythmic activity (Fig. 4, E and F). The time course of events after decentralization was similar to that observed in preparations whose activity was reduced before decentralization by either hyperpolarization or low-Na\(^+\) saline. After muscimol treatment, \( t_{1/2} \) was 0.38 ± 0.27 h (\( n = 6 \)) with no significant difference from control (a 1-way ANOVA was used to compare control, hyperpolarized, low-Na\(^+\)-treated, and muscimol-treated preparations, and all these treatments had indistinguishable effects; \( P = 0.56 \), Table 2). However, the average time to first bout was significantly shorter than in control preparations (\( t_{\text{bout}} = 2.05 \pm 1.60 \text{ h} \), \( n = 6 \); overall 1-way ANOVA \( P = 0.0004 \); post hoc Tukey test \( P = 0.04 \); Table 2). Participation of this ionotropic mechanism in controlling the time course of activity recovery is consistent with the activity-dependent reduction in the delay to the onset of pyloric activity after GABA pretreatment (Table 2).

These experiments demonstrated that when only ionotropic GABA\(_A\) receptors were activated during pretreatment, as in the case with muscimol, only the initial rate of recovery of the network’s activity (\( t_{\text{bout}} \)) was changed and not the rate of activity deceleration after decentralization (\( t_{1/2} \)). On the other hand, if during pretreatment only GABA\(_B\) metabotropic receptors were activated by baclofen, only the rate of activity deceleration after decentralization (\( t_{1/2} \)) was significantly affected but not the delay of initiation of activity recovery (\( t_{\text{bout}} \)). It appears therefore that two independent signaling pathways control the effects of GABA pretreatment on the recovery after decentralization, one activated via GABA\(_A\) and another via GABA\(_B\) receptor. GABA\(_A\) signaling is mediated by an ionotropic effect and thus results in changes in the ionic balance of the cell. We suggest that GABA\(_A\) receptor activation launches an activity-dependent mechanism of network activity recovery, identified by a decreased time to first bout, \( t_{\text{bout}} \). This mechanism is likely also launched by the other activity-altering treatments tested (hyperpolarization and incubation in low-Na\(^+\) saline). We suggest further that GABA\(_B\) signaling, probably acting through a G protein pathway (Duan and Cooke 2000; Swensen et al. 2000), controls the rate of activity deceleration.

To verify the plausibility of such a mechanism, we adapted a computational model of decentralization, previously developed to account for the process of bouting in an untreated pyloric network (Zhang and Golowasch 2007), to include two independent processes of Ca\(^{2+}\) conductance regulation plus the activity-dependent regulation of intracellular Ca\(^{2+}\) sequestration and applied to the model the same experimental manipulations as were used here on the biological preparations to examine their effects on the initial phase of the recovery from decentralization.
Model of activity- and neuromodulator-dependent recovery of pyloric network activity

The schematic diagram of neuromodulator and activity-dependent feedback mechanisms shown in Fig. 1 illustrates the essential components of our model and the hypothesis of activity regulation it represents. In our previous model (Zhang and Golowasch 2007), we used three Ca\(^{2+}\) sensors that operated in an activity-dependent manner. Here our goal was to develop a model that could provide a plausible explanation for the observed effects of modifying both activity and of neuromodulator application prior to decentralization on the recovery of pyloric activity after decentralization. For this, we first developed a simplified form of our previous model by reducing the three activity-dependent sensors to a single one \((S_A)\) regulated only by intracellular calcium ([Ca\(_{\text{cyt}}\)]\(^{\text{*}}\)). While \(S_A\) increases with increasing [Ca\(_{\text{cyt}}\)]\(^{\text{*}}\) (see \(M_i\) in Eq. 3), it has an inverse effect on the maximum Ca\(^{2+}\) conductance, \(G_{\text{Ca}}\), as well as on the intracellular Ca\(^{2+}\) pump activity, \(R_{\text{pump}}\) (Eq. 4). In spite of the simplification in the activity-dependent branch of regulation, this model still adequately captured the activity-dependent aspects of the recovery from decentralization, including the generation of bouting and delayed recovery of stable activity [Fig. 5, compare with Fig. 2B of Zhang and Golowasch (2007)]. As with the biological preparations (Fig. 2D), decentralization was rapidly (<1 oscillation period) followed by a cessation of all rhythmic activity, and the first bout appeared with a delay of 0.31 h (Table 2; this is ~3 h when realistic time constants are used). Bouting activity ensued (Fig. 5, A and B, center) and became regular and sustained after ~4.5 h.
i.e., ~45 h with realistic time constants, Fig. 5A). Bursting activity after recovery was slower than before decentralization also as observed in the biological preparations (compare Fig. 2, B and C, with 5B). When activity terminates following decentralization, the level of the activity sensor $S_A$ drops (Fig. 5A, bottom), which then leads to recovery of activity via enhancement of $G_{Ca}$ (Eqs. 2–4) (Zhang and Golowasch 2007).

The effect of neuromodulators is here hypothesized to be independent from activity-dependent effects. Neuromodulator-dependent effects are thus represented by a direct activation of the sensor $S_{NM}$ at increasing neuromodulator concentrations (Fig. 1 and Eq. 6). Activation of $S_{NM}$ only affects the maximum calcium conductance, $G_{Ca}$ (Eq. 8), but does not affect the activity-dependent sensor $S_A$ or the intracellular Ca$^{2+}$ pump activity (Fig. 1). Two different sets of neuromodulator receptors, GABA$_B$ receptors and other metabotropic receptors such as those sensitive to proctolin, are modeled (Fig. 1). GABA$_B$ is known to weakly activate a K$^+$ conductance in crab STG neurons (Swensen et al. 2000), and this effect is not included in the model, only the slower effect on $S_{NM}$ (Eq. 6). $R_{GABA}$ activation is also assumed to activate $I_{GABA}$, but for simplicity, the ionotropic GABA$_A$ and the metabotropic GABA$_B$ receptors are combined together here. Proctolin and other peptidergic neuromodulators are known to activate a distinct conductance (Buchholtz et al. 1992; Golowasch and Marder 1992; Swensen and Marder 2000), and this effect is here represented by the direct activation of $G_{NM}$. We suggest that activated proctolin receptors also have slow effects on the sensor represented by $S_{NM}$ (Fig. 1, Eq. 6). In fact, in our model, both GABA and proctolin are assumed to have identical effects on this sensor (Eq. 6). However, they have distinct ionotropic effects (Eqs. 5 and 1, respectively). Finally, IP$_3$RCa, represents the Ca$^{2+}$-dependent release of Ca$^{2+}$ from intracellular stores via an IP$_3$-dependent mechanism (Zhang and Golowasch 2007). We hypothesize that during the normal, ongoing pyloric rhythm the activity level of the sensor $S_{NM}$ is very low (in fact, with [NM] = 2, $S_{NM}$ = 0.27), and thus a decrease in $S_{NM}$ activity contributes very little to the postdecentralization events (Fig. 5A, middle). Instead before decentralization, the neuromodulator-activated current, $I_{proc}$, predominantly drives the generation of oscillatory activity.

We represent the effect of decentralization by setting the neuromodulator proctolin concentration [proc] to zero. This brings $G_{proc}$ to almost zero, turning $I_{proc}$ off and inactivating the neuron’s pacemaking capability (see Zhang and Golowasch 2007). Activity deceleration after decentralization decreases the value of $S_A$, which initiates the increase in $G_{Ca}$ that will eventually lead to the recovery of bursting activity (Zhang and Golowasch 2007).

**Model neuron hyperpolarization reduces time to first bout with no effect on deceleration rate**

Introduction of a small negative current ($I_{ext} = -0.5$ nA, Eq. 1) to the model neuron caused its hyperpolarization and inhibited bursting activity. In our experiment, the model neuron was hyperpolarized for 45 min before decentralization, then hyperpolarization was stopped, and the model neuron resumed bursting almost immediately. Two minutes later, decentralization was performed. In this experiment, the bursts stopped instantaneously after decentralization (Fig. 6, Table 2), similar to what was seen in the control case. However, it took 0.18 h to produce the first bout, which is almost half as short as the time required in control conditions (Fig. 5, Table 2). As described before, here the activity sensor $S_A$ tracked changes in activity (Fig. 6, bottom) while the neuromodulator sensor $S_{NM}$ had no appreciable change. These results matched the results of
biological experiments, suggesting that activity-dependent regulation alone can regulate the initial rate of recovery after decentralization as measured by the time to produce the first bout.

Low-Na\(^+\) saline incubation (experimental results shown in Fig. 3) reduces the activity of the STG pacemaker neuron in a similar manner as hyperpolarization. Note however, that low-Na\(^+\) treatment in the biological system is likely to also hyperpolarize the neuromodulator-containing axonal terminals from the input stm nerve and thus also reduce the release of neuromodulators. Hence, low-Na\(^+\) saline incubation was also modeled here by simultaneously setting neuronal activity and both [GABA] and [Proc] to zero. The results from this treatment are virtually indistinguishable from those of hyperpolarization treatment alone (\(t_{1/2} \approx 0\), \(t_{\text{bout}} = 0.18\) h). When the model cell was only hyperpolarized without affecting the neuromodulator levels, we observed an almost indistinguishable effect to those observed when the neuromodulators are also inactivated.

“GABA pretreatment” of the model neuron both slows the kinetics of pyloric rhythm deceleration and reduces time to first bout.

Activation of the hyperpolarizing GABA current (Eq. 5) in the model neuron for 45 min before decentralization completely inhibited the bursting activity even if all other currents, including \(I_{\text{proc}}\), were left intact (Fig. 7A). After 45 min of exogenous application of GABA at a level two times larger than the endogenous level ([GABA] = 2, as explained in METHODS; corresponding to simultaneous GABA\(_A\) and GABA\(_B\) receptor activation), [GABA] was briefly reduced to control levels ([GABA] = 1) before it was set to zero (together with [proc] = 0) to simulate decentralization. During \(R_{\text{GABA}}\) activation, the activity of both \(S_A\) and \(S_{\text{NM}}\) was affected with \(S_A\) tracking the neuron’s activity and turning off (Fig. 7A, bottom), which increased \(F_{\text{g}(S_A)}\), and with \(S_{\text{NM}}\) increasing to a high level (Fig. 7A, middle), which increased \(F_{\text{n}(S_{\text{NM}})}\). On GABA removal, bursting activity rapidly resumed at an increased

**Fig. 6.** Recovery process in the model neuron hyperpolarized before decentralization. Before decentralization, the model neuron was hyperpolarized or “incubated” with low-Na\(^+\) solution for 45 min (horizontal bar). Both treatments have nearly identical effects. Decentralization corresponds to time \(t = 0\) (up arrow). After decentralization the neuron rapidly stops bursting. The 1st bout is indicated by down arrow. The **bottom 2 traces** show the changes of sensors \(S_{\text{NM}}\) and \(S_A\) as a function of time. \(S_A\) tracks the activity changes instantaneously, whereas \(S_{\text{NM}}\) does not change during hyperpolarization or decentralization.

**Fig. 7.** Recovery process in the model neuron treated with GABA before decentralization. A: bursting frequency in the model neuron “pretreated” with GABA for 45 min before decentralization (horizontal bar). Decentralization (up arrow) corresponds to time \(t = 0\). A significant delay in the deceleration of rhythmic activity occurs after decentralization. The 1st bout is marked by down arrow. The **bottom 2 traces** show the changes of sensors \(S_{\text{NM}}\) and \(S_A\) as a function of time. \(S_A\) tracks the activity changes instantaneously, whereas \(S_{\text{NM}}\) slowly tracks the changes in GABA concentration. B: membrane potential traces of the model neuron activity before GABA incubation, before decentralization but after GABA was removed, and at half-time (\(t_{1/2}\)) after decentralization.
frequency relative to control, similar to the observations we have made in biological experiments using baclofen or GABA (compare Fig. 7, A and B, with 4, C and D, which shows the metabotropic GABAergic effects; although not shown in Fig. 4A, we have observed this effect in 30% of the experiments with GABA bath applications). While the activity sensor $S_A$ rapidly increased, which reduced $F_C(S_A)$, the $S_{NM}$ sensor only slowly returned to its low baseline level. This kept $F_C(S_{NM})$ high for a relatively longer period than $F_C(S_A)$; Fig. 7A), thus maintaining $C_{Ca}$ at an elevated level. When decentralization was performed a moment later, the bursting activity remained high after decentralization due to this increased $C_{Ca}$ level, only gradually decreasing over the course of ~20 min ($t_{1/2} = 0.18$ h), compared with a nearly instantaneous inactivation in control conditions (Table 2). At the same time, it took a slightly shorter time (0.28 h) for the model cell to produce the first bout compared with 0.31 h in control conditions (Table 2). These results closely matched the effects of equivalent manipulations with GABA observed in biological experiments (Fig. 4A).

**Effects of pretreatment with proctolin are consistent with the activation of $S_{NM}$ and $S_A$**

Our decentralization modeling experiments suggest that the application of a neuromodulator, e.g., proctolin, that can enhance activity due to its activation of an inward current (Buchholtz et al. 1992; Golowasch and Marder 1992; Swensen and Marder 2000) and has metabotropic effects (Swensen and Marder 2000), would simultaneously activate $S_{NM}$ and $S_A$. In contrast with the effects of GABA application, the enhanced sensor $S_A$ levels would lead to the reduction of $C_{Ca}$ levels, but the enhanced sensor $S_{NM}$ levels would have a similar effects as GABA with an the opposite effect on $C_{Ca}$ levels. As a consequence, the delay of initiation of activity recovery ($t_{bout}$) would be reduced but not the rate of activity deceleration ($t_{1/2}$) after decentralization.

Thus we tested the effects of proctolin on bursting activity recovery first in our model and then in the biological preparation. In model experiments exogenous proctolin application at levels up to two times larger than the endogenous level (1 unit, as explained in METHODS) was maintained for ~45 min before decentralization. After this time, [Proc] was briefly reduced to control levels before it was set to zero to simulate decentralization. Bursting activity terminated almost instantaneously (Table 2). However, it took ~25% less time (0.23 h) for the first bout to be produced than in control (Fig. 8A, Table 2).

A similar protocol was applied to biological pyloric network preparations: they were incubated in 1 μM proctolin for 1–18 h before decentralization (no difference in effects was observed over this wide range). In all proctolin-pretreated biological preparations, rhythmic activity was slightly (but not statistically significantly) increased on proctolin bath application (from $0.99 \pm 0.42$ to $1.21 \pm 0.42$ Hz; $P = 0.213$, $n = 7$, Student’s $t$-test; Fig. 8B). On subsequent decentralization, the pyloric rhythm rapidly ceased ($t_{1/2} = 0.16 \pm 0.14$ h), a delay that was not significant different from control preparations ($P = 0.79$, $n = 7$, Student’s $t$-test; Fig. 8B, Table 2). However, the initiation delay of activity recovery was significantly shortened to $t_{bout} = 2.34 \pm 1.88$ h compared with control ($P = 0.048$, $n = 7$, Student’s $t$-test; Fig. 8B, Table 2).

We conclude from these modeling and experimental results that although both neuromodulators proctolin and GABA could have identical metabotropic signaling effects, the interactions with their distinct ionotropic effects, result in significant phenotypic differences in their effects on the initial phase of rhythmic activity recovery from decentralization.

**Separation of activity-dependent from neuromodulator-dependent pathways**

The low-Na⁺ and muscimol treatments we performed were designed to reduce activity without interfering with the puta-

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**FIG. 8. Recovery of pyloric activity after decentralization in the proctolin treated preparations. A: model neuron. Before decentralization, the proctolin concentration was increased for ~45 min to 3 times the endogenous level present before decentralization. After this, the concentration was reduced back to the standard endogenous level briefly before it was set to 0 to simulate decentralization (↑). The bottom 2 traces show the changes of sensors $S_{NM}$ and $S_A$ as a function of time. $S_A$ tracks the activity changes instantaneously, whereas $S_{NM}$ slowly tracks the proctolin concentration changes. Decentralization takes place at $t = 0$. The 1st bout is indicated by ↓. B: biological preparation. Pyloric frequency vs. time of this representative preparation in which proctolin was bath applied at 1 μM for ~2 h then washed until the pyloric frequency returned to control levels. The preparation was then decentralized at $t = 0$ (↑) and activity recorded for the next 10 h, during which the 1st bout occurred (↓).**
tive neuromodulator-dependent pathways. Because the axons of the projection neurons that release neuromodulators in the STG enter the ganglion, these treatments could reduce the level of release even if the applications were restricted strictly to only the STG. In fact, it has recently been shown that some of these terminals hyperpolarize in response to GABA (Stein et al. 2007). To exclude the confounding possibility that the reduced activity of the neuromodulator-releasing terminals (and thus reduced neuromodulator release) may be responsible for what we call activity-dependent effects, we applied the neuromodulator proctolin \((5 \times 10^{-7} \text{ M})\) onto the STG only while simultaneously reducing the pyloric network activity by hyperpolarizing both PD neurons (with \(-3\) to \(-6\text{nA}\) current injection). After 5 h of simultaneous hyperpolarization and proctolin application, current injection was discontinued (but the cells left impaled with the electrodes for \(\geq 1\) additional hour) and proctolin was washed for \(\sim 10\) min before the preparations were decentralized. We obtained stable impalements in five of nine preparations. We measured \(t_{1/2}\) and \(t_{\text{bout}}\) under the hypothesis that the first bout would occur earlier compared with hyperpolarization treatment alone due to the added positive effect of the neuromodulator on the initiation of recovery. According to the predictions of the model, we hypothesized also that an increased delay in the rhythm deceleration after decentralization might be observed. We found that, in fact, the time to first bout occurs significantly faster in proctolin + hyperpolarization treated preparations than in hyperpolarization-only treated preparations \((0.22 \pm 0.17, n = 5, \text{compared with } 1.60 \pm 1.72, n = 12, P = 0.049, \text{Student’s } t\text{-test}, \text{Table 2})\). On the other hand, the time it takes for the preparations to decelerate and turn off after decentralization was not significantly affected relative to hyperpolarization-only treated preparations (Table 2). This lack of effect on \(t_{1/2}\) may be due to the short-duration of the applications, which was obligatory due to the difficulty in maintaining neurons stably impaled for \(>6-7\) h.

**Discussion**

Many different types of neurons possess activity-dependent homeostatic mechanisms that stabilize or recover their lost activity patterns after a perturbation or disruption (Davis 2006; Davis and Bezprozvanny 2001; Frick and Johnston 2005; Turrigiano 1999; Turrigiano and Nelson 2003; Zhang and Linden 2003), including neurons from the crustacean pyloric network (Golowasch et al. 1999a; Haedo and Golowasch 2006; Turrigiano et al. 1994). Previous experimental and modeling work suggested that the recovery of rhythmic activity of pyloric network neurons after the complete removal of neuromodulatory input by decentralization could be accomplished by the readjustment of intrinsic properties solely mediated by activity-dependent mechanisms (Golowasch et al. 1999b; LeMasson et al. 1993; Liu et al. 1998; Luther et al. 2003; Zhang and Golowasch 2007). Recently, however, Khorkova and Golowasch (2007) showed the existence of slow regulatory effects on conductances by neuromodulators, in particular proctolin. The present study was designed to examine the hypothesis that in the crab pyloric network the process of pyloric activity recovery after decentralization is determined not only by an activity-dependent mechanism but also by a parallel neuromodulator-dependent pathway. For this, we experimentally altered the network activity before decentralization in various ways, which included both specific (ionotropic) activity-reducing methods as well as by (metabotropic) neuromodulator bath applications, and then examined the initial phase of this recovery experimentally and in a model of the pyloric network pacemaker. We measured two hallmark features of this initial phase of recovery: the delay to the beginning of the generation of bouting activity and the rate of deceleration of rhythmic activity after decentralization. The effects of all predecentralization treatments (i.e., hyperpolarization, low-Na\(^+\) saline, GABA\(_A\) and GABA\(_B\) receptor agonists, and proctolin application) applied to both the biological pyloric network preparations and our simplified model match closely. Therefore we conclude that distinct activity- and neuromodulator-dependent signaling mechanisms may regulate ionic conductances such as \(G_{Ca}\) in the pacemaker of the pyloric network.

In our computational model, these two signaling mechanisms were represented by two sensors: \(S_A\), the activity-sensitive mechanism already described in previous theoretical (Golowasch et al. 1999b; LeMasson et al. 1993; Liu et al. 1998; Zhang and Golowasch 2007) and experimental work (Golowasch et al. 1999a; Haedo and Golowasch 2006; Turrigiano et al. 1994), and \(S_{NM}\), a neuromodulator-dependent mechanism. The activity-dependent mechanism homeostatically regulates neuronal activity by upregulating activity when it is low and by downregulating it when it is high. This, we have suggested before, takes place via the control of an intracellular signaling pathway activated by cytosolic calcium, which regulates Ca\(^{2+}\) and K\(^+\) conductances (Zhang and Golowasch 2007). Our present modeling work (cf. Zhang and Golowasch 2007) shows that the regulation of just one Ca\(^{2+}\) conductance is sufficient to explain the compensatory changes that lead to the recovery of rhythmic activity after decentralization. In fact, we have evidence (not shown in this study) that either the neuromodulator activated current \(I_{pro}\) or \(I_K\) could each be the sole targets of the activity-dependent regulatory pathway if parameters of this pathway in our model are slightly adjusted and lead to bouting and stable recovery of rhythmic activity. However, as has been shown in other systems (Desai et al. 1999b; Linsdell and Moody 1994; MacLean et al. 2003), more than a single conductance is likely to be simultaneously regulated in biological systems, perhaps leading to a richer and more complex range of activity and activity regulation patterns. Together with this regulation of an ionic conductance, the simultaneous regulation of an intracellular Ca\(^{2+}\) pump by the same \(S_A\) sensor (see diagram in Fig. 1) accounts for all observed characteristics of this recovery process, including the period and dynamics of bouting activity as well as the delayed recovery of stable but slower pyloric activity. The second long-term mechanism of neuronal activity regulation revealed by our experimental data appears to depend on slow-acting neuromodulatory input. We propose that this mechanism, represented in this model by the \(S_{NM}\) sensor, only regulates (positively) the voltage-gated Ca\(^{2+}\) conductance, and, as a consequence, it is not homeostatic in nature.

Neuromodulators in the stomatogastric nervous system have been thought to predominantly have acute effects on network activity. However, some the effects of these neuromodulators and of the activation of neuromodulator-containing projection neurons to the STG can be very long-lasting (Beenakker and
Nusbaumer 2004; Beenakker et al. 2004; Dickinson 2006). Slowly developing neuromodulatory effects in the pyloric network were recently reported in which the slow effects of proctolin were shown to be radically different from those resulting from acute proctolin application (Khorkova and Golowasch 2007). It is possible that acute neuromodulatory effects in this system operate either via different signaling mechanisms [probably a calmodulin-dependent mechanism (Swensen and Marder 2000)] than the slow-acting effects or that the sensitivities to neuromodulator or signaling molecules are very different in both cases. Here we show new slowly developing neuromodulatory effects by GABA (via a GABA_B receptor) and proctolin that are unrelated to their acute effects. GABA and proctolin require several hours of incubation to reveal their effects on pyloric activity deceleration or the appearance of the first bout of pyloric activity after decentralization. Whether the signaling pathways that mediate the effects we have here described on the initial phases of the activity recovery after decentralization and those responsible for the long-term effects on ionic currents described by Khorkova and Golowasch (2007) are the same remains to be examined. Nevertheless, our model suggests that they need not be different. Having identical metabotropic effects (as in our model), the different overall effects of GABA and proctolin on the initial phases of the recovery of rhythmic activity after decentralization can be accounted for on the basis of their distinct ionotropic effects: GABA having a strong inhibitory effect on pyloric activity (GABA_B agonists having a weak inhibitory effect), and proctolin having a weak excitatory effect on pyloric activity. Under these conditions, the net overall effect of a predecentralization GABA application is a strong enhancement of G_Ca. In contrast, a predecentralization proctolin bath application produces a weaker enhancement of G_Ca.

According to this model, the voltage-dependent and neuromodulator-activated inward current I_proct acts as a pacemaker current, and its removal by decentralization hyperpolarizes the pacemaker cell and consequently deactivates I_Ca. This leads to the drop in [Ca]_cyt and to a rapid decrease of the activity sensor S_A. As a consequence, G_Ca increases with time constant \( \tau_g \) until I_Ca is high enough to sustain temporary bursting activity, i.e., bouts. The neuronal activity is suppressed with hyperpolarizing current, low-Na\(^+\) solution or muscimol before decentralization, \( S_A \) is decreased, which in turn increases \( F_G(S_A) \). Even though \( F_G(S_A) \) begins its return to its control level when the treatment is removed, \( G_Ca \) still remains elevated compared with its control level. If decentralization occurs at this time, or shortly after the inhibitory treatment is removed, \( G_Ca \) will already be at an elevated level and closer to the level that will sustain bouts, reducing the time to generate the first bout.

A similar effect on the time to begin bouts can be obtained with proctolin preincubation. During proctolin pretreatment the neuron becomes depolarized due to the activation of G_proct/(V). This leads to the increase of the neuromodulator-sensitive sensor \( S_{NNM} \) directly and to the increase of the activity sensor \( S_A \) indirectly due to enhanced \( Ca^{2+} \) influx. The ultimate effect on activity is the result of the opposing effects of these sensors on \( G_Ca \): \( S_A \) downregulates [via \( F_G(S_A) \), Eq. 2] and \( S_{NNM} \) upregulates [via \( F_S(S_{NNM}) \), Eq. 8] \( G_Ca \). In contrast, GABA application leads to a hyperpolarization of the neuron via activation of \( G_{GABA} \). This leads to the indirect decrease of \( S_A \) due to a reduction in \( Ca^{2+} \) influx and an increase of \( S_{NNM} \) by the activated GABA_B receptors and to an upregulation of \( G_Ca \) by both mechanisms [via \( F_S(S_A) \) and \( F_S(S_{NNM}) \)]. Due to the dependence of \( G_Ca \) on both \( S_A \) and \( S_{NNM} \) (Fig. 1), these two cases of neuromodulator action will have different effects on the activity of the neuron. In one case (GABA effect), \( G_Ca \) will be sufficiently enhanced to be at a supra-threshold level for the generation of rhythmic activity at the time of decentralization, whereas in the other (proctolin effect), \( G_Ca \) will stay below this threshold. As a consequence, GABA (or baclofen) pretreatment leads to a slow deceleration (after decentralization) of a rhythm activated beyond the threshold for stable activity to be produced. The slow deceleration of activity in this case is due to slowly decaying \( Ca^{2+} \) conductance to below the threshold level. Proctolin, however, cannot upregulate \( G_Ca \) enough to surpass this threshold and therefore has no effect (compared with control) on the deceleration rate of the rhythm when decentralization occurs. However, both GABA and proctolin will reduce the time to the generation of the first bout.

The operation of the neuromodulator-dependent mechanism (represented by the \( S_{NNM} \) sensor) was assumed here to only affect one ionic conductance, \( G_Ca \), which was sufficient to explain the observed effects of neuromodulators on the rate of deceleration of activity \( (t_{1/2}) \) after decentralization. A direct effect of neuromodulators proctolin and GABA on \( Ca^{2+} \) conductances such as those proposed here remains to be experimentally demonstrated. It is of course possible that the neuromodulator effects that we have described involve a number of additional conductances. In fact, our model can be modified to include the regulation of several conductances by activity and by neuromodulators adding a richer repertoire of activity and its regulation. Indeed the fact that boutting activity can sometimes be observed among a background of nonzero pyloric activity may be due to different signaling pathways, sensitive to different features of activity, or activated by distinct signaling mechanisms that could be responsible on the one hand for regulating the background pyloric activity and on the other underlie boutting activity.

A number of different conductances are often regulated by the same neuromodulator (Khorkova and Golowasch 2007), and there is growing evidence that many conductances may be coordinately regulated within and between network neurons (Khorkova and Golowasch 2007; MacLean et al. 2003; Schulz et al. 2007), suggesting possible interactions between the ion channels directly or regulatory interactions at the transcription or at some posttranscriptional level (Kaczmarek 2006; Schulz et al. 2007). The possibility that activity of the target network regulates the release of neuromodulators, thus making the neuromodulator-dependent regulation indirectly activity-dependent, also needs to be considered and experimentally tested. Wood et al. (2004) have shown the existence of a feedback circuit from the pyloric network to projection neurons. However, in this case, the pyloric network does not appear to be influenced back; instead a different rhythm-generating network in the STG, the gastric mill network, is affected.

The results of our experiments, in which proctolin was applied simultaneously with the reduction of pyloric network activity (via PD neuron hyperpolarization), indicate that these effects can operate independently. However, incontrovertible proof is not easy to obtain in this case because a reliable method to separate activity-dependent from neuromodulator-
dependent effects over the very long time that is required for the process of pyloric activity recovery to occur does not exist. Furthermore, we cannot completely exclude the possibility of such interactions because it has been shown before that the growth factor BDNF, for example, is released by neurons in an activity-dependent fashion and then acts on the releasing neuron as well as other adjacent neurons to homeostatically regulate their activity (Desai et al. 1999a). However, in the crustacean stomatogastric system, no growth factors are presently known, and only one peptidergic neuromodulator has been shown to be produced by neurons within the STG. However, this modulator is not produced by neurons of the pyloric network itself (Skiebe et al. 2002), and it is thus unlikely to act as “activity messenger” in the system.

Testing the validity of our model will be complicated by several possible factors that we have, for the sake of simplicity, disregarded in our model. Thus multiple conductances rather than a single one may be regulated, multiple sensors of activity and receptor activation may exist, and interactions between activity- and neuromodulator-dependent mechanisms may be present. Additionally, we have modeled the process of activity recovery of the entire pyloric network assuming that they are generated by the changes triggered in a single pacemaker neuron. It is certainly possible that all neurons and synapses in the pyloric network are potential targets for similar forms of regulation. It is therefore now crucial to experimentally determine the molecular details of the signaling pathways activated by the different neuromodulators in this system, at the very least those activated via GABAB and proctolin receptors. This should allow the testing of our proposed model by separately modifying intracellular Ca\(^{2+}\) and Ca\(^{2+}\) sensor levels, and the levels of the signaling molecules activated by these neuromodulators.

In summary, our results demonstrate that the process of recovery after decentralization is likely governed by complex regulatory mechanisms, integrating both activity- and neuromodulator-dependent inputs. Multiple and redundant regulatory mechanisms are very common in biological systems and could be an evolutionary conserved way to ensure the uninterupted function of vitally important processes in the face of constantly changing environmental conditions.

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**References**


