Ionic Mechanism Underlying Recovery of Rhythmic Activity in Adult Isolated Neurons

Rodolfo J. Haedo and Jorge Golowasch

Departments of Biological Sciences, Rutgers University; and of Mathematical Sciences, New Jersey Institute of Technology, Newark, New Jersey

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INTRODUCTION

Neuronal excitability regulation is important for the maintenance of stable activity patterns in individual neurons (Davis and Bezprozvanny 2001; Desai et al. 1999; Franklin et al. 1992; Hong and Lennicka 1995; Li et al. 1996; Lindsell and Moody 1994; Turrigiano et al. 1994) and in neuronal networks (Galante et al. 2001; Luther et al. 2003; Turrigiano and Nelson 2004). This homeostatic plasticity allows neuronal and network activity to remain within functional limits during their normal operation and in response to perturbations or injury. Regulation of neuronal excitability and intrinsic properties can be induced by electrical stimulation (Cudmore and Turrigiano 1999a; Li et al. 1996; Turrigiano et al. 1994), ionic conductance perturbations (Desai et al. 1999; Lindsell and Moody 1994), development and growth (Spitzer et al. 2002), and trauma (Darlington et al. 2002) and may play a role in memory formation (Daoudal and Debanne 2003; Marder et al. 1996; Zhang and Linden 2003).

The rhythmical activity pattern of the pyloric network in the stomatogastric ganglion (STG) of crustaceans is conditional on neuromodulatory release from neurons located in central ganglia. When these inputs are permanently removed, activity ceases but a stable new activity pattern spontaneously develops within hours to days (Golowasch et al. 1999b; Luther et al. 2003; Thoby-Brisson and Simmers 1998). It has been suggested that this recovery of rhythmic activity occurs via up- and downregulation of voltage-dependent ionic conductances and the consequent acquisition of oscillatory properties by some of the network components (Golowasch et al. 1999b; Mizrahi et al. 2001; Thoby-Brisson and Simmers 2002). Neuromodulators may also have suppressive trophic effects on the excitability of STG neurons in the intact network, suppression that may be released after removal of neuromodulatory input by decentralization (Le Feuvre et al. 1999; Thoby-Brisson and Simmers 1998, 2000).

Neuronal intrinsic excitability is affected by patterned electrical activity in isolated STG neurons in culture (Turrigiano et al. 1994) and in neurons in situ (Golowasch et al. 1999a). Excitability changes can also occur spontaneously both in isolated STG neurons in culture in response to cell dissociation (Turrigiano et al. 1995) or in the intact ganglion in response to decentralization (Golowasch et al. 1999b; Mizrahi et al. 2001; Thoby-Brisson and Simmers 2002). To understand how network activity evolves in response to persistent perturbations (such as the removal of central inputs essential for the generation of the activity), it is essential to understand the dynamics and plasticity of the voltage-dependent ionic currents of its component neurons. In cultured STG neurons, spontaneous activity changes have been correlated with changes in a TEA-sensitive K⁺ current and in various inward currents (Turrigiano et al. 1995). However, the conductance changes induced by prolonged rhythmic stimulation (Turrigiano et al. 1994) have not been identified.

Here we study the ionic mechanisms involved in spontaneous and activity-induced recovery of oscillatory activity in

Address for reprint requests and other correspondence: J. Golowasch, Dept. Mathematical Sciences, NJIT, University Heights, Newark, NJ 07102 (E-mail: Jorge.P.Golowasch@njit.edu).

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adult dissociated crab STG neurons. We find the same ionic currents to be modified in both cases, although probably via different signaling pathways. Dynamic-clamp experiments confirm that the ionic currents modified are sufficient to produce the observed activity changes. Surprisingly, during the first 10 days after dissociation, neurons from the pyloric and gastric networks cannot be distinguished on the basis of neuronal activity, ionic conductance changes, or response to stimulation.

METHODS

Animals and solutions

Adult male crabs Cancer borealis (carapace length >15 cm) were obtained from local fish markets (Newark, NJ) and maintained in saltwater aquaria at 12–14°C. The following solution compositions were used (concentrations all in mM): standard Cancer saline solution (440.0 NaCl, 11.0 KCl, 13.0 CaCl₂, 26.0 MgCl₂, 5.0 Maleic acid, and 11.2 Trizma base, pH 7.4–7.5); salt supplement solution (743.7 NaCl, 16.4 KCl, 24.7 CaCl₂, 50.2 MgCl₂ and 10.0 HEPES, pH 7.4); 0 Ca²⁺/10 mM Mg²⁺ saline solution (440.0 NaCl, 11.0 KCl, 12.9 BaCl₂, 0.1 CaCl₂, 26.0 MgCl₂ 5.0 Maleic acid, and 11.2 Trizma base, pH 7.4–7.5). Mn²⁺ saline was identical to barium saline with Mn²⁺ substituting for Ba²⁺. All chemicals were obtained from Fisher Scientific (Fairlawn, NJ) unless otherwise indicated. The sodium channel blocker Tetrodotoxin, TTX (EMD, Biosciences) was used at 0.1 µM.

Cell dissociation

Adult STG neurons were cultured following a protocol similar to those used by Turrigiano and Marder (1993), Glowik et al. (1997), and Swensen and Marder (2000). Crabs were anesthetized by cooling during 15–30 min on ice. The foregut was removed, and the STG, with a portion of the nerves attached, was isolated as previously described (Selverston et al. 1976) in a sterile laminar flow hood. The ganglia were pinned down in sterile silicone coated inside with goat serum (Invitrogen, Carlsbad, CA) and with fire-polished tips. Dissociated neurons were plated individually onto uncoated 35-mm plastic Nunclon culture dishes in sterile salt supplement solution diluted 1:1 with sterile Leibowitz L-15 medium (In-vitrogen) and then placed in an incubator at 12–14°C for the duration of the culturing period. Saline was not replaced during this time.

FIG. 1. Dissociation preserves biophysical properties. A: extracellular recordings from the lateral ventricular nerve (lvn) showing the action potentials of 3 main pyloric motor neurons [lateral pyloric (LP), pyloric constrictor (PY), and pyloric dilator (PD)]. Top: (control before treatment) from a freshly dissected preparation. Middle: (control, 24 h) recorded in normal saline after protease treatment (METHODS). Bottom: (pilocarpine) was recorded immediately after the middle trace was recorded, showing a strong neuromodulatory response to 100 µM pilocarpine after enzymatic treatment. B: recordings from 2 stomatogastric ganglion (STG) neurons 24 h after enzymatic treatment and mechanical dissociation (METHODS). Neurons were impaled with 1 electrode and stimulated with a +0.1-nA current pulse (top traces). Voltage responses before (control) and after application of the neuromodulator pilocarpine (5 µM) are shown below. Neuronal input resistance was >10 times the electrode resistance and thus no attempt was made to subtract electrode resistance in these recordings. 1861 MECHANISM OF RHYTHMIC ACTIVITY RECOVERY • VOL 96 • OCTOBER 2006 • www.jn.org

Cell identification

A subset of the experiments was performed with identified neurons of the STG. Because the gastric network is known to oscillate at a frequency several times slower than the pyloric network (Nusbaum and Beenhakker 2002), we hypothesized that gastric neurons, if they become oscillatory in dissociated culture, would oscillate at a lower frequency than dissociated pyloric neurons. Thus we aimed to distinguish only between neurons belonging to the pyloric network, the gastric network and other neurons not belonging to either of these networks. For this purpose, we followed the established protocol for cell identification (Selverston et al. 1976). A map of the STG neurons is shown in the top trace. After 24 h of proteolytic treatment (control—24 h, middle), a clear effect of the enzymatic treatment can be seen by comparing the top two traces. Immediately before the mechanical dissociation was performed, the pyloric network’s main motor nerve (the lateral ventricular nerve, lvn). Control before enzymatic treatment is shown in the top trace. As a second test, we applied 1–10 µM pilocarpine to isolated STG neurons 1–5 days after enzymatic treatment and mechanical dissociation. Approximately 50% of the neurons responded by changing their activity pattern (Fig. 1B). Note that not all neurons in the intact STG respond to pilocarpine (Swensen and Marder 2001). Thus both of these tests confirm that neurons retain many of their biophysical properties through the proteolytic and mechanical dissociation procedure, as has been reported previously (Panchin et al. 1993; Turrigiano and Marder 1993).
Alexa Fluor 568 (Invitrogen) by dipping the back of the electrode in the dye solution for 1–2 min and backfilling the rest of the micropipette with 1 M KCl. Neurons identified as pyloric were filled with the other dye. After this, the dissociation procedure was followed as described above.

Electrophysiological recordings

Single- or two-electrode voltage clamp (SEVC or TEVC) performed with an Axoclamp 2B amplifier (Molecular Devices, Union City, CA) was used to measure ionic currents. Data were digitized and then analyzed using the pClamp9.0 software (Molecular Devices). Recordings were obtained using Citrate-filled microelectrodes (4 M K-citrate +20 mM KCl). Current injection electrodes had resistances of 12–18 MΩ and voltage recording electrodes of 15–25 MΩ. The preparation was grounded using an Ag/AgCl wire connected directly to the bath or via an agar bridge (4% agar in 0.6 M K2SO4)

K-citrate

preparation was grounded using an Ag/AgCl wire connected directly to the bath or via an agar bridge (4% agar in 0.6 M K2SO4) (Figs. 6–8), at +10 mV for Is (Figs. 6 and 7) and +20 mV for Ica tail (Fig. 6C). We chose these voltages to maximize the numbers of cells we could use for our analyses because not all cells could be voltage clamped to higher levels. The leak conductance was measured either as 1/Rin, where Rin is the input resistance measured with small hyperpolarizing current steps in current clamp, or as the slope of the I–V curve in the voltage range where no voltage-dependent currents appear to be activated (–40 to –60 mV).

Neurons were allowed to rest for 15–20 min after impalement before data acquisition was initiated. Prolonged neuronal stimulation was started only after ionic currents showed stable amplitudes for several minutes; these were then used as control.

Capacitance was determined from the areas of the capacitive current transient resulting from 10-ms-long hyperpolarizing membrane potential steps from a holding voltage of –40 or –50 mV. In some neurons, we estimated the electrotonic length, L, by applying a low-amplitude hyperpolarizing current pulse around the resting potential of the cells. This was repeated 20 times, and the traces averaged to reduce noise. Double-exponential fits to the voltage change produced the membrane time constant, τ0, and an equalizing time constant, τ1, which were used to determine L according to

\[ L = \pi \sqrt{\tau_0/\tau_1} - 1 \]

(Rall 1977).

Stimulation protocols

Neuronal activity was altered by applying 1-s-long hyperpolarizing current pulses at 0.33 Hz, driving neurons to a membrane potential of approximately –120 mV (peak response, see Fig. 4E). This protocol has been found to be effective in modifying the spontaneous activity of cultured lobster STG neurons before (Turrigiano et al. 1994). Current level was adjusted often during the stimulation period to maintain this level of hyperpolarization. Control ionic current measurements were recorded in voltage clamp immediately prior to the beginning. Currents were measured again after a 45- to 60-min stimulation period.

Analysis

SigmaStat (Aspire Software International, Leesburg, VA), Origin (OriginLab, Natick, MA), and CorelDraw (Corel) software packages were used for statistical and graphical analysis. ANOVA tests were performed using either a standard two-way ANOVA, the nonparametric Kruskal-Wallis ANOVA on ranks for nonnormally distributed populations, or a repeated-measures (RM) ANOVA. Results of statistical analysis were considered significant if the significance level P was below α = 0.05. All error bars shown and the reported variability around the averages correspond to SDs.

Dynamic-clamp experiments

A NI PCI-6070-E board (National Instruments, Austin, TX) was used for current injection in dynamic clamp experiments. Data acquisition was performed using the Digidata board and pClamp software, as described in the preceding text. The dynamic-clamp software was developed by Farzan Nadim and collaborators (available for download at http://stg.rutgers.edu/software.htm) in the LabWindows/CVI software environment (National Instruments, Austin, TX) on a Windows XP operating system. Ionic currents that showed an approximately average effect in response to prolonged rhythmic stimulation were recorded and fitted with Hodgkin and Huxley-type equations. We used the measured parameters that produced the best fit to reproduce ionic conductances that were then added to or subtracted from a neuron
TABLE 1. Ionic current parameters used for dynamic-clamp experiments

<table>
<thead>
<tr>
<th>Current</th>
<th>(\tau_{mX})</th>
<th>(\tau_{hX})</th>
<th>(V_{1/2mX})</th>
<th>(V_{1/2hX})</th>
<th>(s_{mX})</th>
<th>(s_{hX})</th>
<th>(q)</th>
<th>(E_{\text{max}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I_{Ca})</td>
<td>1</td>
<td>300</td>
<td>-13</td>
<td>-16</td>
<td>-9</td>
<td>+10</td>
<td>1</td>
<td>+100</td>
</tr>
<tr>
<td>(I_{KSO})</td>
<td>100</td>
<td>NA</td>
<td>-20</td>
<td>NA</td>
<td>-9</td>
<td>NA</td>
<td>0</td>
<td>-80</td>
</tr>
<tr>
<td>(I_{KTr})</td>
<td>1</td>
<td>70</td>
<td>-23</td>
<td>-34</td>
<td>-4</td>
<td>4</td>
<td>1</td>
<td>-80</td>
</tr>
</tbody>
</table>

\(g_{\text{max}}\) is specific for. \(V_{m}\) is the membrane potential, and \(E_{i}\) is the equilibrium potential of ion \(i\) that each current is specific for. \(\tau_{mX}\) and \(\tau_{hX}\) are the time constants with which the \(m\) and \(h\) gates, respectively, evolve in time toward their respective steady states \(m_{\text{st}}\) and \(h_{\text{st}}\). These steady states are governed by each two voltage-dependent parameters \(V_{1/2m}\) and \(s_{m}\) that are listed in Table 1. \(q\) is an exponent that takes value 1 if the ionic current exhibits voltage-dependent inactivation and value 0 if it does not.

**RESULTS**

**Spontaneous activity changes with time in culture**

Our dissociation procedure typically yielded around 20–40% of the 25–26 STG neurons found (Kilman and Marder 1996) in each ganglion and, with careful suction, the soma and a relatively long segment of the major neurite could be removed. Figure 2, B–D, illustrates the morphology of three typical neurons with differing neurite lengths immediately after dissociation (days 0 and 1) and 6 days later. By the sixth day in culture, some neurons grew wide lamellipodia (Fig. 2B), others tended to grow one or more long processes with smaller lamellipodia extending from the ends (Fig. 2D), whereas others showed a combination of relatively wide lamellipodia and long processes (Fig. 2C). Any significant outgrowth originated almost exclusively from the neurite stump (Fig. 2, C and D) however short it may have been (see Fig. 2B). Only very small extensions were occasionally observed growing directly out of

![Image](http://jn.physiology.org/)

**FIG. 2.** Neuronal activity classification and spontaneous activity. A1: intracellular recordings with 2 microelectrodes of the neuronal activity of a tonic firing cell on day 6 in response to a +0.1-nA current pulse. TTX (0.1 μM) abolished all spiking in this and 6 other similar neurons (n = 7). A2: similar recording as in A1 of an oscillatory neuron on day 5 after dissociation in response to a +0.4-nA current pulse. TTX (0.1 μM) abolished all spiking but not the slow oscillations in this and 8 other similar neurons, whereas Mn2+ saline (METHODS) abolished all oscillations. n = 9. B–D: dissociated STG neurons were photographed and impaled with 1 electrode at different days in culture. The neurons showed little growth at day 0–1. B: at day 1 in culture, this neuron is silent, becomes tonically active by day 4, and develops oscillatory activity by day 6 with action potentials riding on the slow oscillations of the membrane potential. On day 6, this neuron shows an extensive lamellipodium with many small processes growing at its edges. C: this neuron shows incipient growth at the end of the neuritic stump on day 1 that grows into a broad lamellipodium by day 6 from which several projections with extensive dendritic processes originate. The neuron is silent on day 1 and begins to generate membrane potential oscillations classified as oscillatory activity (high duty cycle) that increases in frequency at day 6. D: on day 6, this neuron extends 2 long projections from the original stump (day 0) and a relatively modest dendritic outgrowth emerging from them. Oscillatory activity appears immediately after dissociation, becomes silent by day 4 and then resumes oscillatory by day 6.
the soma (Fig. 2C, day 6). No obvious correlation was observed between the length of the original neurite and subsequent outgrowth morphology or electrical activity.

Neurons were classified according to the pattern of activity they expressed. Three types of electrical activity were readily distinguishable from the first day in culture when the cultured neurons were depolarized with low-amplitude current injection: silent, tonic, or oscillatory. Neurons that responded passively to all depolarizing and hyperpolarizing current injection levels were classified as silent (day 1 in Fig. 2, B and C). Tonic neurons were those that exhibited fast, transient depolarizations having a duty cycle ≤0.2 (Fig. 2A1, top, and B, day 4) and that could be blocked with 0.1–1 μM TTX (Fig. 2A1, bottom). Oscillatory activity was defined as slow, low-amplitude depolarizations having a duty cycle >0.2 (Fig. 2A2, top and middle, also see day 6 in Fig. 2, B–D), resistant to TTX treatment (Fig. 2A2, middle) and sensitive to Ca2+ current blockers such as Mn2+ (Fig. 2A2, bottom) or Cd2+ (not shown). The oscillations recorded on days 4 and 6 of Fig. 2C are considered to be at a transition between tonic and oscillatory states and were classified as oscillatory. Duty cycle was defined as the duration of a depolarizing event (action potential or slow oscillation) relative to the period of an oscillation measured at 50% of the maximum oscillation amplitude.

None of the neurons recorded during the initial 10 days in culture expressed spontaneous activity without some depolarizing current, and all neurons were tested with depolarizing current steps of various amplitudes. On day 0, most neurons (81%) are silent, 14% of the neurons showed tonic firing of action potentials, and a very small percentage (>5%) of the nearly 350 neurons we recorded from expressed oscillatory activity (Fig. 2A1). To understand the contributions of ionic currents to the evolution of five different ionic conductances over successive days in cell culture, and the percentage of each category is plotted as a function of time. A progressive decrease of silent neurons and increase in both tonic and oscillatory neurons is observed. B: Ca2+ and high-threshold K+ conductance density changes. The changes observed in these conductances over the first 6 days in culture are statistically significant (gCa: P = 0.002, n = 38; gK: P = 0.047, n = 65). C: A, h, and leak current conductance changes. Peak gA was measured at 20–30 ms after the onset of a depolarizing pulse to 0 mV. gA was measured at the end of 4-s-long hyperpolarizing voltage steps to −120 mV. The values of gA were scaled up 10-fold to increase visibility. No statistically significant change in either gA or gK could be detected (gA: P = 0.654, n = 62; gK: P = 0.313, n = 59). gMax showed a statistically significant increase with time in culture (P < 0.001, n = 139), but this disappeared when the density values were considered (not shown). All statistical comparisons were made using the Kruskal-Wallis one-way ANOVA on ranks. Data show means ± SD.

Spontaneous conductance changes with time in culture

To determine whether any of the activity changes correlate with STG neuron identity, we recorded from 37 additional neurons, classified into pyloric and gastric type (see METHODS). Of these, 25 produced oscillations as defined in the preceding text at days 2–8 in culture. The average oscillation frequency of the identified pyloric neurons was 3.65 ± 2.01 Hz (n = 14), whereas the average oscillation frequency of the identified gastric neurons was 2.95 ± 1.43 Hz (n = 11). Thus the average oscillation frequency of the gastric neurons was somewhat lower than that of the pyloric neurons but this difference was not statistically significant (P = 0.342, Student’s t-test).

FIG. 3. Spontaneous progression of activity and ionic conductances in culture. A: neurons classified as silent, tonic, or oscillatory were counted on successive days in cell culture, and the percentage of each category is plotted as a function of time. A progressive decrease of silent neurons and increase in both tonic and oscillatory neurons is observed. B: Ca2+ and high-threshold K+ conductance density changes. The changes observed in these conductances over the first 6 days in culture are statistically significant (gCa: P = 0.002, n = 38; gK: P = 0.047, n = 65). C: A, h, and leak current conductance changes. Peak gA was measured at 20–30 ms after the onset of a depolarizing pulse to 0 mV. gA was measured at the end of 4-s-long hyperpolarizing voltage steps to −120 mV. The values of gA were scaled up 10-fold to increase visibility. No statistically significant change in either gA or gK could be detected (gA: P = 0.654, n = 62; gK: P = 0.313, n = 59). gMax showed a statistically significant increase with time in culture (P < 0.001, n = 139), but this disappeared when the density values were considered (not shown). All statistical comparisons were made using the Kruskal-Wallis one-way ANOVA on ranks. Data show means ± SD.
density changes over time rather than simply conductance changes to remove possible effects of growth. Only two of these showed statistically significant changes over that period (Fig. 3B) after normalizing by the capacitance of the cell: \(g_{Ca}\), which increased by 159% \((P = 0.002, n = 38)\), and the high-threshold outward current \(g_K\) that includes both a delayed rectifier and a \(Ca^{2+}\)-dependent \(K^+\) current (Golowasch and Marder 1992), which decreased by 54% \((P = 0.047, n = 65, \text{Fig. 3B})\). \(g_{leak}\) increased by 79% \((P < 0.001, n = 139, \text{Fig. 2C})\) but when normalized by cell capacitance, the increase was reduced to only 15% and was not statistically significant \((P = 0.777, n = 32)\). Thus the change in leak conductance can probably be explained simply by the growth of the cell, whereas both \(g_{Ca}\) and \(g_K\) changes appear to be related to changes in neuronal activity as these changes correlate with the progression from silent to tonic to oscillatory activity (Fig. 3A). In contrast, \(g_A\) and \(g_h\) (Fig. 3C) increased by \(-30\%\) between days 1 and 10 in culture, but these changes were not statistically significant \((P = 0.654, n = 62; P = 0.313, n = 59)\), respectively; all the preceding reported statistical tests were performed using the Kruskal-Wallis one-way ANOVA on ranks). We did not measure the capacitance in most of the neurons in which \(g_A\) and \(g_h\) were measured. However, an increase of conductance density of these two currents with age in culture is not likely because the average capacitance during this period increased by the same amount as these conductances (~25%). Surprisingly, the increase in capacitance during the first week in culture \((c_m\) on day 1 = 0.437 ± 0.154 nF, \(c_m\) on day 7 = 0.548 ± 0.238 nF) was not statistically significant \((P = 0.106, n = 87)\) similar to what was observed by Turrigiano et al. (1995). Furthermore, the electrotonic length of these neurons is relatively short (thus neurons are electrotonically compact) and not significantly different between day 1, when there is virtually no growth \((L = 1.67 ± 0.61, n = 16)\), and day 6, when extensive growth is apparent (see Fig. 2, B–D; \(L = 1.73 ± 0.781, n = 8, P = 0.842\), Student’s t-test). From these results, we conclude that neurons at these stages grow by extensively stretching the existing membrane rather than by incorporating significant amounts of new membrane.

To determine whether the conductance changes described in the preceding text correlate with STG neuron identity, we recorded \(g_{Ca}\) from seven pyloric and seven gastric neurons, ages 1 and 6 days in culture. We found no statistically significant difference between pyloric and gastric neurons \((P = 0.841, \text{Student’s t-test})\). However, we found a statistically significant difference between ages 1 (0.007 ± 0.002 \(\mu S\)) and 6 days (0.024 ± 0.014 \(\mu S\); \(P = 0.012\), Student’s t-test) of the pooled data, confirming the results described in the preceding text for unidentified cells. We also measured \(g_K\), \(g_A\), and \(g_h\) from 30 additional identified neurons (16 pyloric neurons and 14 gastric neurons). We grouped all neurons aged 5–7 days in culture and compared pyloric versus gastric neurons. No statistically significant difference in any of the three conductances between the two cell types was observed \((P = 0.366, 2\text{-way ANOVA}), with \(g_K = 0.30 ± 0.34 \mu S\) (pyloric) versus 0.23 ± 0.15 \(\mu S\) (gastric), \(g_A = 0.93 ± 0.65 \mu S\) (pyloric) versus 0.77 ± 0.53 \(\mu S\) (gastric), \(g_h = 0.020 ± 0.009 \mu S\) (pyloric) versus 0.018 ± 0.008 \(\mu S\) (gastric).
seen in all four examples shown in Fig. 4E, there was no major change in PIR properties or amplitude during the course of the stimulation.

Interestingly, none of the recorded neurons showed a transition from a silent to a tonic (or from a tonic to a silent) pattern of activity, suggesting a lack of sensitivity to patterned stimulation of those currents responsible for the generation of action potentials. Alternatively, in these cells some of the intermediate signaling molecules leading from the detection of membrane potential changes to the modification of the ionic currents responsible for the changes in activity may be absent. In all cases where recordings could be held long enough (2–4 h), reversal of the activity pattern was always significantly slower than the induction phase and almost always partial. We rarely observed a complete reversal of these effects. Figure 4, B and C, shows the only two examples (of 28 cases) we obtained of almost complete reversal of activity, and in both it took ~2.5 h to complete.

It is important to note that the ability for neurons to regulate activity in an activity-dependent manner did not appear to be related to the age of the neurons in culture but rather to their activity state at the time of stimulation. A similar fraction (40%) of “young” neurons (ages 1–4 days in culture) and “old” neurons (31%, ages 5–8 days in culture) could be induced to change their state of activity. However, most stimulated tonic (average age = 3.5 days) and oscillatory (average age = 1.1 days) neurons were older than stimulated silent (average age = 1.1 days) cells simply because of the spontaneous progression in activity observed in culture (see Fig. 3A).

Within the relatively simple activity pattern categories we have classified these neurons into, there is a wide range of variability in terms of action potential frequency, duration, and amplitude, slow-wave oscillation frequency and amplitude, threshold current required to elicit patterns of activity, etc. We reasoned that this variability may be related to the expression of large, variable amplitude outward currents (Golowasch et al. 1999a) and that reducing their amplitude may make the activity patterns more uniform and reveal a more consistent effect of rhythmic stimulation on neuronal activity. When outward K+ current amplitudes were reduced with 20 mM TEA in the bath, we did indeed observe a reduction in the variability of the activity states (Fig. 5). As shown before by Turrigiano and Marder (1993) in lobster isolated STG neurons, in the presence of TEA, most crab STG neurons showed a tendency to generate slow oscillations with relatively long depolarizations (Fig. 5B, control, n = 9/15) while all others were silent (Fig. 5A, control, n = 6/15) before rhythmic stimulation was begun. When these neurons were rhythmically stimulated, 100% of the initially silent neurons developed slow and large-amplitude oscillations (Fig. 5A, after stim), 100% of the initially oscillatory neurons increased the duration of the slow depolarizations by 83% from 319 ± 138 to 584 ± 369 ms (P = 0.030, n = 9, Fig. 5B, after stim), increased their amplitude by 40% from 24.4 ± 14.3 to 34.1 ± 14.2 mV (P < 0.001, n = 8, Fig. 9B, left), and also increased their oscillation period by 170% from 473 ± 314 to 1284 ± 936 ms (P = 0.046, n = 6, see Figs. 5B and 9B, left). Period was calculated on longer recordings than shown in Fig. 5. Comparisons were made with unpaired Student’s t-tests.

These changes indicate that, in general cultured STG neurons increase their excitability with patterned stimulation (from

![Image](http://jn.physiology.org/)

**FIG. 4.** Rhythmic stimulation-induced neuronal activity changes. Examples of activity transitions resulting from rhythmic stimulation for 45–60 min with hyperpolarizing current pulses (METHODS). Fifty neurons were recorded in normal saline before (control) and after stimulation (after stim). A: silent to oscillatory transition. Silent activity (control) changed to rapid oscillatory activity after 60 min of rhythmic stimulation. We classify the stimulus-induced state as oscillatory because of the high duty cycle. The activity shown was elicited with +0.1-nA current injection. B: oscillatory to tonic transition. Oscillatory activity (with bursts of 2–3 action potentials per oscillation) was induced by a +0.4-nA current injection (control). Sixty minutes of stimulation led to a change to tonic firing (after stim). Reversal to oscillatory activity took ~2 h of no stimulation. C: Tonic to oscillatory transition; +0.4-nA depolarizing current was used in all traces. Tonic firing (control) changed to oscillatory activity after 60 min of rhythmic stimulation (after stim). The pattern reversed to tonic firing after ~2 h of no stimulation (reversal). D: percentage of neurons (from a total of 50 cells) that changed activity among silent (S), tonic (T), and oscillatory (O) after 45–60 min of rhythmic stimulation. E: voltage changes during the period of hyperpolarizing stimulation used to induce activity changes. F: activity in the period of hyperpolarizing current injection (left; **P = 0.005, n = 6, paired Student’s t-test) and in neurons that showed no difference in activity pattern (right; P = 0.375, n = 6, paired).

found that neurons that do not show a change in activity induced by patterned stimulation generate a PIR on average almost twice as large (4.5 ± 2.7 mV, n = 30) as those that do (2.7 ± 3.5 mV, n = 16), but this difference is not statistically significant (P = 0.363, unpaired Student’s t-test). As can be
after stimulation, under these conditions and have the ability to also increase either silent or weak oscillatory to robust oscillatory activity. We found no statistically significant difference in the changes induced by stimulation between these three measures. We observed a statistically significant decrease in high-threshold outward currents. Neurons were bathed in 20 mM TEA to reduce the depolarizing stimulation. Rhythmic hyperpolarizing stimulation, the neuron displays larger amplitude and lower frequency bursts (after stim). Figure 5: Rhythmic stimulation-induced changes in neuronal activity in reduced outward currents. Neurons were bathed in 20 mM TEA to reduce the dominant outward currents. In the presence of TEA 40% of the cells were initially silent and 60% were initially oscillatory in response to a small depolarizing current pulse. A: example of 1 of the 6/15 neurons that shifted their activity from silent (control) to oscillatory (after stim) on 40 min of rhythmic hyperpolarizing stimulation, \( \Delta -67 \) mV. Current injection: \( +0.1 \) nA. B: example of 1 of the 9/15 neurons that show oscillatory behavior on depolarization (+0.4 nA) in control conditions. After 45-min rhythmic hyperpolarizing stimulation, the neuron displays larger amplitude and lower frequency bursts (after stim). \( \Delta -60 \) mV. Bottom traces in A and B show the depolarizing current steps (baseline = 0 nA).

either silent or weak oscillatory to robust oscillatory activity) under these conditions and have the ability to also increase their excitability in normal saline conditions (Fig. 4, A, C, and D). This can be interpreted as a homeostatic mechanism for the long-term recovery of oscillatory properties that complements the spontaneous tendency for neurons to become oscillators (Fig. 3A). However, once oscillatory activity is achieved, a certain degree of flexibility remains and neurons can revert to tonic (but not to silent) activity depending on the intrinsic properties of the cell (see also Turrigiano et al. 1994) and (see Effect of stimulation protocol) the characteristics of the input.

To determine if the different activity changes observed in response to patterned stimulation correlate with neuronal identity, we looked at the responses of identified pyloric versus gastric neurons in the presence of 20 mM TEA. Of a total of eight pyloric neurons and three gastric neurons, all expressed oscillatory activity. We measured oscillation amplitude (32.1 ± 19.2 mV), cycle period (590 ± 450 ms), and duty cycle (0.71 ± 0.28) and measured stimulus-induced changes in these three measures. We found no statistically significant difference in the changes induced by stimulation between pyloric and gastric cells in any of these three measures (P = 0.411, 2-way RM ANOVA).

Conductance changes induced by patterned stimulation

In neurons stimulated with rhythmic hyperpolarizing pulses, we observed a statistically significant decrease in high-threshold K\(^+\) conductance, \( g_K \), recorded at +10 mV in normal saline of ~45% (0.58 ± 0.07 \( \mu \)S before stimulation, 0.31 ± 0.04 \( \mu \)S after stimulation, \( P = 0.005, n = 6 \), paired Student’s t-test; Fig. 4F, left). In those neurons in which the activity was not modified, no change in \( g_K \) was observed (0.65 ± 0.17 \( \mu \)S before stimulation; 0.68 ± 0.15 \( \mu \)S after stimulation, \( P = 0.375, n = 6 \), paired Student’s t-test; Fig. 4F, right). These results are consistent with an increased excitability of those neurons sensitive to prolonged stimulation as would be expected for neurons that switched activity from a silent or tonic pattern to an oscillatory pattern (Fig. 4, A and C). These results appear to be inconsistent with the change in activity from oscillatory to tonic firing that we saw in a small subset of stimulated neurons (12%, Fig. 4D), which under our definition, would be considered due to a reduction of excitability (see dynamic clamp results below and DISCUSSION).

STG neurons in situ (cf. Golowasch and Marder 1992; Graubard and Hartline 1991) and in culture (Turrigiano et al. 1995) express large outward currents dominated by a TEA-sensitive \( I_{K(Ca)} \). In contrast with the complete block of \( I_{K(Ca)} \) by TEA in situ (Golowasch and Marder 1992; Graubard and Hartline 1991), TEA has been reported to incompletely block \( I_{K(Ca)} \) in cultured STG neurons (Hurley and Graubard 1998). In our hands, 20 mM TEA eliminates 84 ± 6% (n = 6) of the total high-threshold outward current in cultured STG neurons. \( I_{K(Ca)} \) constitutes ~20% of the outward current recorded in 20 mM TEA (defined as the current additionally blocked by 200 \( \mu \)M Cd\(^{2+}\); the high-threshold current remaining in the presence of Cd\(^{2+}\) corresponds to a delayed rectifier current, \( I_{K_d} \)). In the presence of 20 mM TEA, we observed that prolonged patterned stimulation induced a significant reduction of the outward current for all voltages above ~20 mV (\( P = 0.004, n = 6 \), 2-way RM ANOVA, Fig. 6A) but with no apparent effect on the voltage dependence of activation (Fig. 6A; \( V_{1/2} \) before stimulation = −9.4 ± 17.6 mV, \( V_{1/2} \) after stimulation = −10.4 ± 14.7 mV, \( P = 0.900, n = 6 \), paired Student’s t-test). We estimated the maximum conductance from the current averages measured at 0 mV. Before stimulation, the estimated maximum conductance was 0.12 ± 0.06 \( \mu \)S, which decreased by a statistically significant 22% to 0.09 ± 0.06 \( \mu \)S (\( P = 0.022, n = 7 \), paired Student’s t-test). In the absence of known specific K\(^+\) current inhibitors, these data suggest that most of the spontaneous and stimulation-induced changes of \( I_K \) could be attributed to \( I_{K(Ca)} \) because 87% of the total \( I_K \) corresponds to \( I_{K(Ca)} \), as defined by K\(^+\) current blockade with Cd\(^{2+}\) (Golowasch and Marder 1992), and 13% to a delayed rectifier K\(^+\) current. Thus the 45% stimulation-induced reduction in the total K\(^+\) current that we observe cannot be accounted for by effects on the delayed rectifier only. Furthermore, because we observe a stimulation-induced reduction of 22% of the remaining total outward current in the presence of 20 mM TEA and 20% of that current can be further blocked with Cd\(^{2+}\) (and thus corresponds to \( I_{K(Ca)} \)), we conclude that an effect of rhythmic stimulation exclusively on \( I_{K(Ca)} \) is in principle sufficient to account for all our observations of activity-dependent effects on outward currents.

We performed similar measurements on six identified pyloric neurons and five identified gastric neurons to confirm if any of these changes are neuron type-specific. We compared the high-threshold K\(^+\) current I-V relationship difference (after normalization) before and after 0.33-Hz hyperpolarizing stimulation for pyloric and gastric neurons separately as was done with unidentified neurons before (see Fig. 6A, bottom). A two-way RM ANOVA reveals no statistical significant differ-
ence in the effect of rhythmic stimulation between identified pyloric and gastric neurons ($P = 0.546$).

We isolated $I_{Ca}$ as described in METHODS (Fig. 6B). Hyperpolarizing stimulation for as little as 15 min induced a marked increase in $I_{Ca}$ (Fig. 6B, top). Figure 6B also shows the peak current-voltage relationship before (control, ■) and after rhythmic stimulation of STG neurons (after stim, □). A statistically significant increase for all voltages above −20 mV is observed ($P = 0.011, n = 15, 2$-way RM ANOVA followed by Bonferroni post hoc analysis), with the peak conductance measured at +10 mV growing 2.5-fold from 0.014 ± 0.031 to 0.036 ± 0.031 $\mu$S ($P = 0.046, n = 7$, paired Student’s t-test). We observed no significant effect of stimulation on the voltage dependence of activation of this current (Fig. 6B; the half-maximal activation voltage, $V_{1/2}$, before stimulation = −2.5 ± 10.0 mV, $V_{1/2}$ after stimulation = −5.5 ± 30.2 mV, $P = 0.099, n = 7$, paired Student’s t-test).

As can be seen in the current traces in Fig. 6B (top), $I_{Ca}$ measurements are slightly contaminated with $I_k$, although the very early measurements of $I_{Ca}$ mimicked this contamination. In 20 mM TEA −20% of that $K^+$ current corresponds to $I_{KCa}$, the conductance of which we argue is reduced by rhythmic hyperpolarizing stimulation. To eliminate this possibility, we first tested the effect of 100 mM TEA on $I_k$. We found that the cells were not adversely affected by this extremely high concentration. We also found that adding 200 $\mu$M Cd$^{2+}$ does not produce an additional block of $I_k$ (not shown). We take this as evidence that 100 mM TEA completely eliminates $I_{KCa}$, leaving intact only part of the delayed rectifier conductance. As a further way to isolate $I_{Ca}$, we measured the Ca$^{2+}$ tail currents (in the presence of 100 mM TEA + 0.1 $\mu$M TTX) at −80 mV after activating $I_{Ca}$ with 800-ms-long depolarizing pulses in the range −60 to +30 mV (Fig. 6C). The K$^+$ equilibrium potential in these neurons is close to −80 mV. Thus these tail currents should be almost entirely free of contaminating K$^+$ currents. Under these conditions, we observe a highly significant increase in $I_{Ca}$, at all voltages above −20 mV ($P = 0.001, n = 5, 2$-way RM ANOVA followed by Bonferroni post hoc analysis). At +20 mV, we calculate a highly significant Ca$^{2+}$ conductance increase from 0.040 ± 0.047 to 0.061 ± 0.056 $\mu$S ($P < 0.001, n = 10$, paired Student’s t-test).

In contrast with the effect on $I_k$ and $I_{Ca}$, the leak conduc-
tance showed no change in conductance in these experiments (0.004 ± 0.003 $\mu$S before stimulation and 0.005 ± 0.002 $\mu$S after stimulation, $P = 0.712, n = 7$, paired Student’s t-test). Leak conductance was determined in these experiments from the current changes elicited in response to the voltages steps from −40 to −50 (or −60) mV. Additionally, the transient $I_A$ current (measured in TEA to minimize other K$^+$ currents and with $I_k$ and leak current subtracted) is completely unaffected by patterned stimulation in cultured crab STG neurons (Fig. 6D, $P = 0.918$, 2-way RM ANOVA over the activation range: −40 to +30 mV). Specifically, the average peak conductance $g_A V_{1/2}$, and s values measured at +10 mV were $g_A = 0.32 ± 0.11$ $\mu$S, $V_{1/2} = 24.9 ± 5.6$ mV, and $s = 9.5 ± 3.5$ mV before stimulation, and $g_A = 0.33 ± 0.13$ $\mu$S, $V_{1/2} = 23.5 ± 5.3$ mV, and $s = 7.9 ± 2.4$ mV after stimulation were likewise

FIG. 6. Effect of patterned activity on ionic currents. A, top: raw traces of high-threshold K$^+$ currents, $I_k$, recorded at +10 mV in the normal Ca$^{2+}$ saline +20 mM TEA before (control) and after 60 min of 0.33-Hz hyperpolarizing stimulation. Middle: a raw voltage trace used to elicit the control current trace above. †, −40 mV. Bottom: the mean ± SD I-V relationship of normalized currents recorded at steady state (†) and shown as above. $P < 0.004, n = 6$, $I_k$ was normalized by currents measured in control conditions at 0 mV. B, top: typical recordings of a Ca$^{2+}$ current at 0 mV in Cancer saline containing 20 mM TEA. 0.1 $\mu$M TTX. The neurons were also TEA and Cs$^+$ loaded to reduce K$^+$ currents to a minimum. The transient inward current (control) increased in amplitude after 30 min of rhythmic stimulation (after stim). Middle: raw voltage trace used to elicit the control current trace above. †, −40 mV. Bottom: control I-V plots of mean ± SD peak normalized $I_{Ca}$ measured at 20–30 ms after pulse onset (†), and the I-V plot after −30 min 0.33-Hz hyperpolarizing stimulation. $P = 0.011, n = 15$. $I_{Ca}$ was normalized by currents measured in control conditions at +10 mV. C, top: raw current traces at the end of an 800-ms-long depolarizing test pulse to 0 mV followed by a voltage drop to −80 mV before (control) and after 30-min 0.33-Hz hyperpolarizing stimulation. Currents were recorded in same conditions as in B. Middle: test voltage trace. †, −80 mV. Bottom: I-V plots (control and after stimulation) of mean ± SD normalized tail $I_{Ca}$ recorded at 20 ms after the beginning of the hyperpolarizing voltage step (†). $P = 0.001, n = 10$. $I_{Ca}$ tail was normalized by currents measured in control conditions at +10 mV. D, top: raw traces of a typical transient A. $I_{Ca}$ current elicited at 0 mV before (control) and after 60-min stimulation. Middle: raw voltage trace used to elicit the control current trace above. †, −80 mV. Bottom: I-V plot of the mean ± SD normalized peak $I_{Ca}$ recorded at the peak as shown in top panel (†). $P = 0.918, n = 7$. $I_{Ca}$ was normalized by currents measured in control conditions at 0 mV. † in top panels of A–D indicate 0 nA. All currents are leak subtracted. All statistical comparisons were performed with 2-way RM ANOVAs. Bonferroni post hoc analysis was used for multiple pair-wise comparisons: **$P < 0.001$; *$P < 0.05$. J Neurophysiol • VOL 96 • OCTOBER 2006 • www.jn.org
FIG. 7. Role of Ca\textsuperscript{2+} influx on activity-dependent conductance regulation. Unidentified STG neuron current recordings in normal Ca\textsuperscript{2+} saline before and after stimulation with 0.33-Hz hyperpolarizing current pulses was carried out under conditions that eliminate Ca\textsuperscript{2+} influx. \(I_{Ca}\) was recorded in 100 mM TEA saline after loading cells with TEA and Cs\textsuperscript{+} (METHODS). High-threshold K\textsuperscript{+} current, \(I_{K}\), was recorded in normal saline + 20 mM TEA. Top: a sample raw current trace recorded under each of the applied test conditions, arrowheads indicate 0 nA. Middle: raw voltage trace used to elicit the current traces above. Lower panels: I-V plots of normalized currents averaged (±SD) across cells before (control) and after stimulation. \(I_{Ca}\) and \(I_{K}\) were normalized by currents measured in control conditions at +10 mV. \(I_{K}\) was normalized by currents measured in control conditions at 0 mV. All statistical comparisons were performed with 2-way repeated measures (RM) ANOVAs. A. top and middle: initial 370 ms of the Ca\textsuperscript{2+} current (top) elicited by a depolarizing step from -40 to +10 mV (middle) and recorded in normal Ca\textsuperscript{2+} conditions. Control (■) trace was recorded before stimulation began, Stim in Cd\textsuperscript{2+} (△) trace was recorded after 30 min of stimulation in the presence of 200 \(\mu\)M Cd\textsuperscript{2+} and washout of Cd\textsuperscript{2+}: Re-Stim in Cd\textsuperscript{2+} (×) trace was recorded after washout of Cd\textsuperscript{2+} and additional 40 min stimulation in normal Ca\textsuperscript{2+} conditions. Bottom: average I-V plots of the normalized peak \(I_{Ca}\) measured 25 ms after the onset of the depolarizing test pulse, recorded before stimulation (control) and after stimulation in (and after washout of) Cd\textsuperscript{2+} (Stim in Cd\textsuperscript{2+}). \(P = 0.308, n = 5\). Inset: example of a single cell recorded under the 3 conditions indicated above. B. top and middle: noninactivating \(I_{K}\) (top) elicited by a test pulse from -40 mV to +10 mV (middle) before (control) and after 45 min of rhythmic stimulation (after stim). Bottom: I-V plot of the steady-state average (±SD) normalized currents. \(P = 0.300, n = 5\). C. top and middle: \(I_{K}\) (top) elicited by a test pulse from -40 to 0 mV (middle), before (control) and after 40 min of rhythmic stimulation in (and washout of) 200 \(\mu\)M Cd\textsuperscript{2+} (Stim in Cd\textsuperscript{2+}). Bottom: I-V plot of the steady-state average (±SD) and normalized currents. \(P = 0.618, n = 5\).

not statistically significantly affected by stimulation (\(P = 0.938, n = 7\), 2-way RM ANOVA).

The hyperpolarization-activated current’s voltage-dependence (i.e., steady-state activation curve) is strongly shifted to more negative values than those observed in cultured lobster STG neurons or crab’s LP neuron in situ (Golowasch 1992 #11; Turrigiano et al. 1995) before \((V_{1/2} = -104.1 ± 4.4\) mV, slope factor \(s = 8.3 ± 3.7\) mV, \(n = 7\)), and neither of these values nor the maximum conductance were affected by patterned stimulation. The maximum conductance measured at -120 mV was 0.015 ± 0.007 \(\mu\)S before stimulation, and 0.018 ± 0.010 \(\mu\)S after stimulation (\(P = 0.140, n = 8\), paired Student’s t-test).

Role of calcium influx in activity-dependent regulation of conductances

The conductance changes reported in the preceding text most likely occurred in response to the experimentally imposed activity pattern and were not due to effects of the electrode-filling solutions (K-citrate, K\textsubscript{2}SO\textsubscript{4}, or TEA/Cl + CsCl) or of different bathing solutions (TEA, TTX, Cs\textsuperscript{+}), and occurred in the absence of any known growth factors or neuromodulators. For activity to be responsible for these changes, neurons need to be able to detect changes in their own patterns of activity. A plausible candidate for such a gauge of activity is intracellular Ca\textsuperscript{2+} (Bito et al. 1997; De Koninck and Schulman 1998; Liu et al. 1998; Schulman et al. 1995). Indeed, in our neurons the only conditions that block the effects of rhythmic stimulation are those that interfere with Ca\textsuperscript{2+} influx (neither the K\textsuperscript{+} current blocker TEA nor the Na\textsuperscript{+} current blocker TTX do). Figure 7A shows peak \(I_{Ca}\) recorded in 100 mM TEA and C shows \(I_{K}\) recorded in 20 mM TEA, before (control) and after 0.33-Hz hyperpolarizing stimulation in the presence of 200 \(\mu\)M Cd\textsuperscript{2+} (stim in Cd\textsuperscript{2+}). Cd\textsuperscript{2+} is known to block the high-threshold \(I_{Ca}\) in STG neurons (Golowasch and Marder 1992; Graubard and Hartline 1991; Turrigiano et al. 1995), and we reasoned that stimulation in the presence of a blocker of Ca\textsuperscript{2+} influx should eliminate the effect of stimulation on \(I_{K}\) and \(I_{Ca}\). Indeed we observed that 0.33-Hz hyperpolarizing stimulation in the presence of Cd\textsuperscript{2+} eliminates the enhancing effect on \(I_{Ca}\) (\(P = 0.308, n = 5\)) and the depressing effect on \(I_{K}\) (\(P = 0.618, n = 5\), 2-way RM ANOVA). The inset in Fig. 7A (re-stim in Ca\textsuperscript{2+}) also shows a strong enhancement of \(I_{Ca}\) recorded in one of (2) cells in which we succeeded to wash out Cd\textsuperscript{2+} and further stimulate with hyperpolarizing pulses for ~30 additional minutes with Ca\textsuperscript{2+} influx restored. Furthermore, Fig. 7B shows an inward current that shows virtually no inactivation when Ca\textsuperscript{2+} is replaced with Ba\textsuperscript{2+} in the extracellular solution. With Ca\textsuperscript{2+} influx thus minimized, no change in the amplitude of the inward current now carried by Ba\textsuperscript{2+} (control) was observed in response to prolonged patterned stimulation (after stim, Fig. 7B). No significant changes were recorded over the voltage-dependent activation range (~30 to +30 mV) of this current (\(P = 0.300, n = 5\), 2-way RM ANOVA).

Effect of stimulation protocol

The results shown thus far suggest that rhythmic neuronal activity plays an important role in determining both activity and ionic conductance changes. The stimulation protocol used thus far has been hyperpolarizing 1-s-long pulses every 3 s...
Figure 8. Effect of stimulation pattern. Unidentified neuron high-threshold K⁺ current recordings were performed in normal Ca²⁺ saline + 20 mM TEA. I-V plots were made with current measurements obtained at the end of 800-ms-long voltage steps (see Fig. 6A) from a holding voltage of −40 mV. All currents were normalized by the current measured in control conditions at 0 mV. All statistical comparisons were performed with 2-way RM ANOVAs. A: control measurements were obtained before any stimulation (black squares). Neurons were then stimulated with 8-s hyperpolarizing pulses every 9 s for ~25 min (0.11-Hz hype, gray triangles), followed by ~40 min of the standard 0.33-Hz hyperpolarizing stimulation (0.11→0.33-Hz Hype, open gray squares). The pairwise statistical comparisons between the 3 treatments is shown as P values (n = 6). Pairwise statistical comparisons of the origin of the differences among the treatments were used the Bonferroni post hoc test and is indicated as **P < 0.001; *P < 0.05. B: control current measurements were obtained before stimulation (control, black squares) and after stimulation with hyperpolarizing 1-s-long pulses at 0.5 Hz (0.5-Hz Stim, open gray squares). P = 0.779, n = 5. C: control current measurements were obtained before stimulation (control, black squares) and after stimulation in voltage-clamp with depolarizing 1-s pulses from −40 to −10 mV at the standard 0.33-Hz frequency (depolar stim, open gray squares). P = 0.526, n = 5. In 2 occasions, depolarizing stimulation was followed by 40-min stimulation with the standard 1-s hyperpolarizing pulses at 0.33 Hz. Measurements of 1 of these cells is shown in the inset (then → Hype Stim, open gray triangles).

(Methods). Does the effect of rhythmic stimulation on activity and ionic currents depend on the properties of the stimulus? We tested this by measuring the high-threshold K⁺ current, Iₖ (Fig. 8). Cells were hyperpolarized rhythmically at a slower rate (8 s pulses every 9 s, i.e., 0.11-Hz Hype, Fig. 8A) and at a slightly faster rate (1-s pulses every 2 s, i.e., 0.5 Hz, Fig. 8B) than the standard stimulation frequency of 0.33 Hz. Because our several controls on identified pyloric and gastric neurons have shown no correlation of any of the measurements of activity or conductance with neuronal cell type, we performed this experiment on unidentified neurons. Figure 8A shows the effects on Iₖ of applied 0.11-Hz hyperpolarizing stimulation for ~25 min, followed by ~35–45 min of standard 0.33-Hz hyperpolarizing stimulation. Compared with the results shown in Fig. 6A, where 0.33-Hz stimulation induces a clear and statistically significant decrease in amplitude of Iₖ at all voltages above ~40 mV, 0.11-Hz hyperpolarizing stimulation instead induces a statistically significant increase of the current amplitude at voltages ≥0 mV (P = 0.002, n = 6, 2-way RM ANOVA; Fig. 8A, solid gray triangles). When 0.11-Hz hyperpolarizing stimulation was followed by 0.33-Hz stimulation (Fig. 8A, open gray squares), we observed a statistically significant decrease of the current amplitude at voltages above ~20 mV (control vs. 0.33 Hz stim: P = 0.011; 0.11-Hz Hype vs. 0.33-Hz Hype: P < 0.001, n = 6, 2-way RM ANOVA) comparable to our previous observations (Fig. 6A). To establish if this is a specific effect on the high-threshold K⁺ current, we measured the effects of the same stimulation sequence on Iₐ, which we have already shown to be unresponsive to 0.33-Hz stimulation (Fig. 6D). No statistical significant effect of either stimulus pattern was observed on Iₐ (P = 0.869, n = 5, 2-way RM ANOVA). On the other hand, only a slightly higher hyperpolarizing stimulation frequency (0.5 Hz) compared with the standard 0.33-Hz frequency results in no significant change in amplitude of Iₖ at any voltage (Fig. 8B, P = 0.779, n = 5). Iₖ was also not affected by 0.5-Hz hyperpolarizing stimulation (P = 0.254, n = 5, 2-way RM ANOVA). The fact that hyperpolarizing stimulation within a voltage range in which no Ca²⁺ current has been directly detected in STG neurons in culture or in situ raises the question of what the path may be for cytoplasmic Ca²⁺ increase. Because Iₖ,Ca is strong at ~10 mV, we stimulated STG neurons with depolarizing pulses from ~40 to ~10 mV in voltage clamp to ensure Ca²⁺ influx through a path known to be present in these cells. Figure 8C shows the results of such an experiment. Depolarizing stimulation had no discernible effect on Iₖ (P = 0.526, n = 5, 2-way RM ANOVA). To demonstrate that this result is specific to depolarizing stimulation, in two instances, we were able to maintain the cells impaled long enough to stimulate them with 0.33-Hz hyperpolarizing pulses for additional 40 min. Figure 8C, inset, shows that the characteristic downregulating effect of hyperpolarizing stimulation could be clearly elicited in this neuron. Finally, depolarizing stimulation also had no significant effect on Iₐ (P = 0.898, n = 5, 2-way RM ANOVA).

Additionally, as would be expected from the effects of 0.11-Hz hyperpolarizing stimulation on Iₖ mentioned in the preceding text, 0.11-Hz stimulation had a statistically significant effect on the amplitude of the slow oscillations recorded in the six neurons that we stimulated (50.2 ± 14.0 mV before stimulation, 31.7 ± 17.8 mV after stimulation; P = 0.016, Student’s t-test). We could not measure either burst duration or cycle period because 0.11-Hz stimulation had the additional effect of drastically reducing the number of oscillations measured with a current pulse of any levels, in most cases to only one or zero oscillations. These results, although not quantifiable, are consistent with an enhancement of a high-threshold K⁺ current.

Dynamic-clamp experiments

The effects of prolonged stimulation on voltage-dependent currents and neuronal activity only partially reversed during the time we could maintain the recordings (2–4 h). To verify if
the spontaneous and stimulation-induced conductance changes ($g_K$ decrease and $I_{Ca}$ increase) are indeed sufficient to account for the observed alterations in neuronal patterns of activity, we introduced negative or positive outward and inward conductances with dynamic clamp. We fitted with Hodgkin and Huxley-type equations the high-threshold $K^+$ current difference and separately the $Ca^{2+}$ current difference of neurons sensitive to patterned stimulation in normal saline. For these fits, we chose neurons that responded to patterned stimulation with high-threshold $K^+$ and $Ca^{2+}$ current changes close to the population average. The best fit to the $K^+$ current could be obtained with two conductance components, one transient ($g_{Ktr}$) and one sustained ($g_{Kst}$), whereas the $Ca^{2+}$ current could be fitted well with a single component ($g_{Ca}$). The conductance parameters thus obtained and used for our dynamic-clamp experiments are given in Table 1. Because the voltage dependence of these currents does not appear to be affected by patterned stimulation, we modified only the maximum conductances to mimic the changes observed on these two currents. Figure 9A shows the results of one such experiment on naïve unstimulated neurons recorded in normal saline. Activity was elicited by small depolarizing current injections. Conductance values indicated above the arrows correspond to the maximum conductance values of each dynamic-clamp current. Many different patterns of activity could be produced by relatively small changes of the maximum conductance values, which depended on the particular neuron recorded (Fig. 9A). However, we could repeatedly induce oscillatory activity by reducing the outward currents (negative conductance) and/or increasing the inward current (positive conductance, Fig. 9A). It was relatively easy to also find combinations of maximum conductance values within the range of the spontaneous or stimulation induced conductance changes described before that could produce tonic firing in a neuron that was originally oscillatory (Fig. 9A, bottom). Figure 9B shows in more detail that some properties induced by prolonged rhythmic stimulation cannot only be mimicked by adding $g_{Ca}$ and subtracting $g_K$ but also reversed by subtracting $g_{Ca}$ and adding $g_K$ after they were induced by stimulation. Before rhythmic stimulation was started, the neuron shown in Fig. 9B (top left) was bathed in 20 mM TEA to induce slow, large-amplitude oscillations. Adding $g_{Ca}$ and subtracting $g_K$ with dynamic clamp increased the amplitude and slightly decreased the frequency of oscillations (Fig. 9B, top right). A similar but more pronounced effect was later observed after rhythmically stimulating the neuron for 20 min with dynamic clamp discontinued. Finally, the enhanced oscillation amplitude and reduced oscillation frequency induced by rhythmic stimulation was partially reversed by subtracting $g_{Ca}$ and adding $g_K$ with identical levels as used immediately before to induce the changes with dynamic clamp (Fig. 9B, bottom right).

**Discussion**

Long-term regulation of neuronal excitability and intrinsic properties can play a key role in maintaining activity patterns of single neurons and networks within stable and operational ranges in response to a variety of perturbations (Davis and Bezprozvanny 2001; Desai et al. 1999; Franklin et al. 1992; Galante et al. 2001; Hong and Lennicka 1995; Li et al. 1996; Lindsell and Moody 1994; Luther et al. 2003; Turrigiano and Nelson 2004; Turrigiano et al. 1994). Here we have used adult cultured STG neurons of the crab *C. borealis* to identify mechanisms that underlie the recovery and stabilization of rhythmic activity after dissociation. We find that downregulation of the high-threshold $K^+$ current, $I_K$, and upregulation of a $Ca^{2+}$ current, $I_{Ca}$, is called into play during the spontaneous regulation of activity after dissociation. Prolonged 0.33-Hz rhythmic stimulation with hyperpolarizing pulses induces similar changes in the same two currents. However, low-frequency
hyperpolarization (0.11 Hz) had the opposite effect on $I_K$ and slightly higher frequency (0.5 Hz) had no effect at all (Fig. 8A), indicating that crab STG neurons are sensitive to the temporal properties of the stimulus as previously suggested by Turrigiano and collaborators. As a consequence STG neurons may be tuned to the oscillating frequencies of the network each of these neurons are normally part of (Turrigiano et al. 1994). From this it would be expected that neurons belonging to networks the natural frequency of which is not the same would respond differently to the same pattern of stimulation or, alternatively, develop a different frequency of oscillation in culture. Surprisingly, however, we found that identified neurons belonging to both the pyloric and the gastric mill networks, which typically oscillate in situ at 5- to 10-fold different frequencies, developed oscillations in culture with indistinguishable frequencies. We further found that gastric and pyloric neurons cannot be distinguished during the culture period studied (≤10 days) on the basis of their activity changes or the ionic mechanism involved in their responses to stimulation. Preliminary ongoing experiments indicate that in culture pyloric neurons appear to retain the properties developed during the first week in culture, whereas gastric neurons appear to slowly differentiate from a pyloric neuron phenotype in a time scale of weeks via an unknown mechanism.

We find that excitability cannot only be reduced by patterned stimulation as has previously been reported (Turrigiano et al. 1994) but can also be heightened with the same type of stimulation, an excitability increase being three times more likely in crab neurons than an excitability reduction. The apparent difference in response to rhythmic stimulation of lobster neurons, which in the study of Turrigiano and collaborators were not shown to be able to enhance their excitability, may stem from the fact that in their study only bursting neurons were selected for stimulation and analysis (Turrigiano et al. 1994). We find that oscillating neurons can only respond to stimulation by switching to either tonic firing (similar to lobster neuron responses) or not at all. In their study, Turrigiano et al. (1994) apparently did not analyze bursting neurons that did not respond to stimulation.

**Spontaneous activity changes**

The progressive decrease in the proportion of silent STG neurons in primary culture and the increase in the proportion of neurons expressing tonic and, later, mostly oscillatory activity, suggests a predetermined tendency to oscillate and the existence of homeostatic mechanisms that allow neurons to restore their oscillatory activity after it is lost (see also Turrigiano et al. 1995). Pyloric network neurons can behave as oscillators when acutely isolated from the network but, with the exception of the single pacemaker AB neuron, all oscillate irregularly (Bal et al. 1988). These oscillations occur only when neuromodulatory inputs to the STG are intact (Nusbaum and Beenhakker 2002). In the absence of these neuromodulatory inputs, all STG neurons, including the AB neuron, lose the ability to oscillate (Bal et al. 1988). Our results and those of Turrigiano et al. (1995) indicate that most (if not all) STG neurons in time evolve robust oscillatory activity when isolated from network and from neuromodulatory influences.

In our culture conditions, neuromodulators are completely absent and the recovery of rhythmic activity of isolated STG neurons is reminiscent of the recovery of rhythmic activity in the intact pyloric network after the permanent removal of neuromodulatory inputs (Luther et al. 2003; Thoby-Brisson and Simmers 1998). In lobsters, this latter recovery is accompanied by the development of oscillatory properties by some identified pyloric network neurons within a few days (Thoby-Brisson and Simmers 2002). Thus the acquisition of oscillatory properties we and Turrigiano et al. (1995) have observed in isolated STG neurons in culture provides a plausible mechanism for the recovery of rhythmic activity in the network. It is interesting, however, that the conductances that have been shown to be spontaneously regulated in the intact network after decentralization are not the same as those regulated in dissociated neurons in culture (Thoby-Brisson and Simmers 2002) (see Ionic mechanism of activity regulation).

Two hypotheses (as of yet not tested) may explain the suppression of endogenous oscillatory properties in neurons within the intact STG. One, oscillatory properties may be suppressed by neuromodulators exerting trophic effects (Le Feuvre et al. 1999; Thoby-Brisson and Simmers 2000). Two, activity itself may regulate the expression of endogenous oscillatory properties (Golowasch et al. 1999b; Turrigiano et al. 1994). Our results are consistent with both hypotheses. In the intact network, neuromodulators may somehow suppress the expression of endogenous oscillatory properties in most neurons. Once this input is eliminated (by cell dissociation or by removal of neuromodulatory inputs in situ), constraints on the expression of these properties are removed and oscillatory activity can be expressed by up- and downregulation of ionic currents such as those characterized in this work and those characterized previously by Turrigiano et al. (1995). Spontaneous emergence of oscillatory properties in culture conditions may be genetically determined as suggested by the spontaneous changes in activity and ionic currents described in this study and by Turrigiano et al. (1995). However, this possibility does not exclude the possibility that activity itself may also regulate neuronal electrical properties (Desai et al. 1999; Golowasch et al. 1999a; Liu et al. 1998).

**Stimulation-dependent effects on activity**

Our results show that isolated STG neurons are sensitive to prolonged rhythmic stimulation. Neurons displaying either silent or tonic firing activity can be induced to oscillate, whereas neurons that show oscillatory activity remain able to revert to tonic firing (but not to the silent) states (Fig. 4).

The large variability of the effects on activity of prolonged stimulation (Fig. 4D) may partly be due to the heterogeneity of our sample of (mostly unidentified) neurons. However, when outward currents were reduced in the presence of TEA, 100% of the stimulated neurons increased their excitability (Fig. 5). Furthermore, 100% of the neurons in which outward currents were measured in the presence of TEA showed a decrease of the remaining TEA-resistant $I_K$, and 100% of those neurons in which $I_{Ca}$ was measured showed an increase in conductance. The homogeneity of these effects, although variable in their extent, suggests to us that all STG neurons, irrespective of cell type, can regulate their excitability in an activity-dependent manner and via the same ionic mechanism, namely by down-regulation of a $I_K$ current and an upregulation of $I_{Ca}$. This conclusion is further supported by the similarity of the results...
of our measurements of change in \( I_K \) in identified pyloric and gastric neurons.

**Ionic mechanism of activity regulation**

Only two ionic currents appear sensitive to prolonged patterned stimulation in dissociated *C. borealis* STG neurons, \( I_{Ca} \) and \( I_K \). The same two currents are responsible for the spontaneous changes of activity seen in these neurons. It has previously been shown in cultured lobster STG neurons that, in addition to these currents, changes in two Na\(^{+}\) currents also correlate with spontaneous changes of activity from silent to tonic firing but that only \( I_{Ca} \) continues to increase as neurons become bursters (Turrigiano et al. 1995). We cannot exclude the participation of Na\(^{+}\) currents in cultured crab STG neurons. However, tonic firing becomes gradually less and less prevalent among neurons recorded up to day 6 in culture (Fig. 3A) and almost completely absent after 3 wk in culture (not shown). Also, silent cells could never be induced to fire action potentials with prolonged hyperpolarizing (patterned or constant) stimulation. These observations suggest that Na\(^{+}\) currents in crab STG neurons, apparently in contrast with their lobster counterparts, may not be subject to long-term upregulation or to activity-dependent regulation but may only spontaneously and transiently be upregulated during the first few days in culture. Additionally, Turrigiano et al. (1995) observed a drastic reduction in the transient A current, \( I_A \), as neurons transition between silent, tonic, and oscillatory states. In contrast, we have seen no change in \( I_A \) at any time in culture (Fig. 3C), and also no changes in \( I_A \) are induced by prolonged stimulation (Fig. 6D and see RESULTS). The lack of effects of prolonged hyperpolarizing stimulation on \( I_A \) in crab cultured STG neurons is consistent with the lack of effects of similar hyperpolarizing stimulation on \( I_A \) in two different identified crab pyloric neurons in situ (Golowasch et al. 1999a). However, although \( I_A \) is strongly affected by depolarizing stimulation of IC\( \text{\textit{C}} \) neurons in situ (Golowasch et al. 1999a), \( I_A \) was not affected in any neurons in culture (this study). On the other hand, in situ hyperpolarizing stimulation of these same cells did also not affect the high-threshold K\(^{+}\) currents (Golowasch et al. 1999a) that we find to be affected in our STG cultured neurons. Finally, the hyperpolarization-activated current \( I_h \) did not change either spontaneously or in response to stimulation in our study, but it does increase spontaneously after decentralization in lobster PD neuron [Thoby-Brisson and Simmers 2002; it was not measured in lobster cultured neurons (Turrigiano et al. 1995)]. It could be argued that these differences in spontaneous changes and response to rhythmic stimulation may stem from the fact that our cultured neurons were not precisely identified to the level of individual cell types (but only to the level of network identity). Although this is possible, we find that, as stated in the previous section, all the neurons stimulated respond, and all respond in the same manner at the level of ionic conductance changes, suggesting that neuronal identity appears not to be a factor. It is possible that in situ the high-threshold K\(^{+}\) currents sensitive to stimulation represents a small current relative to the total high-threshold K\(^{+}\) current and thus activity changes, or total conduction changes, could not be unambiguously discerned. Alternatively, the process of dissociation in which cells lose most of their dendritic structure, the special culture conditions (artificial saline, complete absence of neuromodulators, etc) and the absence of synaptic network interactions may reveal a new, or alter an existing activity-sensing mechanism.

The 2.5-fold stimulation-induced \( I_{Ca} \) increase was achieved with 30–60 min of stimulation (Fig. 6B). \( I_{Ca} \) increases spontaneously also by \( \sim 2.5\)-fold but only after 4 days in culture (Fig. 3B). Similarly, the high-threshold K\(^{+}\) conductance was reduced by 45% in normal saline with 45–60 min of rhythmic stimulation but changes spontaneously by only 25% over 6 days in culture. Thus although the same currents appear targeted during spontaneous and induced activity changes, these effects may occur via two different but probably overlapping pathways. Slow, spontaneous changes may predominantly require gene transcription and protein synthesis, similar to the transcription requirement for spontaneous but slow pyloric activity recovery in the intact pyloric network after the permanent removal of neuromodulatory inputs (Thoby-Brisson and Simmers 2000).

Our dynamic-clamp experiments confirm that an increase in Ca\(^{2+}\) conductance, \( g_{Ca} \), and a decrease in K\(^{+}\) conductance, \( g_K \), are sufficient to explain all the observed changes in activity. We were able to induce a switch from either silent or tonic firing to oscillatory activity by modifying the same conductances \( (g_{Ca}, g_K) \), within the conductance ranges observed physiologically, that we showed to be affected during spontaneous activity changes and by patterned stimulation (Fig. 9A). Although relatively small \( g_K \) changes were sufficient to generate significant activity changes, the \( g_{Ca} \) levels we needed to use to induce activity changes were comparable to the \( g_{Ca} \) changes observed in response to prolonged patterned stimulation. Although increasing \( g_{Ca} \) with dynamic clamp always increased the tendency of neurons to burst, it is important to remember that this current is not a Ca\(^{2+}\) current in the biological sense because no Ca\(^{2+}\) influx occurs. An increase in Ca\(^{2+}\) influx due to an augmented biological \( g_{Ca} \) will be accompanied by an increase in \( I_{K_{Ca}} \), which can reduce the excitability of a neuron and thus induce tonic firing. However, even a dynamic-clamp-reduced K\(^{+}\) current in a background of high \( I_{Ca} \) can switch an oscillatory to a tonically firing neuron (Fig. 9A, bottom), similar to 41% of our rhythmically stimulated oscillatory neurons (Fig. 4, C and D). Consistent with our observations of stimulation-induced activity changes, we could never induce tonic firing in silent cells by manipulating \( g_{Ca} \) and \( g_K \). Unlike Thoby-Brisson and Simmers (2002), we observed no spontaneous or stimulation-induced changes in \( I_h \) and did not test the effects of this current with dynamic clamp. This current is well known to be involved in the generation of oscillatory activity (Forti et al. 2006; Maccaferri and McBain 1996; McCormick and Bal 1997; Noble et al. 1992) and may be responsible for the enhancement of oscillatory properties observed in decentralized lobster preparations (Thoby-Brisson and Simmers 2002). Our dynamic-clamp results show that changes in \( I_{Ca} \) and \( I_h \) are sufficient to explain our observations but certainly do not exclude the possible participation of \( I_h \) under other conditions or in other species.

Our observations suggest that spontaneous changes in Ca\(^{2+}\) and high-threshold K\(^{+}\) currents likely play a central role in homeostatic recovery of rhythmic function in the decentralized pyloric network of the crab STG. Decreases in K\(^{+}\) currents as a result of decreased neuronal drive are common to most if not all systems in which a homeostatic recovery of excitability can
be identified (Zhang and Linden 2003). Although among inward currents sometimes Na⁺ currents are also affected (Desai et al. 1999; Mee et al. 2004), more commonly Ca²⁺ currents are strengthened due to decreased activity (Chung et al. 1993; Garcia et al. 1994; Su et al. 2002) or reduced due to enhanced neuronal activity (DeLorme et al. 1988; Franklin et al. 1992; Hong and Lnenicka 1995; Li et al. 1996). The effects we have observed of very slow frequency hyperpolarizing stimulation (0.11 Hz) do not appear to be consistent with the concept of homeostatic regulation of function. Rather a different mechanism that regulates these same conductances in a frequency-dependent manner and with a faster time course than the spontaneous recovery appears to be active in parallel. A solid conclusion on this will need to await further experiments to rule out possible unphysiological effects of the strong hyperpolarization used during stimulation in our study.

Intracellular signaling

We show that activity-dependent Ca²⁺ and K⁺ conductance changes depend on Ca²⁺ influx into these cells. We suggest that a Ca²⁺-dependent intracellular mechanism modifies the conductances of both I_K and I_Ca. Ca²⁺ influx appears to be crucial also in other systems in mediating activity-dependent conductance and activity changes during development (Spitzer et al. 2002) and in the adult (Zhang and Linden 2003). Several possible Ca²⁺-dependent mechanisms may be involved, including transcription regulation (Spitzer et al. 2002; West et al. 2002), ion channel downregulation (Klein et al. 2003), and posttranslational modifications (Cudmore and Turrigiano 2004), which can be sensitive to the exact pattern and path of Ca²⁺ entry (Bito et al. 1997; De Koninck and Schulman 1998; Dolmetsch et al. 1998; Fields 1994; Li et al. 1996). We observed no correlation of stimulation-induced activity changes with expression of post inhibitory rebound (PIR) properties. This lack of correlation of activity or conductance changes with the measured PIR may lie in that PIR is a rather indirect measure of Ca²⁺ influx. If contaminated with a simultaneous activation of I_K, a small Ca²⁺ influx may still take place with little or no measurable PIR. Second, we observed the opposite effect on the levels of I_K and on activity when the 0.33-Hz rhythmic pattern of hyperpolarizing stimulation was replaced with low-frequency (0.11 Hz) hyperpolarization, i.e., an increase instead of a decrease in I_K (Fig. 8A) and a reduction in the amplitude of the membrane potential oscillations. This is consistent with the possibility that a PIR-like mechanism during which a small but sufficient influx of Ca²⁺ takes place as suggested by Turrigiano et al. (1994). Prolonged hyperpolarization would simply close Ca²⁺ channels that are open near the resting potential and maintain them closed longer, thus preventing Ca²⁺ influx into the cells and bringing intracellular Ca²⁺ levels below the levels of the unstimulated neurons. Although this mechanism is plausible, it is harder to reconcile with our observation that only a slightly higher frequency of hyperpolarizing stimulation completely eliminates the effect of patterned stimulation on I_K (Fig. 8B). It is conceivable, however, that the kinetics of Ca²⁺ influx can sensitively interact with the kinetics of intracellular Ca²⁺ uptake and of intracellular activation of Ca²⁺-dependent molecules involved in the activity-sensing mechanism to yield a highly frequency-specific transduction mechanism (Dolmetsch et al. 1998; Gorunova and Spitzer 2002; Soderling et al. 2001). A more serious challenge to this hypothesis comes from the results showing a complete lack of effect of depolarizing stimuli (that ensure Ca²⁺ influx through I_Ca) on I_K (Fig. 8C). These results strongly suggest that it is not merely Ca²⁺ influx that determines the effect on these currents. An interaction of the localization of the voltage-sensitive plasma membrane channels required for Ca²⁺ influx and the intracellular localization of the appropriate transducing signaling molecules would be required. Such an interaction has been observed in many other systems (Hobai and Levi 1999; Parekh 2006; Wu et al. 1999). The question still remains: what is the path of Ca²⁺ entry? Some forms of L-type Ca²⁺ channel, although they typically carry a high-threshold current, have a finite conductance at membrane potentials low enough (i.e., −70 mV) to contribute to the resting potential (Smith et al. 1993). As Fig. 6C shows, however, we observe no inward current at voltages near or below the resting potential of cultured STG neurons (i.e., −60 to −40 mV). No direct report to date exists of Ca²⁺ current mediating a Ca²⁺ influx at low membrane potentials in STG neurons, but such an influx has been inferred from the voltage dependence of synaptic release in these cells and from Ca²⁺ imaging data showing a Ca²⁺-sensitive low-threshold Ca²⁺ influx (Kloppenburg et al. 2001). A very-low-level Ca²⁺ influx via these Ca²⁺-sensitive channels could go undetected and could trigger Ca²⁺ release from intracellular stores thus amplifying the Ca²⁺ signal. This mechanism would account for the sensitivity to Cd²⁺ at the same time as the absence of measurable transmembrane Ca²⁺ current but would require postulating two different Ca²⁺ channels, with distinct voltage dependence and cellular localization and both with similar Cd²⁺ sensitivity. With the exception of Cd²⁺ sensitivity, such distinct voltage dependence and cellular localization is not uncommon (cf. Ivanov and Calabrese 2006a,b). It has been shown that hyperpolarization-activated channels can be permeable to Ca²⁺ (Yu et al. 2004). Because most cultured STG neurons express an I_K this is a possibility that remains to be tested. Another candidate pathway for Ca²⁺ influx are gap junction-forming hemi-channels. Gap junctions are known to be permeable to Ca²⁺ (Saez et al. 1989), and many STG neurons form gap junctions. Hemi-channels may also be permeable to Ca²⁺ although to our knowledge this has not been established for innexins.

In conclusion, we suggest that, consistent with our observations and those of Turrigiano et al. (1995), adult STG neurons have a natural predetermined tendency to oscillate independent of neuronal type. Activity can, however, be regulated in an activity-dependent manner over this natural tendency. Only two ionic currents, I_Ca and I_K, appear to be involved in both the spontaneous development of oscillations and in stimulation-induced changes of activity in crab STG neurons. Our dynamic-clamp experiments show that regulation of only these two currents is sufficient to produce different changes in activity. The spontaneous changes in I_Ca and I_K can explain the homeostatic recovery of pyloric network activity after removal of neuromodulatory input to the ganglion in situ, whereas the rhythmic stimulus-induced conductance changes appear to be to some extent at odds with the concept of homeostatic recovery of function. We also conclude that, at least during approximately the first week in culture, no discernible differences in the process of recovery of activity or the mechanisms involved
can be made between neurons belonging to the two main functional neural networks (pyloric and gastric) found in the STG. Differences in the generation of oscillatory properties in pyloric versus gastric neurons in situ may thus be due to either synaptic interactions within the STG or to intrinsic mechanisms operating at longer time scales than 1 wk after dissociation.

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