Long-Term Neuromodulatory Regulation of a Motor Pattern–Generating Network: Maintenance of Synaptic Efficacy and Oscillatory Properties

MURIEL THOBY-BRisson AND JOHN SIMMERS
Laboratoire de Neurobiologie des Réseaux, Université Bordeaux 1 and Centre National de la Recherche Scientifique, Unité Mixte de Recherche 5816, 33405 Talence, France

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Thoby-Brisson, Muriel and John Simmers. Long-term neuromodulatory regulation of a motor pattern-generating network: maintenance of synaptic efficacy and oscillatory properties. J Neurophysiol 88: 2942–2953, 2002; 10.1152/jn.00482.2001. Rhythm generation by the pyloric motor network in the stomatogastric ganglion (STG) of the spiny lobster requires permissive neuromodulatory inputs from other central ganglia. When these inputs to the STG are suppressed by cutting the single, mainly afferent stomatogastric nerve (stn), pyloric neurons cease to burst and the network falls silent. However, as shown previously, if such a decentralized quiescent ganglion is maintained in organ culture, pyloric network rhythmicity returns after 3–4 days and, although slower, is similar to the motor pattern expressed when the stn is intact. Here we use current- and voltage-clamp, primarily of identified pyloric dilator (PD) neurons, to investigate changes in synaptic and cellular properties that underlie this transition in network behavior. Although the efficacy of chemical synapses between pyloric neurons decreases significantly (by ≈50%) after STG decentralization, the fundamental change leading to rhythm recovery occurs in the voltage-dependent properties of the neurons themselves. Whereas pyloric neurons, including the PD, lateral pyloric, and pyloric cell types, are unable to generate burst-producing membrane potential oscillations in the short-term absence of extrinsic modulatory inputs, in long-term decentralized ganglia, the same cells are able to oscillate spontaneously, even after experimental isolation in situ from all other elements in the pyloric network. In PD neurons this reacquisition of rhythm is associated with a net reduction in outward tetraethylammonium-sensitive ionic currents that include a delayed-recurrent type potassium current (IdrK) and a calcium-dependent K+ current, IKCa. By contrast, long-term STG decentralization caused enhancement of a hyperpolarization-activated inward current that resembles Ih. These results are consistent with the hypothesis that modulatory inputs sustain the modulation-dependent rhythmogenic character of the pyloric network by continuously regulating the balance of membrane conductances that underlie neuronal oscillation.

INTRODUCTION

The operation of central neural networks responsible for the generation of rhythmic motor behavior derives from an interplay of the intrinsic membrane properties of constituent neurons and their synaptic interactions (Marder and Calabrese 1996). As well as producing basic network rhythmicity, these cellular and synaptic properties are targets of extrinsic neuromodulatory inputs that, via their actions on a wide array of both classical synaptic conductances and voltage-dependent channels implicated in neuronal oscillation, are able to shape network activity to satisfy immediate and changing behavioral demands (Calabrese 1998; Harris-Warrick and Marder 1991; Stein et al. 1997).

In addition to such short-term adaptive instruction, modulatory inputs may exert long-term regulatory effects on their target networks. In the developing nervous system, for example, modulatory inputs have been found to play a critical role in the maturation of motor networks, either by facilitating the progressive emergence of adult neuronal properties (Sillar et al. 1992) or by actively repressing the adult circuit phenotype until appropriate stages in development (Le Feuvre et al. 1999).

There is also strong evidence, derived mainly from the experimental suppression of innervating pathways, that presynaptic inputs continue to exert persistent and long-term regulatory influences on their postsynaptic targets in the mature nervous system. In addition to the well-described innervation-dependent regulation of receptor/channel expression and distribution in muscle (Angelides 1986; Fambrough 1979; Lupa et al. 1995), synaptic inputs have been found to regulate transmitter/receptor biosynthesis (Hyatt-Sachs et al. 1993; Kirsch and Betz 1998), neuronal structure (Kossel et al. 1997), and gene expression (Fawcett et al. 2000; Martinou and Merlie 1991; Weiser et al. 1994). Furthermore, recent evidence suggests that central synaptic and neuromodulatory inputs also play an important role in regulating and maintaining the biochemical properties of neurons in the adult networks they modulate. For example, in the spinal cord of the adult turtle, the membrane properties of deafferented motoneurons gradually alter and revert to an embryonic phenotype after several days in organ culture (Perrier and Houngsaard 2000). The persistent extrinsic regulation of intrinsic excitability that this implies under normal conditions could be mediated by two different but complementary mechanisms: either indirectly by altering the ongoing activity of target neurons to allow activity-dependent regulation of their membrane conductances (Desai et al. 1999; Golowasch et al. 1999; Turrigiano et al. 1995) or directly via a trophic control of second messenger cascades that ultimately lead to changes in ion channel expression in these neurons (Jonas and Kaczmarek 1999).

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Address reprint requests to: J. Simmers.
In the stomatogastric nervous system (STNS) of the adult spiny lobster, *Jasus lalandii*, the well-described pyloric motor network in the stomatogastric ganglion (STG) fails silent when modulatory inputs from anterior ganglia are eliminated by cutting or blocking the STG input nerve. However, after 3–4 days in organ culture, the decentralized network gradually recovers a pattern-generating capability that no longer depends on these inputs (Thoby-Brisson and Simmers 1998, 2000; see also Golowasch et al. 1999). Thus the prolonged absence of central modulatory inputs to the pyloric network allows the emergence of a modulation-independent rhythmogenic property that is maintained in a modulation-dependent state when these inputs are present. The reacquisition of network rhythmicity requires new gene transcription occurring in a critical period at the time the modulatory inputs are first eliminated (Thoby-Brisson and Simmers 2000), and recent evidence from the lobster *Homarus gammarus* has indicated that pyloric circuit decentralization induces neuron-specific alterations in gene expression of at least one ion channel, the transient potassium current, *I\textsubscript{A}* (Mizrahi et al. 2001).

The aim of the present study was to further explore the electrophysiological basis for the recovery of pyloric network operation following long-term decentralization. Specifically, to assess the extent to which modulatory inputs might normally sustain the conditional nature of pyloric network operation, we examined the effects of modulatory input deprivation on pyloric neuron properties in organ cultures of the spiny lobster STNS by comparing these properties in short- and long-term isolated STG. In addition to analyzing decentralization-induced changes in synaptic conductances within the pyloric network, alterations in membrane properties of individual pyloric neurons were assessed through voltage clamp investigation of three currents known to play crucial roles in shaping oscillatory membrane behavior in a number of neuronal networks (Calabrese 1998): a sustained voltage-activated K\textsuperscript{+} current (*I\textsubscript{Kd})*, a calcium-activated K\textsuperscript{+} current (*I\textsubscript{KCa})*, and a hyperpolarization-activated inward current (*I\textsubscript{h})*. Our data indicate that the pyloric network responds to the prolonged absence of extrinsic modulatory innervation by a substantial decrease in the strength of its synaptic connections and selective conductance changes that lead to an overall increase in the intrinsic oscillatory capacity of individual pyloric neurons.

**METH ODS**

Experiments were performed on adult *J. lalandii* purchased from commercial suppliers and kept in laboratory tanks of fresh circulating sea water until used. Before dissection, lobsters were cold anesthetized in ice for 30 min. To set up in vitro preparations of the STNS (Fig. 1A), the STG, still attached via the stomatogastric nerve (stn) to the esophageal (OG) and bilateral commissural ganglia (CoGs), was dissected from the foregut wall and pinned out under lobster saline in a silicone elastomer-lined (Sylgard 184; Dow Corning) petri dish, as previously described (Thoby-Brisson and Simmers 1998). The saline composition consisted of (in mM) 480 NaCl, 12.75 KCl, 3.9 MgSO\textsubscript{4}, 13.7 CaCl\textsubscript{2}-2H\textsubscript{2}O, 5 HEPES, pH 7.45. For long-term in vitro survival of preparations, the saline was sterile-filtered and contained glucose (1 g/l), penicillin (35 µg/ml), and streptomycin (50 µg/ml). Such organ cultures, which remained viable for ≥8–10 days, were maintained at 15°C and the bathing saline was renewed daily. The STG was disconnected from extrinsic modulatory inputs arising in the OG and CoGs by cutting the stn at the approximate midpoint between STG and OG.

**FIG. 1.** The lobster stomatogastric nervous system (STNS) and pyloric neural network. A: schematic of isolated STNS. B: pyloric neurons and their synaptic connections. Resistance symbols indicate electrotonic synapses; stick and ball symbols: filled, glutamatergic inhibitory synapses; clear, cholinergic inhibitory synapses. Numbers of each cell type are indicated. Hatching denotes main pyloric neurons. IC and PD neurons control the cardiopyloric valve. AB, anterior burster interneuron; Ach, acetylcholine; CoG, commissural ganglion; Glu, glutamate; IC, inferior ventricular neuron; LP, lateral pyloric neuron; lp-pyn, nerve with LP and PY axons; OG, esophageal ganglion; PD, pyloric dilator neuron; pdn, nerve with PD axons; PY, pyloric neuron; STG, stomatogastric ganglion; stn, stomatogastric nerve; VD, ventricular dilator neuron.

Electrophysiological procedures were as routinely used for the STNS in vitro (Harris-Warrick et al. 1992). Extracellular recordings were made from motor nerves with Vaseline-isolated platinum electrodes while intracellular recordings were made from somata within the desheathed STG with glass microelectrodes (tip resistance 10–20 MΩ) filled with 3 M KCl. Single electrode recordings and current injection were achieved via the bridge circuits of WPI electrometers. In a series of experiments, an Axoclamp 2A amplifier (Axon Instruments) was used for both current- and voltage-clamp recordings. Voltage-clamp experiments were performed in two-electrode mode using pClamp 6 software from Axon Instruments. The degree of space clamp obtained in these neurons under our conditions is unknown, although the finding that the rate and voltage-dependence of the conductances we investigated were smooth and continuous functions of the membrane potential suggested that sufficient clamp was obtained to avoid serious error. Conventional techniques were used for display, storage, and transcription of recorded data.

Impaled STG neurons were identified according to their peripheral axonal projections, firing patterns, and synaptic interactions with other pyloric neurons. Sketching the somata layout within a ganglion allowed the same neuron(s) to be recognized and penetrated repeatedly in a given organ culture experiment. In most cases, pairs of pre- and postsynaptic pyloric neurons were impaled to assess the strength of synaptic connections. To isolate neurons of primary interest from their partners within the pyloric network, known cholinergic presynaptic elements (Fig. 1B) were photoablated with blue light illumination after intrasomatic injection of Lucifer yellow (Miller and Selverston 1979) and remaining glutamatergic inputs were suppressed with bath-applied picrotoxin (PTX, 10⁻⁵ M) (Biday 1980).

In two-electrode voltage-clamp experiments, TTX (10⁻⁷ M) to block action potential generation and tetraethylammonium (TEA, 10⁻⁴ M) to block delayed rectifier K\textsuperscript{+} current were included in the saline.
mM) to block combined outward K⁺ currents (delayed rectifier $I_{K_d}$ plus Ca²⁺-activated current $I_{KCa}$) were introduced to the bathing medium. All averaged data are given as mean ± SD, unless otherwise stated. Student’s $t$-tests were used to assess statistical difference. Significances were accepted at $P < 0.05$.

RESULTS

Recovery of pyloric network rhythmicity after long-term STG decentralization

The pyloric network in the STG of the combined STNS in vitro (Fig. 1) is continuously active, generating a basic output pattern that includes sequential bursting in the lateral pyloric (LP), pyloric (PY), and pyloric dilator (PD) motoneurons (Fig. 2A). In *Jasus*, pyloric rhythm generation depends strictly on permissive modulatory substances released from stn axon terminals, since, after STG inputs from the OG and CoG are prevented by cutting the stn, pyloric network rhythmicity ceases within 10 min (Fig. 2B) (see also Bal et al. 1988). However, as reported previously (Thoby-Brisson and Simmers 1998, 2000), when such decentralized STG are maintained 3 to 5 days in organ culture, a pyloric motor pattern is gradually reexpressed, which, although slower, is comparable in terms of general burst phase relations to that generated by the intact STNS (compare Fig. 2C with 2A). Moreover, in all isolated STG in which rhythm recovery occurred (77% of 93 preparations), spontaneous activity persisted for the remaining survival time (maximum 15 days) of the preparation. Thus, in long-term decentralized STG, the pyloric network is capable of functional recovery from the loss of central inputs on which its activity normally depends.

Since extrinsic factors, such as regeneration of input pathways or residual activity in axotomized STG terminals do not underlie this restoration of pyloric rhythmicity (Thoby-Brisson and Simmers 1998), the recuperative process must derive from the pyloric network itself, and specifically from modifications either in the synaptic interactions between pyloric neurons and/or in their intrinsic membrane properties.

Decentralization-induced changes in synaptic connectivity

Fundamental changes in synaptic wiring within the pyloric network did not occur in the 5-day period after STG decentralization. First, the maintenance of coordinated phase relations between LP, PY, and PD neuron bursts, indicating functionally significant synaptic inhibition between these cells, was clearly evident in the pyloric motor pattern reexpressed by long-term disconnected STG. As seen in Fig. 2C, each PD neuron burst was again associated with synchronous hyperpolarization of postsynaptic LP and PY neurons, with the latter cells tending to fire in alternation (LP neuron bursts again preceding those of the PY neuron) due to their reciprocal inhibitory connection (see Fig. 1B). Second, routine examination with pair-wise intracellular recordings confirmed that all previously established network synaptic connections were conserved and no new synapses were found.

However, closer inspection of synaptic relations did reveal a substantial decline in synaptic strength throughout the decentralized pyloric network. This is illustrated in Fig. 3 where graded synaptic inhibition between PD and PY neuron pairs was examined in the presence and long-term absence of STG inputs. To prevent spike-mediated transmission and membrane oscillations, these experiments were performed in saline containing $10^{-7}$ M TTX. In each case the membrane potential of the presynaptic neuron (PD in Fig. 3) of a stn-intact pyloric network was current clamped to $-50$ mV, and then 3 s depolarizing pulses incrementing in 10 mV steps and repeating at 4 s intervals were applied through a second electrode until the

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**FIG. 2.** Functional recovery of pyloric network rhythmicity after long-term STG decentralization in vitro. A: top, STNS with STG connected via the stn to anterior ganglia. Bottom: pyloric rhythm recorded extracellularly on motor nerves carrying the axons of LP, PY (lp-pyn) and PD (pdn) neurons, and intracellularly from their somata (LP, PY, and PD). B: same recordings 1 h after stn transection. Pyloric neurons no longer burst. C: same recordings from the same preparation after 4 days in organ culture. Spontaneous network rhythmicity has reappeared.

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neuron’s membrane potential reached +20 mV (Fig. 3A2). The graded inhibitory synaptic potential (GSP) was measured with a third electrode placed in the postsynaptic neuron soma (PY in Fig. 3). Examples of control day 1 recordings are illustrated in the lower left panels of Fig. 3, A1 and B1 where two different presynaptic PD neurons were step depolarized from −50 to −10 mV. As for graded synaptic inhibition in STG neurons in general (Graubard 1978; Johnson and Harris-Warrick 1990; Manor et al. 1997), an early peak component that decays to a lower sustained level is evident in the postsynaptic PY neuron responses. In Fig. 3, A1 and B1 for example, the peak and persistent component amplitudes of control PY neuron GSPs on day 1 in vitro were 3.0 and 1.5 mV, respectively. After TTX washout, eight such preparations, including four freshly decentralized STG, were placed in organ culture. After 4 further days in vitro, these preparations were again placed under TTX and the same PD and PY neurons were reimpaled and identical presynaptic voltage steps were reapplied (see lower right panels of Fig. 3, A1 and B1). The amplitude of the PY neuron GSP did not change significantly in the four long-term stn-intact preparations (Fig. 3, A1 and A2), suggesting that synaptic efficacy in the pyloric network with intact modulatory inputs was maintained under our organotypic conditions. In contrast, in long-term decentralized STG, both the peak and persistent component of the PY neuron response showed a significant decrease in amplitude at all presynaptic voltages. In the experiment of Fig. 3B1, the peak amplitude of the PY neuron’s response to PD neuron voltage steps from −50 to −10 mV decreased from 3.11 to 0.85 mV, and, as seen from the normalized data in Fig. 3B2, the mean decline in the PY neuron response to the same presynaptic voltage step was ca. 46% (from 64.3 ± 11.3% of maximal GSP amplitude on day 1 to 17.6 ± 6.6% on day 5 postdecentralization; \( P = 0.023, n = 4 \)).

These data therefore suggest that, in long-term organ culture, the strength of synapses between PD and PY neurons decreases specifically as a result of prolonged modulatory input suppression. Similar observations were also made for the reciprocal inhibitory synapses between the cholinergic PD and glutamatergic neurons.

FIG. 3. Postdecentralization decrease in strength of the graded synapse from the PD to PY neurons. A: PD neuron-evoked graded synaptic potential (GSP) in PY neurons in stn-intact STG on day 1 and day 5 in vitro. A1: inhibitory responses of the same PY neuron to a 40 mV depolarization (from −50 to −10 mV) of a presynaptic PD neuron on day 1 (left) and on day 5 (right). A2: input–output curves (peak PY neuron GSP amplitudes normalized to their respective values at +20 mV, plotted against presynaptic PD potential) pooled from 4 preparations measured on day 1 (open circles) and day 5 (filled circles). B: PD-PY GSP in 4 further STG on day 1 (open circles) and day 5 after STG decentralization (filled circles). B1 and B2: same data treatment as in A1 and A2.

* Mean ± SD corresponding to the single responses illustrated in A1 and B1, respectively.
ergic LP neurons. The LP neuron response to \(-50\) to \(-10\) mV PD neuron voltage steps declined by 49% with decentralization (from 64.2 ± 16.0% of day 1 maximal GSP to 15.5 ± 14.6% on day 5 postdecentralization, \(P = 0.018, n = 3\)), while the LP to PD synapse weakened by ca. 47% (from 76.0 ± 20.3 to 29.9 ± 19.2%, \(P = 0.016, n = 4\)), therefore indicating that decentralization results in a very similar decline in synaptic efficacy between these chemically connected pyloric neurons. It is noteworthy finally that recordings from four PD neuron pairs indicated that STG decentralization did not modify significantly (\(P > 0.15\) for comparison at all voltage steps between \(-120\) and \(+20\) mV from a holding potential of \(-50\) mV) the strength of electrical coupling, at least between the two PD cell types.

**Decentralization-induced changes in oscillatory properties**

The second site from which functional network recovery might arise is the intrinsic excitability of pyloric neurons themselves and, specifically, the extent to which their burst-generating properties remain dependent on modulatory inputs. To explore this possibility we compared the bursting capability of pyloric neurons in stn-intact STG and after short- and long-term network decentralization. Moreover, to eliminate any contribution of synaptic input to recovered neuronal bursting in the absence of modulatory inputs, we examined individual neurons after further experimental isolation from all other pyloric circuit members (see Fig. 1A). For example, to isolate a PD neuron, its electrically coupled ventricular dilator, anterior burster (AB), and PD network partners were photoablated and the glutamatergic synapse with the LP neuron was blocked with \(10^{-5}\) M PTX (see schematic Fig. 4A). After such in situ isolation, but with modulatory inputs still intact, the PD neuron (\(n = 10\)) oscillates spontaneously as in the intact network (Fig. 4A, lower panels). Moreover as for neuronal oscillators in general, this activity is strongly voltage dependent in that cycle frequency increased with tonic depolarizing current injection (top and middle traces) and the phase of oscillation could be reset by brief current pulses (bottom). In contrast, soon after modulatory inputs to the STG were eliminated (Fig. 4B), the same isolated PD neuron now ceased to oscillate, even in response to tonic or pulsed current injection. This is consistent with the cessation of activity in the intact pyloric network immediately following STG decentralization as seen in Fig. 2B and further attests to the modulator dependence of pyloric neuron oscillations (see also Bal at al. 1988).

However, in eight further preparations that had been previously decentralized on day 1 in vitro and then maintained 4 days in organ culture, six of eight PD neurons continued to express strong spontaneous membrane potential oscillations.
when further isolated on day 5 from all detectable synaptic input (Fig. 4C). The frequency of these oscillations again displayed voltage-dependent, regenerative responsiveness to continuous and pulsed current injection. These displayed voltage-dependent, regenerative responsiveness to C input (Fig. 4). The frequency of these oscillations again when further isolated on day 5 from all detectable synaptic input (Fig. 4, A and B) into a chemo-independent burster that oscillates freely without modulatory input (Fig. 4C).

This transition to endogenous bursting seen in isolated PD neurons also occurred in other pyloric cell types. In Fig. 5, for example, which is from a 5-day decentralized STG, a recorded PY neuron was transiently isolated from presynaptic LP and PD neurons by holding the latter silent with tonic hyperpolarizing current injection. In this experiment, moreover, the AB interneuron, which is electrically coupled to the PD neurons, had been previously photoablated following migration of Lucifer yellow into the stn cut stump (note, this procedure also photoablates modulatory stn input terminals in the STG; see Thoby-Brisson and Simmers 1998). Under these conditions, and again in striking contrast to the situation in short-term decentralized STG, the PY neuron continued to oscillate and burst at its inherent frequency, and the phase of its rhythm could be easily reset by brief current pulse injection. When released from hyperpolarization (Fig. 5, right), however, the LP and PD neurons immediately resumed their own oscillatory activity and, via their inhibitory synaptic relations, the three cells again became locked into a coordinated activity pattern. Similar evidence for the acquisition of an endogenous oscillatory capability in the long-term absence of modulatory inputs was obtained for four of five PY neurons examined.

**Day 5 post-decentralization**

![Graph](image)

**FIG. 5.** Endogenous bursting properties in a PY neuron after long-term STG decentralization. Intracellular recordings of three main types of pyloric neuron (LP, PY, and PD) in a 5-day disconnected ganglion. In this preparation the AB interneuron had been previously photoablated on day 2 in vitro (see text). The PY neuron was “isolated” by hyperpolarizing the LP and PD neurons with negative current injection (−2 nA), during which time the PY cell continued to oscillate spontaneously and the phase of oscillation could be reset with a brief hyperpolarization (black squares indicate rhythm phase in absence of resetting). Note that the flat membrane potentials of the PD and LP neurons during experimental hyperpolarization indicate that the second PD neuron had also ceased to oscillate during the current injection.

**Recovery of oscillatory properties in long-term isolated neurons**

In a further strategy, we wanted to determine whether intercellular communication, possibly involving intrinsic modulatory signaling (Katz and Frost 1996), within the pyloric network throughout the decentralized period somehow contributed to the above changes in intrinsic behavior of pyloric neurons. To assess this possibility a selected pyloric neuron was isolated from its network partners on day 1 in vitro (Fig. 6A), and then, after stn transection and 4 further days in organ culture, the same neuron was reimpaled and examined with intracellular recording. The LP neuron was the main focus for these difficult experiments since it is a single neuron type and, of the three main cell types (i.e., PD, PY, and LP), it typically displays the weakest capacity to recover activity after decentralization. As illustrated in Fig. 6, A and B, long-term synaptically isolated LP neuron still acquired the ability to oscillate and burst in the prolonged absence of modulatory input. Interestingly, the voltage sensitivity of this recovered activity is similar to that of a freshly isolated LP neuron in a stn-intact STG, where typically the cell fires tonically at resting potential and oscillates only when slightly hyperpolarized (Bal et al. 1988). Results similar to those shown in the experiment in Fig. 6 were obtained from three LP neurons and four long-term isolated PD neurons (although not shown, it is noteworthy that, unlike the LP type cell, long-term synaptically isolated PD neurons oscillated spontaneously without current injection, in a manner similar to PD neurons freshly isolated on day 5 after STG decentralization as seen in Fig. 4C). Together therefore these data indicate that the postdecentralization reexpression of oscillatory activity derives solely from an intrinsic membrane response of individual pyloric neurons to the lack of extrinsic modulatory inputs, without any significant influence from other neighboring STG neurons.

**Changes in electrical properties**

We next compared basic membrane properties of pyloric neurons before and after long-term STG decentralization. The results summarized in Fig. 7 were obtained from 20 PD neurons. Whereas decentralization induced no significant modification in PD membrane potential (−54.1 ± 1.7 mV day 1, −57.4 ± 1.1 mV day 5; P > 0.05), there was a significant decrease in oscillation amplitude (21.5 ± 0.9 mV day 1, 14.0 ± 0.7 mV day 5, P < 0.001), an increase in spike amplitude (5.8 ± 0.3 mV day 1, 9.6 ± 0.7 mV day 5, P < 0.001), and membrane input resistance (measured under TTX) increased from 17.2 to 25.5 MΩ (P < 0.001). Although the changes in oscillation and spike amplitude were possibly due to motoneuron axotomy, which is known to induce alterations in neuronal excitability (Titmus and Faber 1995), that these changes were not observed in control 5-day-old preparations with intact stns (i.e., with only motor nerves axotomized) argues against this possibility (see also Thoby-Brisson and Simmers 1998).

**TEA-sensitive outward currents**

In a further step, two-electrode voltage clamp was used to measure two potassium currents expressed by PD neurons and to compare them before and after long-term decentralization.
These previously well-described currents, the delayed-rectifier current (I_{Kd}) and a calcium-dependent K^+ current (I_{KCa}), are activated together by membrane potential depolarization above around −40 mV, where a third potassium current, the transient A-type current, is almost completely inactivated (Golowasch and Marder 1992; Graubard and Hartline 1991; Kloppenburg et al. 1999; Tierney and Harris-Warrick 1992). We did not attempt systematically to separate the delayed rectifier and calcium-dependent K^+ current components in our organotypic experiments; therefore, the sum of I_{Kd} plus I_{KCa} is reported throughout the remainder of this paper. To investigate this combined outward current, day 1 STG were bathed with saline containing 10^{-7} M TTX to prevent Na^+ spiking and membrane oscillations and then a series of 10 mV steps from a holding potential of −50 to +40 mV was applied to a recorded PD neuron (Fig. 8Ai). Superfusion of the STG with 10 mM TEA was then used to reversibly block a large proportion of I_{Kd} plus I_{KCa} (Fig. 8Aii) (Hille 1992). The combined TEA-sensitive outward current was thus measured by subtracting currents occurring under TEA (Fig. 8Aii) from those elicited under normal saline (Fig. 8, Ai and Aiii). After washout of TTX, the STG was disconnected from the rostral ganglia, maintained 4 days in vitro, and the identical experiment was again performed on the same PD neuron. As can be seen in Fig. 8B, the TEA-sensitive outward current was still evident after long-term decentralization, but with a much decreased amplitude (compare with Fig. 8A). In six PD neurons examined (Fig. 8Ci), the combined K^+ current following decentralization attained no more than 50–55% of control values over the entire voltage range tested. At +40 mV, for example, the outward current displayed a postdecentralization decrease in peak amplitude from a mean (±SD) of 15.8 ± 1.6 nA on day 1 to 8.3 ± 1.8 nA on day 5 (P < 0.01). Moreover, to assess whether this decrease in current magnitude was uniquely a result of PD neuron decentralization, TEA-sensitive currents were measured in three preparations after 5 days in culture but with their stn intact. Under these conditions, the mean maximal current amplitude elicited by voltage pulses to +40 mV was 12.5 ± 3.0 nA. This magnitude was not significantly different from the conductance recorded on day 1 in culture (P = 0.5), although its higher value compared with long-term decentralized PD neurons did not reach statistical significance with our small sample size 1 (P = 0.1; n = 3). As a consequence, we are unable to reject entirely the possibility that the organ culture conditions per se were not also contributing to the decline in combined outward current (but see following text).

In a further analysis, the voltage-dependence of the combined outward current activation before and after long-term
decentralization (Fig. 8C2) was determined by converting the peak currents evoked by each voltage step to peak conductances, \( g \), using the equation \( g = I/(V_m - E_{rev}) \) (where \( V_m \) is the command potential and \( E_{rev} \) is the potassium reversal potential taken as \(-86 \) mV) (Hartline and Graubard 1992), and the resulting \( g/V \) curve was fitted to a third-order Boltzmann equation of the form

\[
g/V_{max} = 1/(1 + e^{(V-V_{h})/T})^n
\]

where \( n = 3 \), \( V_h \) is the voltage at which half-maximal activation of the individual gating step occurs, and \( s \) is a slope factor. The resulting Boltzmann fit to the conductance–voltage relations gave a \( V_h \) of \(-32.1 \pm 1.6 \) mV under day 1 control conditions, leading to half-maximal activation of the peak current at \(-8.9 \) mV. Although the maximal conductance of the combined outward current in PD neurons decreased significantly from a mean of \( 0.12 \pm 0.04 \) \( \mu \)S on day 1 to \( 0.07 \pm 0.021 \) \( \mu \)S on day 5 postdecentralization, the voltage-dependence of activation did not alter substantially. The small shift in \( V_h \) from \(-32.1 \pm 7.5 \) mV (slope value \(-17.2 \) mV) in control to \(-26.5 \pm 6.6 \) mV (slope \(-16.2 \) mV) in our 5-day decentralized preparations was not significant (\( P > 0.05; n = 6 \)).

**Hyperpolarization-activated inward current**

Inward currents in stomatogastric neurons are notoriously difficult to study because of their soma-distant location in combination with rapid activation properties (Golowasch and Marder 1992; Graubard and Hartline 1991). However, one readily accessible inward current is the slow hyperpolarization-activated \( I_h \) current (Golowasch and Marder 1992; Kiehn and Harris-Warrick 1992) responsible for a slow depolarizing sag toward resting potential, as seen for a PD neuron under TTX and current clamp conditions in Fig. 9Ai (arrow). The expression of \( I_h \) in the same neuron under voltage clamp can be seen in Fig. 9Bi, where the current continued to activate with increasing hyperpolarization and with little sign of inactivation. On day 5 after STG decentralization, the voltage response of this neuron to the same current steps as in control conditions revealed a substantially increased depolarizing sag (Fig. 9Bii, arrows). That this was indeed due to an increase in the \( I_h \) conductance can be seen under voltage clamp (Fig. 9Biii, arrows) where the amplitude was increased and the activation kinetics of \( I_h \) were substantially faster in the long-term decentralized neuron (compare with Fig. 9Bi). As evident in the
A- Current-clamp

i) Day 1

ii) Day 5

B- Voltage-clamp

i) Day 1

ii) Day 5

FIG. 9. Changes in hyperpolarization-activated inward $I_h$ current in PD neurons after long-term decentralization. A: current-clamp recordings of voltage responses (bottom) of the same PD neuron to negative current steps (top) ranging from 0 to $-2.6$ nA (duration 10 s) on day 1 (i) in an stn-intact control STG (under $10^{-7}$ M TTX) and on day 5 (ii) postdecentralization. Arrows indicate voltage- and time-dependent depolarizing sag, which increases after STG decentralization. B: same experimental conditions as in A. Voltage-clamp recordings of sag currents (bottom) in a different PD neuron in response to 10-s voltage steps (top) from a $V_h = -50$ mV to a range between $-120$ to $-60$ mV. Hyperpolarizing voltage steps elicit a slow inward current (arrows), which increases with long-term decentralization.

pooled $I-V$ measurements from seven PD neurons in Fig. 10A. $I_h$ began to activate at around $-60$ mV in both control and postdecentralization conditions, but the net current (i.e., the difference between current amplitudes measured at the beginning and end of each voltage step) was substantially enhanced postdecentralization by $\geq55\%$ at all membrane potentials. At $-120$ mV, for example, $I_h$ measured in PD neurons on day 1 in stn-intact STG was $3.5 \pm 0.5$ nA, while 5 days after stn section the current had increased to $5.8 \pm 0.6$ nA ($P < 0.01$, $n = 7$). In four further control preparations that remained 5 days in culture with an intact stn, mean $I_h$ measured in PD neurons was $4.0 \pm 0.4$ nA, which was not significantly different from the current magnitude on day 1 in culture ($P > 0.05$) but was significantly less than $I_h$ in long-term decentralized neurons ($P < 0.01$). This strongly suggests, therefore, that the alteration in this current was a direct consequence of STG decentralization and not simply due to time in culture.

The voltage dependence of $I_h$ activation before and after long-term decentralization is shown in Fig. 10B, in which peak currents were converted to peak conductances by assuming a reversal potential of $-35$ mV (Golowasch and Marder 1992). These values were then normalized to the calculated $g_{\text{max}}$ (mean $0.04 \pm 0.01$ $\mu$S on day 1, $0.07 \pm 0.01$ $\mu$S on day 5 postdecentralization) and the resulting conductance-voltage curves were fitted to a first order Boltzmann equation (Eq. 1; $n = 1$). These Boltzmann fits (Fig. 10B) gave a mean voltage for half-maximal activation of $-91.0 \pm 0.6$ mV (with a slope value of 9.0 mV) under control conditions, which was not significantly different ($P > 0.05$) from the half-activation voltage ($-89.5 \pm 0.7$ mV; slope value 9.8 mV) measured on day 5 postdecentralization.

Whereas the voltage dependence of $I_h$ activation remained unchanged after STG decentralization, the activation time constant for $I_h$ decreased uniformly by almost 20% over the voltage range tested (Fig. 10C). As is typical for $I_h$ (e.g., Harris-Warrick et al. 1995; McCormick and Pape 1990), the activation of this current was very slow and was best fitted by a single exponential function that produced time constant values that accelerated with increasing hyperpolarization, producing a mean value of $3.9 \pm 0.2$ s at $-120$ mV under day 1 control conditions. On day 5, however, the same voltage step activated $I_h$ with a time constant of $2.9 \pm 0.2$ s, commensurate with a significantly ($P < 0.01$) faster activation of this inward current after long-term STG decentralization.

DISCUSSION

In a previous extracellular study we reported that, in the spiny lobster, elimination of extrinsic modulatory inputs rapidly leads the pyloric network to fall silent, but after 4–5 days the network recovers the capacity to be rhythmically active (Thoby-Brisson and Simmers 1998). Therefore the prolonged absence of modulatory inputs allows the expression of a rhythmogenic capability that is normally maintained in a strictly conditional state when these extrinsic influences are present. One source of recovery could be a fundamental reorganization of pyloric network circuitry, involving changes in the strength of preexisting connections or the formation of entirely new synapses. Alternatively, rhythm recovery may derive from specific changes in the membrane properties of individual pyloric neurons (Thoby-Brisson and Simmers 2000). The goal of the present study was to examine both possibilities.

Decentralization-induced modifications in synaptic efficacy

Compensatory changes in synaptic connectivity is a well-known mechanism for functional recovery following lesions to innervating pathways. Examples range from restoration of auditory function in cricket 4–6 days following sensory deprivation (Brodieuerer and Hoy 1988), topographical reorganization of the cat visual cortex in response to retinal lesions (Darian-Smith and Gilbert 1994), and deafferentation-induced synaptic plasticity in the motor cortices of cat (Keller et al. 1990) and humans (Ziemann et al. 1998). In these cases, restoration of function derives from the formation of new functional connections and/or changes in strength or unmasking of preexisting synapses.
Our finding that the efficacy of pyloric synapses decreases substantially after suppression of modulatory input, without the formation of new synapses, does not readily comply with a major contribution of changes in network interactions to pyloric rhythm recovery. Although postdecentralization synaptic formation of new synapses, does not readily comply with a substantially after suppression of modulatory input, without the is unclear. One possibility is that STG decentralization eliminates certain modulatory inputs that are normally responsible for sustaining and reinforcing synaptic efficacy in the pyloric network. For example, amnergic up-modulation of synaptic connectivity is well known in both vertebrates (see, for example, Knapp and Dowling 1987; Perada et al. 1992) and invertebrates, including lobster stomatogastric circuits (Ayali et al. 1998; Johnson and Harris-Warrick 1990; Johnson et al. 1995). In the latter, however, different STG synapses may be affected differently or even in opposite ways by the same or different stn input modulators, so it is difficult to see why removal of the entire modulatory input ensemble should uniformly decrease the strength of chemical synapses throughout the pyloric network.

A further explanation for the postdecentralization decrease in network synaptic efficacy may reside with changes in the intrinsic membrane properties of pyloric neurons themselves. For example, in a manner equivalent to the postdecentralization reduction in voltage-dependent K⁺ currents found in the present study (see following text), an apparent decline in inhibitory synaptic strength could arise from a reduction of other ionic currents that are directly involved in presynaptic transmitter release or postsynaptic responsiveness. An additional interesting possibility is that, as seen in mammalian neocortical cultures (Rutherford et al. 1997), the reduction in synaptic inhibition may derive from an activity-dependent decrease in neurotransmitter expression due to lowered levels of pyloric network activity, particularly during the first few days after STG decentralization.

Decentralization-induced modifications in membrane properties

The most convincing evidence that pyloric rhythm recovery arises from changes in the intrinsic excitability of individual pyloric neurons derived from experiments performed on single cells after isolation in situ from all other intraganglionic input. Such acutely isolated neurons still eventually reacquired a strong burst-generating oscillatory capability, therefore indicating a fundamental alteration in their bioelectrical character; namely the transition from chemo-dependent (conditional) oscillators that are unable to burst without central inputs into endogenous (nonconditional) oscillators that operate without extrinsic input. As previously argued by Thoby-Brisson and Simmers (1998, 2000), this response to decentralization indicates a fundamental alteration in their bioelectrical character; namely the transition from chemo-dependent (conditional) oscillators that are unable to burst without central inputs into endogenous (nonconditional) oscillators that operate without extrinsic input.

\[ I_h \] characteristics in PD neurons before and after long-term decentralization. A: maximal \( I_h \) current amplitude versus membrane potential. B: \( I_h \) activation curves. Currents were converted to conductances (assuming a reversal potential of \(-35 \text{ mV}\)) and normalized to the corresponding maximal conductance (\( g_{\text{max}} \)) measured at a voltage step to \(-120 \text{ mV}\). The data were also fitted to a first-order Boltzmann relation with a calculated \( V_{1/2} \) of \(-91.0 \text{ mV}\) and \(-89.5 \text{ mV}\) and slope values of \( 9.0 \text{ mV} \) on day 1 and \( 9.8 \text{ mV} \) on day 5, respectively. C: kinetics of \( I_h \) activation. Activation time constant (calculated by fitting currents at different voltage steps to a single exponential process of the form \( I(t) = I_{0} - I_{\text{max}} e^{-t/\tau} \), where \( I(t) \) is the current at time \( t \), \( I_{\text{max}} \) is the steady state current, and \( I_{0} \) and \( \tau \) are the peak amplitude and time constant of the current) versus membrane potential. For all plots, measurements were made at day 1 in stn-intact STG (unfilled circles) and then on day 5 postdecentralization from the same neurons (black circles). Each value is an average \( \pm \text{SD} \) for 7 different PD cells.

FIG. 10.
This conclusion is further supported by recent direct evidence that network decentralization leads to changes in the expression of genes responsible for the biophysical properties of pyloric neurons (Mizrahi et al. 2001; Thoby-Brisson and Simmers 2000).

In our initial voltage-clamp experiments, which here focused on a single (PD) neuron type, we investigated postdecentralization changes in three conductances that have all been previously reported in STG cells of other crustacean species (Golowasch and Marder 1992; Graubard and Hartline 1991). These channels included two voltage-dependent outward conductances, \( I_{Kd} \) and \( I_{KCa} \), which are well known to be potent regulators of neuronal excitability and firing patterns (Hille 1992). Following removal of STG modulatory inputs, the magnitude of combined TEA-sensitive \( I_{Kd} \) plus \( I_{KCa} \) in PD neurons decreased to only approximately 50% of the current level in stn-intact controls. This substantial decline in outward current decreased to only approximately 50% of the current level in stn-intact controls. This substantial decline in outward current with time in organ culture, which could partially explain the net increase in PD neuron input resistance and would help promote bursting following decentralization, has also been reported in dissociated STG neurons in primary culture (Turrigiano et al. 1995).

The third conductance we investigated in detail, \( I_h \), also plays an important role in oscillatory behavior in a variety of neural networks (for reviews, see Calabrese 1998; Lüthi and McCormick 1998). This ubiquitous conductance is also found in crustacean pyloric neurons (Golowasch and Marder 1992) where its role and short-term modulation has been studied (Harris-Warrick et al. 1995; Kiehn and Harris-Warrick 1992). In our experiments, a comparison between stn intact control and long-term decentralized PD neurons revealed a considerable modification in the expression of \( I_h \), including a significant increase in magnitude and an increase in its rate of activation. In contrast no change was observed in \( I_h \) voltage activation characteristics. This current is important for neuronal pacing by setting resting potential and the occurrence and frequency of rhythmic bursting (Angstadt and Calabrese 1989; McCormick and Pape 1990; Thoby-Brisson et al. 2000). Thus increasing \( I_h \) and speeding its rate of activation in long-term decentralized pyloric neurons would enhance an inward current mechanism that opposes sustained membrane hyperpolarization and facilitates activation of other voltage-dependent conductances that contribute to membrane oscillations.

Taken together, therefore, our results indicate that prolonged deprivation of modulatory inputs to pyloric network neurons induces a change in electrical behavior accomplished by an increase in at least one important inward current, in parallel with a decreased effectiveness of TEA-sensitive outward conductances. In this way, the transition from the conditional to nonconditional oscillator phenotype does not appear to be attributable to changes in any one particular ion channel but rather derives from modifications in the ensemble of preexisting conductances, which in combination lead to the altered rhythmic capacity of pyloric neurons.

Our results from \( J. lalandii \) and those obtained both by Turrigiano et al. (1995) on dissociated STG neurons of the spiny lobster \( Panulirus interruptus \), and by Golowasch et al. (1999) from modeling studies and STS organ cultures of the crab \( Cancer borealis \) are qualitatively similar in a number of important ways. First, in all three cases, pyloric neuron bursting, which normally depends on the release of permissive modulatory substances from STG input terminals, reoccurs after several days in the absence of neuromodulators. Second, the reacquisition of rhythmicity depends on a modification in intrinsic membrane properties, such that a transition from conditional (modulator-dependent) bursting to endogenous bursting occurs. Third, this transition is associated with specific alterations in a variety of membrane conductances. However, the question of whether rhythm recovery is an indirect, activity-dependent response to the long-term absence of modulatory inputs (Golowasch et al. 1999; Turrigiano et al. 1995) and/or is a direct consequence of the removal of a trophic influence from the modulators themselves (Thoby-Brisson and Simmers 1998, 2000) remains unresolved by our data. Nonetheless our results do add to a growing body of evidence that modulatory inputs play a crucial long-term regulatory role not only in the maturation of motor network properties in the developing nervous system (Scrymgeour-Wedderburn et al. 1997; Sillard et al. 1992, 1995), including the lobster STG (Le Feuvre et al. 1999), but also in controlling the expression of neuronal channel properties in the adult STG (Mizrahi et al. 2001). It is also interesting that, after several days in organ culture, deafferented motoneurons in the turtle spinal cord gradually lose their adult biophysical characteristics and reacquire intrinsic response properties normally only seen in immature motoneurons (Perrier and Hounsgaard 2000; Perrier et al. 2000). This further supports the idea that, in addition to governing the short-term operational flexibility of motor networks, central synaptic and modulatory inputs may also be responsible, either directly or indirectly, for the “life long” maintenance of appropriate rhythmogenic properties of their neuronal targets in the mature nervous system.

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Present address of M. Thoby-Brisson: Laboratoire de Neurobiologie Génétique et Intégrative, Institut Alfred Fessard, Avenue de la Terrasse, 91198 Gif sur Yvette, France.

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