Episodic Bouts of Activity Accompany Recovery of Rhythmic Output by a Neuromodulator and Activity Deprived Adult Neural Network

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CAMS Report 0203-21, Spring 2003

Center for Applied Mathematics and Statistics

NJIT

April 13, 2003

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Keywords: central pattern generator, stomatogastric ganglion, crustaceans, homeostasis, plasticity
Running title: Motor function recovers in bouts

ABSTRACT

The pyloric rhythm of the stomatogastric ganglion of the crab, *Cancer borealis*, slows or stops when descending modulatory inputs are acutely removed. However, the rhythm spontaneously resumes after one or more days in the absence of neuromodulatory input. We recorded continuously for days to characterize quantitatively this recovery process. Activity bouts lasting 40 to 900 seconds began several hours after removal of neuromodulatory input and were followed by stable rhythm recovery after 1-2 days. Bout duration was not related to the intervals (0.3 to 800 minutes) between bouts. During an individual bout the frequency rapidly increased and then decreased more slowly. Photoablation of back-filled neuromodulatory terminals in the STG neuropil had no effect on bouting or recovery, suggesting that these processes are intrinsic to the STG neuronal network. After removal of neuromodulatory input the phase relationships of the components of the triphasic pyloric rhythm were altered, and then over time the phase relationships moved towards their control values. Although at low pyloric rhythm frequency the phase relationships among pyloric network neurons depended on frequency, the changes in frequency during recovery could not account for the change in phase seen after rhythm recovery. We suggest that activity bouts represent underlying mechanisms controlling the restructuring of the pyloric network to allow resumption of an appropriate output following removal of neuromodulatory input.

INTRODUCTION

Episodic bouts of activity are thought to be involved in processes controlling the development of neural networks including neurite outgrowth, neuronal phenotype differentiation, pruning of existing synaptic contacts and the formation of new synaptic contacts. Spontaneous, bouts of activity are seen in developing networks in the retina (Meister, Wong et al. 1991; Wong, Chernjavsky et al. 1995; Wong 1999), spinal cord (O'Donovan and Landmesser 1987; O'Donovan, Chub et al. 1998), hippocampus (Ben-Ari, Cherubini et al. 1989; Garaschuk, Hanse et al. 1998), and cultures of young cortical neurons (Murphy, Blatter et al. 1992; Kamioka, Maeda et al. 1996). In the developing spinal cord, bouts of activity lasting approximately one minute are interspersed with silent periods lasting 10-15 minutes. Individual activity bouts are composed of multiple cycles of action potential discharge that lengthen in duration over the course of the bout (Landmesser and O'Donovan 1984; Landmesser and O'Donovan 1984). Calcium entry during bouts is necessary for the proper development of neuronal phenotype and for the regulation of neurite extension in spinal motor networks (Holliday and Spitzer 1990; Gu, Olson et al. 1994; Gu and Spitzer 1995).

The pyloric rhythm of the crustacean stomatogastric ganglion (STG) consists of a triphasic motor pattern with a canonical frequency of about 1 Hz (Harris-Warrick, Marder et al. 1992). The STG receives descending modulatory inputs that under normal conditions are crucial for the expression of the pyloric rhythm, and removal of these inputs results in either a decrease in frequency or complete loss of the pyloric rhythm (Russell and Hartline 1978; Russell 1979). However, if preparations are maintained in the absence of neuromodulatory inputs for days, the pyloric rhythm recovers (Thoby-Brisson and Simmers 1998; Golowasch, Casey et al. 1999; Thoby-Brisson and Simmers 2000; Mizrahi, Dickinson et al. 2001). In this paper we describe episodic bouts of activity, resembling bouts in developing motor systems, that accompany the recovery of the pyloric rhythm deprived of its modulatory environment, and consequently its activity (Thoby-Brisson and Simmers 1998; Golowasch, Casey et al. 1999; Thoby-Brisson and Simmers 2000; Mizrahi, Dickinson et al. 2001; Thoby-Brisson and Simmers 2002). We suggest that these bouts are a consequence of retuning of cellular and synaptic properties so that the absence of the currents normally evoked by the neuromodulatory inputs is compensated for by other changes in the network (Golowasch, Casey et al. 1999; Thoby-Brisson and Simmers 2002).

METHODS

Animals. All experiments were done using adult rock crabs, *Cancer borealis*, obtained from local seafood suppliers (Commercial lobster, Boston, Massachusetts).

Physiological saline. The physiological saline contained in mM: 440 NaCl, 11 KCl, 26 $MgCl_2$, 13 $CaCl_2$, 12 Trizma base and 5 maleic acid; pH 7.4-7.5.

Organ culture. The stomatogastric nervous system (STNS) (Fig. 1A) consisting of the single STG, two commissural ganglia (CoGs) and single esophageal ganglion (OG) was dissected and the STG was desheathed (Selverston and Moulins 1987). All dissection dishes were autoclaved and dissection tools were cleaned and soaked in 70% ethanol for at least ten minutes. All solutions were sterile filtered (0.2 μm, Nalge Nunc Int. Corp., Rochester, NY). The physiological saline used for the dissection contained 50 μg/ml streptomycin and 50 U/ml penicillin (Sigma, St. Louis, MO). After the dissection, and once a stable recording was obtained, the antibiotic concentration was lowered to 25 μg/ml streptomycin and 25 U/ml penicillin. Antibiotic concentrations were as follows: day 2, 25 μg/25 U streptomycin/penicillin per ml; day three, 33 μg/33 U streptomycin/penicillin per ml; additional days, 100 μg/ml gentamicin (Invitrogen Life Technologies, Carlsbad, CA). Preparations were maintained in a humidified low-temperature incubator at 12° to 13° C, and the saline was exchanged approximately every twelve hours.

Isolation of the STG from descending inputs. In most experiments the STG was isolated from descending inputs by transection of the stomatogastric nerve (stn). In some experiments isolation was accomplished with a Vaseline well containing isotonic sucrose (750mM) and 10^{-6} M tetrodotoxin (TTX). The stn was cut using iridectomy scissors where indicated in Figure 1A. In some cases a Vaseline well filled with isotonic sucrose containing 10^{-6} M TTX was placed on the stn to block action potential transmission through the stn prior to physically cutting the nerve. This procedure resulted in a more rapid loss of the pyloric rhythm than cutting alone.

Photoablation of neuromodulatory terminals. A Vaseline well was made around the stn as described above and filled with isotonic sucrose and 10^{-6} M TTX for ~20 min or until the rhythm began to stop. The sucrose/TTX was then replaced with 10^{-6} M TTX in water and the stn was cut . After several minutes dextran, tetramethylrhodamine 3000 MW (Molecular Probes, Eugene, OR), or lucifer yellow (Sigma Chemical, St. Louis, MO), was added directly to the well and the preparation was maintained overnight to allow the dye to fill the terminals (11-24 hrs). The following day the preparation was illuminated with a 100 watt mercury bulb for 25-45 min. During the initial stages of the photoablation process the preparations exhibited temporary resumption of the pyloric rhythm presumably due to injury discharge-induced modulator release from the dying terminals of the descending axons. After photoablation the preparation was returned to the incubator and recorded from overnight. To ensure that the terminals were ablated the stn was stimulated the next day with 2.5 Hz trains of 3 ms long voltage pulses at amplitudes substantially higher than normally needed to evoke modulator release (up to 100 V). None of the preparations responded to the stn stimulation, indicating that the modulatory terminals were destroyed by the photoablation.

Electrophysiological recordings. Extracellular recordings were performed using monopolar stainless steel electrodes placed into Vaseline wells made around the lateral ventricular nerve (lvn), pyloric dilator nerve (pdn) and pyloric nerve (pyn) (Fig 1A).

Recordings were made continuously throughout the culture period, except during solution exchanges. All electrode and ground leads were soaked for at least ten minutes in 70% ethanol. Signals were amplified using A-M systems 1700 differential amplifiers (Carlsborg, WA). Intracellular impalements were performed using 20-30 MΩ microelectrodes filled with 0.6 M K_2SO_4 and 20 mM KCl. An Axoclamp 2B amplifier (Axon Instruments, Union City, CA) was used in bridge mode to inject DC current into identified pyloric dilator (PD) neurons to alter the pyloric frequency. Signals were recorded to a computer hard drive using pClamp 8 software and a Digidata 1200A or a Digidata 1322A digitizer board (Axon Instruments, Union City, CA).

Data analysis and statistics. Bouts were readily identifiable by eye in extracellular recordings from the lvn and pdn as rapid increases in pyloric rhythm frequency. The rhythm frequency, bout durations and interbout intervals were measured from recordings from the pdn in Clampfit (pClamp 8 software, Axon Instruments,Union City, CA). Analysis of pyloric rhythm frequency and cellular phase relationships were performed using Spike 2 version 4 (Cambridge Electronic Design, Cambridge, England). Phase relationships were defined relative to the onset of the PD burst and were calculated with the following formula: Φ = (X-PDon)/cycle period, where X is the onset or end of the burst of each pyloric network neuron, PD, lateral pyloric (LP) or pyloric (PY) neurons, during the cycle period starting with that PD burst. Statistical analyses were performed using Sigma Stat software package (Jandel Scientific Software, San Rafael, CA). Data are reported as means ± SDs.

RESULTS

The triphasic pyloric rhythm is illustrated in the extracellular recording from the lvn in Figure 1B. The single LP neuron characteristically generates the largest amplitude action potential in such recordings. LP neuron activity is followed by discharge of several PY neurons (there are 5 PY neurons in *C. borealis* (Kilman and Marder 1996)). The PY neuron burst is terminated by discharge of the 2 PD neurons and the anterior burster neuron (AB) (an interneuron with its axon in the stn). Figure 1C shows block diagrams showing the phase of firing of each of the neurons. Together with the AB neuron the two PD cells form the pacemaker kernel for this circuit (Eisen and Marder 1982) and is, for this reason, used as reference for phase analysis. The timing of activity of the LP and PY neurons is set by their intrinsic membrane properties and the synaptic connections within the circuit (Harris-Warrick, Marder et al. 1992).

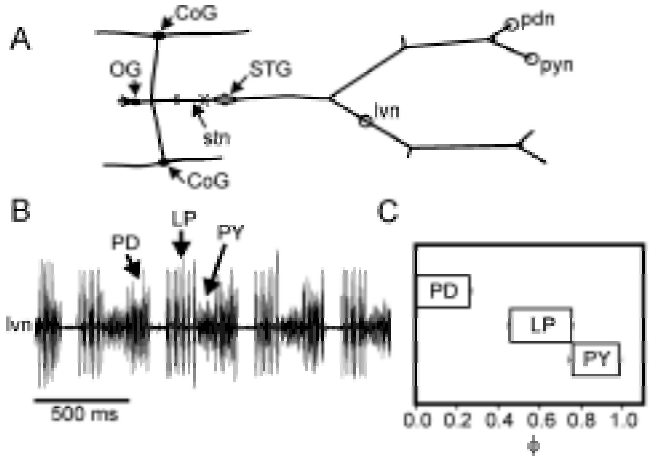

Figure 1. The pyloric rhythm is generated by alternating firing of three groups of neurons in the stomatogastric ganglion (STG), and can be recorded from descending motor nerves. A. Schematic drawing of the stomatogastric nervous system preparation including the paired commissural ganglia (CoG) and the single esophageal ganglion (OG), which give rise to descending neuromodulatory input to the STG through the stomatogastric nerve (stn). To remove neuromodulatory input the stn was cut where indicated by the X. Vaseline wells (circles) were used to make extracellular recordings from three motor nerves, the lateral ventricular nerve (lvn), the pyloric dilator nerve (pdn) and the pyloric nerve (pyn). B. An extracellular recording made from the lvn showing the alternating firing of the pyloric dilator (PD), the lateral pyloric (LP) and the pyloric (PY) neurons. C. A phase plot generated from the recording shown in panel B. Phase (Φ) was defined relative to PD burst onset, and shows the relative timing of bursts in each cell. Box on and end positions are determined with the mean ± SD of the Φ values

The pyloric rhythm ceases when modulatory inputs are removed, but then recovers.

It has been long known that descending inputs from the two CoGs and the single OG are crucial for maintaining a strong and robust pyloric rhythm (Russell 1976; Moulins and Cournil 1982; Selverston and Moulins 1987). In all species studied, acute removal of the influence of these descending inputs always results in a decrease in pyloric rhythm frequency, and often results in a complete cessation of the pyloric rhythm. Figure 2 shows an example of a preparation in which the pyloric rhythm stopped completely after impulse propagation in the stn was blocked. This figure shows the pyloric rhythm frequency plotted as a function of time on an extremely slow time base. At the start of the experiment the anterior modulatory inputs were intact, and the

preparation was rhythmically active at about 1.3 Hz. Shortly after the stn was blocked the rhythm slowed down (inset) and within 20 minutes of the block the rhythm completely stopped.

In this experiment the preparation remained completely silent until at hour 10 it produced a short bout of low frequency activity (Fig. 2). Between then and hour 18 the preparation produced more and more bouts of higher frequency, until by hour 18 a strong pyloric rhythm had recovered. As will be described below, not all preparations became completely silent after the descending inputs were removed. In this paper we include data from seventeen preparations recorded continuously for up to 7 days. In all of these preparations the frequency of the pyloric rhythm decreased to < 10% of control values in the first hours after removal of the modulatory inputs.

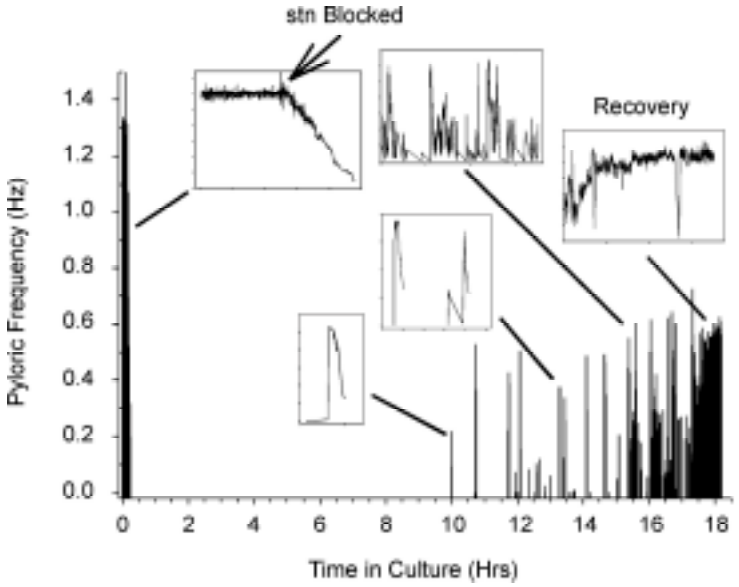

Figure 2. After transection of the stn the pyloric rhythm rapidly slowed, or stopped and was followed by episodic bouts of activity that preceded a period of stable recovery. A plot of instantaneous pyloric frequency over time in culture shows the rapid decrease of the rhythm frequency after blocking the stn (arrow). In this preparation bouts began to occur after about 10 hr in culture (insets). A period of stable frequency followed the bouting events beginning after approximately 18 hr in culture.

The recovery process shows episodic bouts of activity

Figure 3 shows raw data from a preparation that did not become entirely silent after removal of the modulatory inputs, and provides examples of activity patterns at 4 times during the experiment. The top panel shows a control pyloric rhythm with its characteristic LP, PY, PD alternating pattern. The second panel shows a much slower, but canonical pyloric rhythm seen during a bout that occurred 22 hrs after the stn was cut. The third panel shows that during the interbout interval there was spontaneous activity in the LP, PY and PD neurons, which was nevertheless still organized in the characteristic LP, PY, PD pyloric sequence. The fourth panel shows a strong pyloric rhythm recorded after 4.4 days.

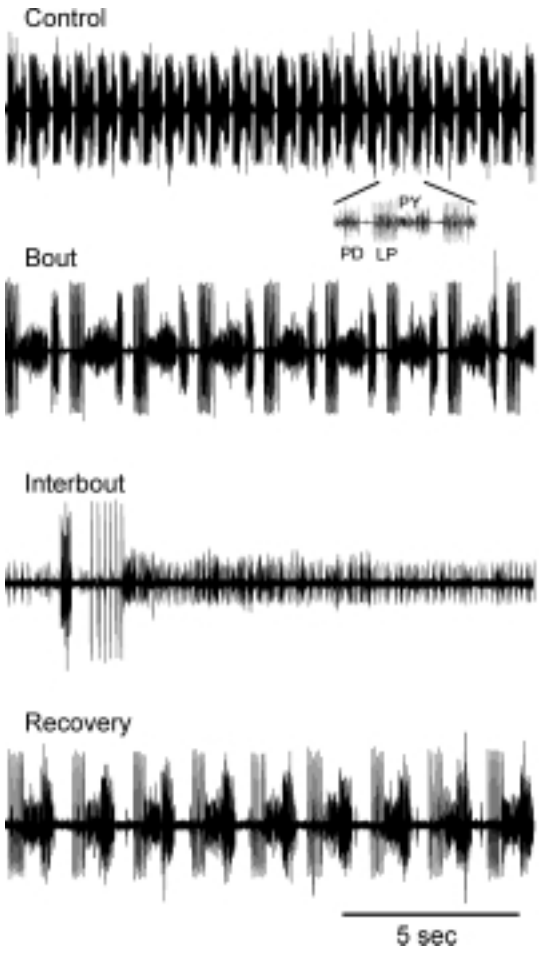

Figure 3. The triphasic pattern of cell bursting of the pyloric rhythm was altered after stn transection, but resumed during bouts and recovery. Recordings from the lvn show the firing of the PD, LP and PY neurons in control (see inset) and during recovery of the rhythm. A triphasic rhythm is seen in recordings from the same preparation during control, bout and during recovery. During interbout periods in this preparation, PD and LP fired periodically with long periods of tonic PY firing, leading to a very low frequency triphasic rhythm.

In 16/17 preparations bouts of activity began 3.16 ± 2.50 hrs after cut or block of the stn, in the other preparation bouting did not begin until 24 hrs after cutting the stn. Bouts occurred in every preparation examined in this study, and ended after 55.9 ± 33.9 hrs, (range: 9-100 hrs), when the rhythm recovered a stable firing pattern. Our criterion for recovery was a strong and stable pyloric rhythm that lasted for at least 5 hours. In 10 preparations the recovered rhythm was stable throughout recordings lasting 5 to 60 hours. In 7 preparations the recovered rhythm was stable for 6 to 50 hours and began to fail after that period.

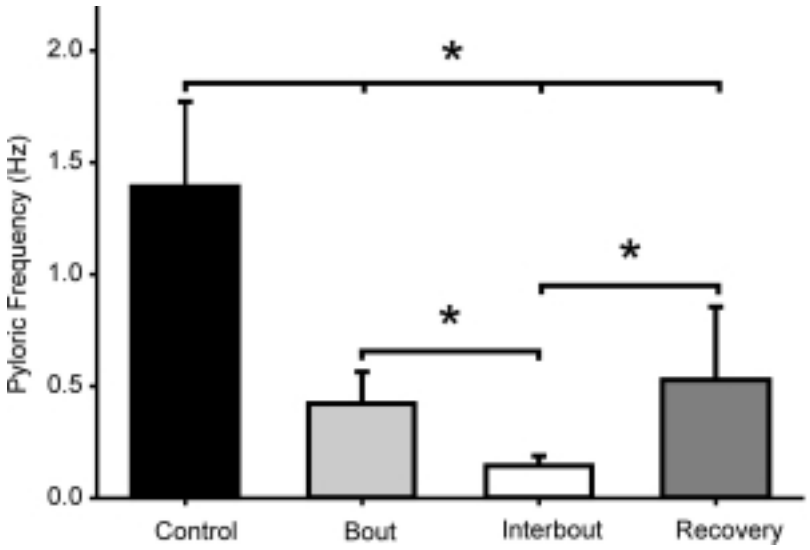

Figure 4. The pyloric rhythm frequency decreased after transection of the stn compared to control, and the frequency increased during bouting and the recovered states compared to interbout periods. Histogram plot of the mean frequency of the pyloric rhythm in control, bout, interbout and recovered states. The control frequency was significantly different from all three other states, but whereas the frequency during bout and recovery states differed from that seen during interbout periods, they did not differ from each other, (ANOVA on Ranks, followed by Dunn's Method; $P < 0.05$). n=17.

Frequency in control, bouts, and recovery

Figure 4 compares the mean pyloric frequency in control, bout, interbout, and recovery. The control pyloric rhythm frequency was 1.38 ± 0.4 Hz (n = 17). Pyloric frequency increased from a mean interbout frequency of 0.14 ± 0.05 Hz to a mean bout frequency of 0.42 ± 0.14 Hz The mean frequency during recovery, 0.53 ± 0.33 Hz, was significantly higher than that during interbouts, but no different from that during bouts. The control frequency was significantly higher than that observed during bout, interbout or recovery states, (ANOVA on Ranks, followed by Dunn's Method; $p < 0.05$).

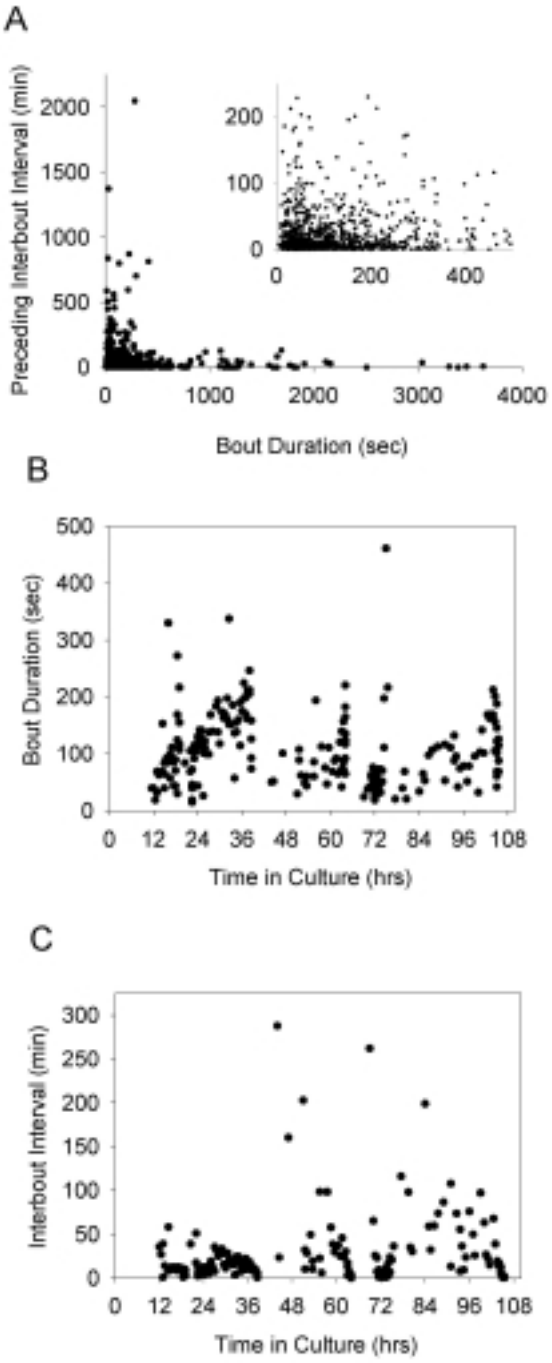

Figure 5. Bouts of activity did not occur with a clear temporal pattern. A. A plot of the bout duration versus the preceding interbout interval shows no obvious correlation between the two. Most bouts lasted less that 500 sec, and most interbout intervals were shorter than 250 min. This section of the plot is shown in detail (inset). B. Bout durations showed no temporal relationship during recovery. This preparation showed a relatively even distribution of bouts under 500 sec spread over the entire recovery period. C. Interbout intervals did not show a temporal relationship during recovery. This plot of interbout interval versus time in culture, made from the same preparation as in B, showed a relatively even distribution of intervals less than 300 min over the course of recovery.

Temporal pattern and time course of bouts

The pattern of bouting varied widely between preparations. The bout duration was not directly correlated to the preceding interbout interval (Fig. 5A) or the subsequent interbout interval (not shown). The total number of bouts varied from 0.16 to 2.35 bouts/hr across preparations (mean 0.96 ± 0.80 bouts/hr). Bout durations varied both within and between preparations and showed no correlation with the total number of events, and no trend over time. Mean bout durations varied from 38 to 883 seconds across preparations, and varied between 8 to 230 fold within preparations. Neither bout duration nor interbout interval showed a trend over time in culture (Fig. 5B,C). Some preparations exhibited a cluster of bouts at the beginning or ending of the culture period, (the preparation shown in Fig. 2 showed bouts clustered near the end of the culture period), while other preparations exhibited a relatively even distribution over time, as for the preparation in Fig. 5.

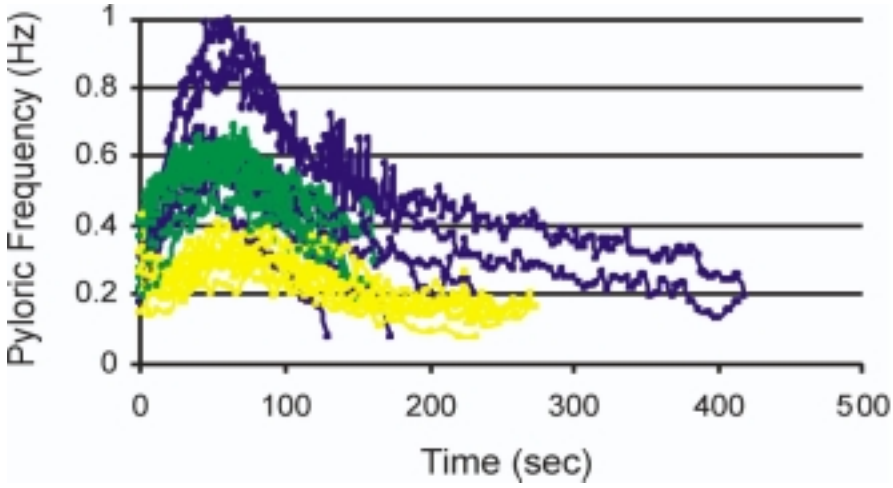

Figure 6. Individual bouts displayed a rapid rise to peak frequency followed by a slower decrease back to the interbout baseline frequency. Plots of instantaneous pyloric frequency versus time show that bouts rose to peak frequency more quickly than they decayed to baseline. Several individual bouts are plotted for each of three preparations (colored plots).

Although varying widely in duration, individual bouts typically showed a relatively fast increase in frequency followed by a slower decay back to the interbout frequency. Figure 6 shows the pyloric frequency profiles of individual bouts from three different experiments. These experiments and bouts were chosen randomly, but represent the gamut of data seen across preparations. In all bouts the rate of frequency increase was more rapid than its decay, and the peak frequency range displayed during the bout varied from 2 fold to almost 5 fold. This phenomenon was observed in all preparations; however the rates of rise and decay of individual bouts varied between and within preparations.

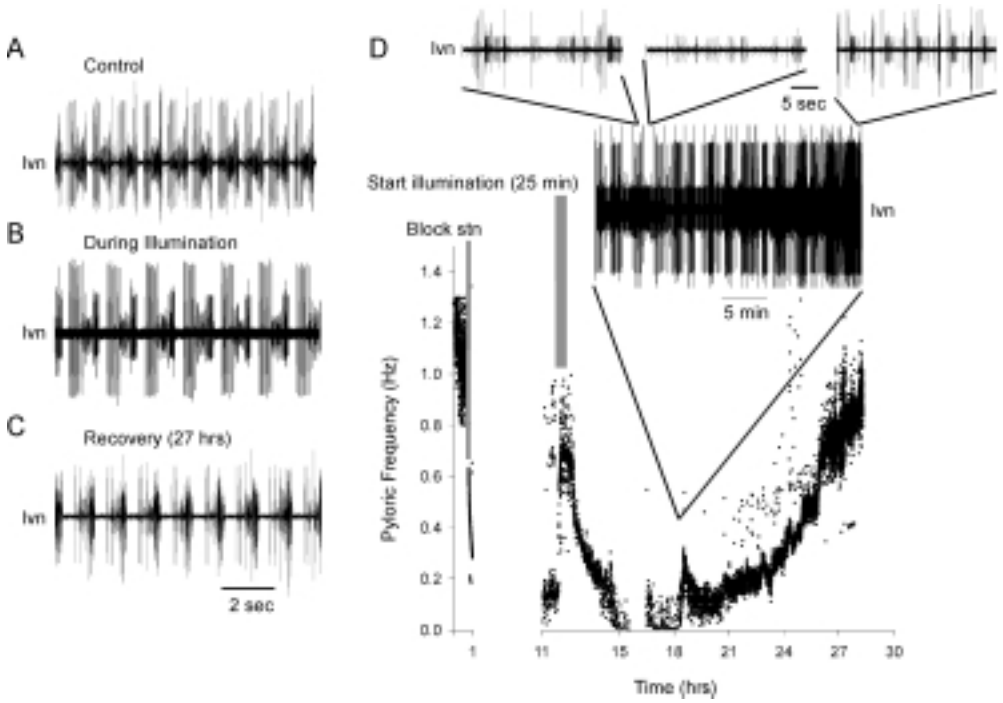

Figure 7. Photoablation of neuromodulatory terminals in the STG neuropil did not stop bouting or recovery. A. A trace showing the triphasic pyloric rhythm output recorded extracellularly from the lvn under control conditions with the stn intact. The stn was subsequently cut in this preparation and the neuromodulatory terminals were back-filled overnight with Rhodamine-dextran beads. B. During illumination, damage-related release of neuromodulators caused a resumption of the pyloric rhythm as seen in this extracellular recording from the lvn. C. After 27 hrs in culture this preparation regained a triphasic pyloric rhythm output, but at a lower frequency than control. D. A plot of instantaneous pyloric frequency versus time in culture shows that after blocking and cutting the stn, (gray bar, left), the pyloric rhythm rapidly stopped. The neuromodulatory terminals were back-filled in this preparation for 11 hrs. Upon illumination, (gray bar, right), the pyloric output regained a typical triphasic pattern and increased in frequency due to damage-related neuromodulator release. Within 3 hrs after photoablation this preparation exhibited bouts of activity, (shown in expanded extracellular recordings from the lvn), and subsequently recovered a stable triphasic pyloric output.

Photoablation of neuromodulatory terminals had no effect on bouting.

Because transected axon terminals can be slow to degenerate in crustacean species (Royer 1987; Bittner 1991; Parnas, Dudel et al. 1991; Parnas, Shahrabany-Baranes et al. 1998) it is conceivable that pyloric activity bouts occur due to intermittent release of neuromodulators from axon terminal stumps in the STG neuropil. To determine if this was the case, we back-filled neuromodulatory axon terminals overnight with rhodamine-conjugated dextran beads or Lucifer yellow, and illuminated the STG the next day to photoablate the terminals (Miller and Selverston 1979). We then recorded from the preparations overnight to determine if they bouted and recovered. Figure 7 shows extracellular recordings from the lvn at different times during one of these experiments. The graph shows pyloric frequency over time in organ culture. This preparation was illuminated for 25 min after 12 hrs in culture when the STG neuropil was clearly dye-filled. Illumination resulted in the damage-related release of neuromodulators from terminals in the STG neuropil, and consequently, an increased pyloric frequency and resumption of a typical triphasic output. After illumination this

preparation became briefly silent around hour 16 and subsequently bouted for several hours and followed by recovery. Similar results were seen in 6 preparations. High voltage stimulation of the stn stump with 2.5 Hz trains of 3 ms pulses had no effect on the recovered rhythm in any preparation, suggesting that the terminals were no longer active. This stimulation protocol evoked modulator release in both stn-intact and in recovering preparations that had not undergone photoablation (not shown).

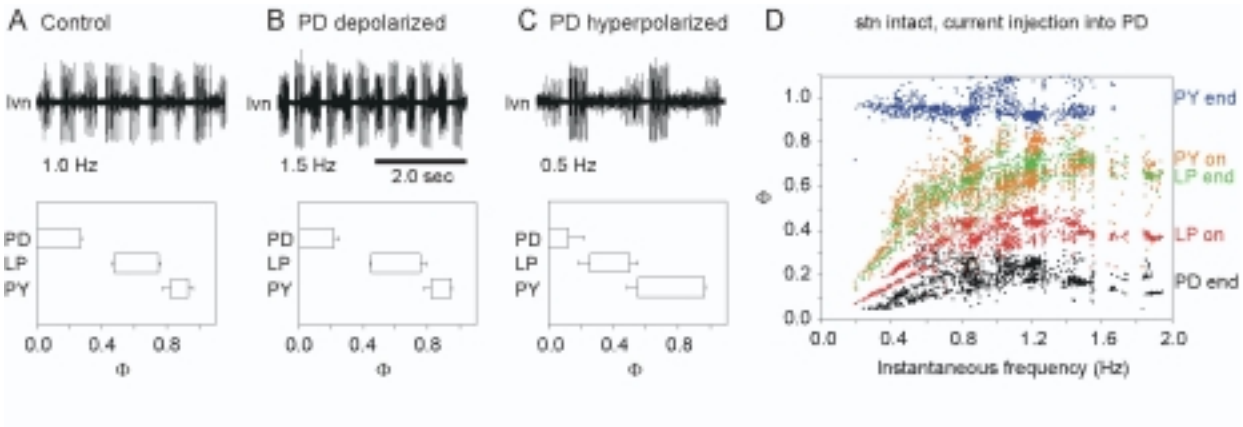

Figure 8. Phase relationships for bursting in PD, LP and PY neurons are dependent on pyloric cycle frequency in intact STNS preparations. A. Bursts of action potentials generated by PD, LP and PY occur with a stereotypical frequency (~ 1 Hz) and phase relationship (Φ) under control conditions, shown in the extracellular recording from the lvn (top) and in the phase plot (bottom). B. By depolarizing a PD neuron with intracellular current injection the pyloric frequency can be increased (top). Phase relationships remain similar to control (bottom). C. Hyperpolarizing a PD neuron decreases pyloric frequency (top), and results in an advance of phase relationship values (bottom). D. A plot of phase values versus a wide range of cycle frequencies were generated by injecting hyperpolarizing or depolarizing current into a PD neuron and recording extracellularly from pyloric nerves (n = 7). Phase values for PDend, LPon, LPend and PYon were strongly frequency dependent below 0.8 Hz. Phase was analyzed for 200 blindly chosen cycles from each preparation

Dependence of phase on neuromodulatory input.

In addition to the burst frequency, the phase relationships and duty cycles of the pyloric network neurons are used to characterize the motor patterns of the network. As the phase relationships of network neurons depend on a variety of synaptic and intrinsic properties (Hartline and Gassie 1979; Eisen and Marder 1984; Harris-Warrick, Coniglio et al. 1995; Harris-Warrick, Coniglio et al. 1995), these can provide indications of the state of the processes that underlie network dynamics. However, the firing phase and duty cycles of pyloric network neurons also vary as a function of neuromodulatory condition (Eisen and Marder 1984; Hooper and Marder 1987; Harris-Warrick, Coniglio et al. 1995) and previous work on the lobster, *Panulirus interruptus*, showed that some of the pyloric network phase relationships are frequency dependent (Hooper 1997; Hooper 1997). To compare phase relationships between control, bouting, interbout and recovered states, the changes in frequency in these states had to be taken into account. Therefore, we first examined the effect of frequency on the onset and end phase of the LP and PY neuron firing and the end phase of PD in the control condition, by injecting current into one of the two PD neurons. Figure 8A-C shows raw data and phase plots of the pyloric

rhythm in a preparation in which the frequency was altered over a range from 0.5 to 1.5 Hz by current injection into a PD neuron. This individual example shows that at the higher frequencies there was little change in phase, but when the preparation was slowed, the phase of firing of the LP and PY neurons was altered. The pooled data from 7 preparations (Fig. 8D) shows that at frequencies below 0.8 Hz the PD neuron terminated its burst at an earlier phase, and the LP neuron (on and end) and the PY neuron onset phases were also advanced. The PY termination (PYend) did not show this frequency dependence because the PY neurons tend to stay active until being inhibited by the next PD burst. Thus PYend phase is always close to 1.

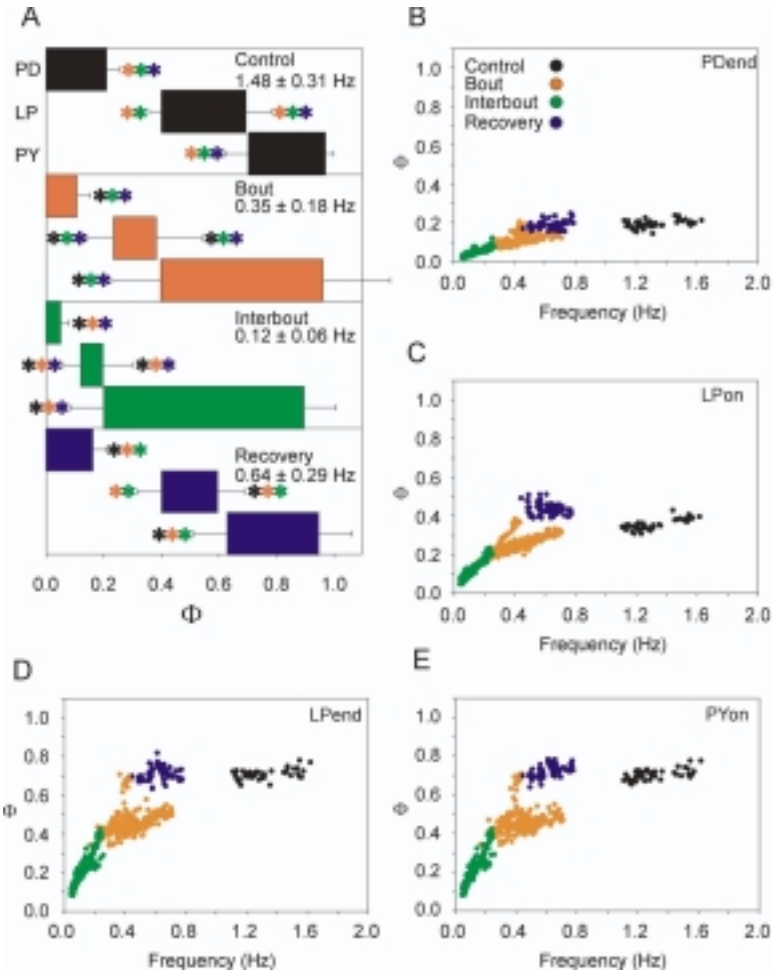

Figure 9. The phase relationships of bursting in PD, LP and PY are smaller during bouts and interbouts as compared to control and recovery. A. Phase plots are shown from eleven preparations with unambiguously analyzable data in each state: control (black), bouting (orange), interbout (green) and recovered (blue). Phase relationships were significantly decreased in bout and interbout states as compared to control, then phase recovered toward control values. Data were compared using Kruskal-Wallis ANOVA on Ranks followed by Dunn's Method; * $P < 0.05$. B.-E. Plots of phase relationships versus instantaneous pyloric frequency from a single preparation. In each case, (PDend in panel B, LPon in panel C, LPend in panel D and PYon in panel E), both phase and frequency was decreased in bouting (orange circles) and interbout (green circles) states as compared to control (black). However, in the recovered state (blue circles), phase recovered to near control values while the frequency remained similar to values observed during bouts.

Figure 9 provides comparisons of the phase relationships of the PD, LP, and PY neurons in control (stn intact), bouting, interbout and recovered states. Figure 9A shows the mean phase relationships for eleven preparations that showed clear and unambiguously analyzable pyloric rhythms in each state. The phase of PDend, LPon, LPend and PYon were significantly decreased during bout and interbout states compared to control, and increased to near control values during recovery, despite the fact that frequency recovered to only 43% of control (0.64 vs. 1.48 Hz). Figure 9B-E shows plots of phase versus instantaneous pyloric frequency for an individual preparation for control, bout, interbout and recovered states (colored circles). The phase relationships for control (black) and recovered (blue) states are similar despite having different frequency ranges, whereas bout (orange) and recovered (blue) have different phase relationships, but have overlapping frequency ranges. This suggests that the delay in phase seen in pyloric rhythm recovery is not only a consequence of changes in bursting frequency.

To separate better the change in phase during recovery from the confound arising from the fact that phase is frequency dependent, we compared phase relationships for PDend, LPon, LPend and PYon in seven preparations that displayed overlapping frequencies in bouting and recovered states. For each preparation, data were selected from a 0.1 Hz bin that showed overlap between pyloric frequency in bouts and during recovery such that the frequencies were not statistically different. There was not sufficient overlap in frequency to include data from control or interbout intervals. Figure 10A-D shows histogram plots of phase values for each of the seven preparations, demonstrating that for LPend, LPon and PYon, at the same frequency, phase was delayed during recovery relative to that during bouts. In each preparation this reached statistical significance ($P < 0.001$), except in one case for LPend (Fig. 10C). Mean values for each neuronal phase relationship were: PDend, 0.12 ± 0.01 Hz vs. 0.14 ± 0.02 Hz; LPon 0.28 ± 0.02 Hz vs. 0.37 ± 0.03 Hz; LPend 0.45 ± 0.05 Hz vs. 0.56 ± 0.05 Hz; PYon 0.48 ± 0.04 Hz vs. 0.60 ± 0.04 Hz, for bout vs. recovered, respectively. Interestingly, the phase relationship for PDend did not differ greatly between bout and recovery in most preparations, which suggests that the differences seen in phase for the other cells are not simply due to changes in frequency.

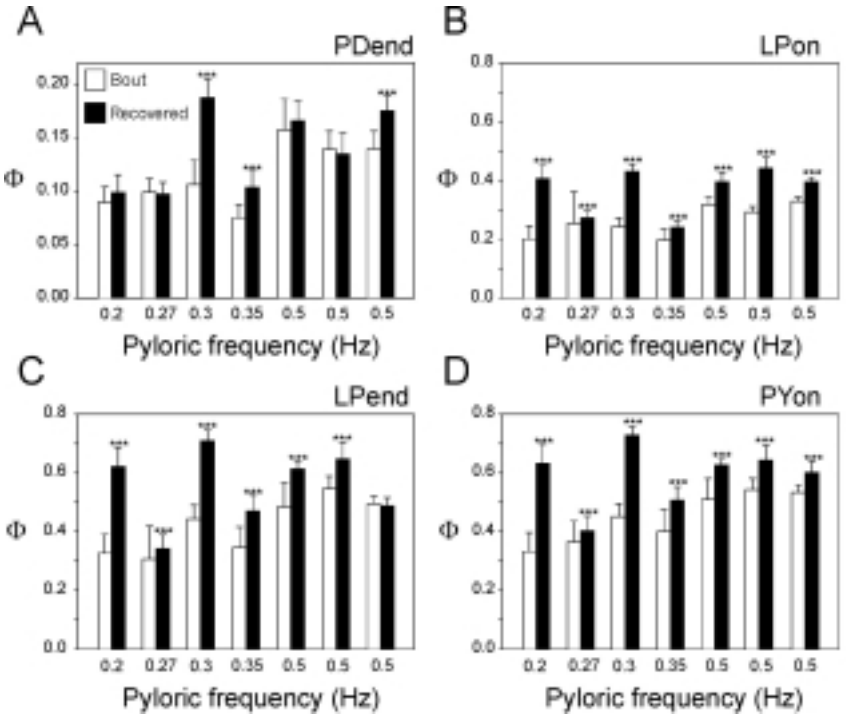

Figure 10. Neuronal phase relationships differ between recovery and during activity bouts at a given pyloric frequency. Frequency-controlled data from bouting and recovered states were compared for 7 preparations. A. A histogram plot of phase (Φ) for PDend versus frequency for the seven preparations. Open bars represent bouting and filled bars represent the recovered states. In four preparations PDend was similar, but in three preparations PDend was significantly larger in recovery than during bouts. B-D. Histogram plots of phase versus frequency for LPon, LPend and PYon for the same preparations as in A. In every case except one, (see 10C), phase values were larger after recovery than during bouts (Data were compared using either Student's t-test for normally distributed data sets or the Mann-Whitney rank sum test for non-normally distributed data; ***, $P < 0.001$).

Blockade of glutamatergic synapses in the STG does not prevent bouting or recovery.

An alteration of the pyloric network that results in compensation for loss of neuromodulation and resumption of output could arise through changes in the intrinsic properties of individual neurons and/or changes in synaptic properties of the pyloric network. Glutamate is the principal transmitter at many synapses between STG neurons (Eisen and Marder 1982). We applied 10^{-5} M picrotoxin (PTX) to block graded and spike-mediated glutamatergic synaptic transmission among STG neurons (Eisen and Marder 1982) (Bidaut 1980). The PTX was applied shortly before transection of the stn and allowed to remain until the preparation recovered. Six out of nine preparations bouted and recovered in PTX. In the presence of PTX the pyloric cellular phase relationships were altered in stn-intact and recovered preparations, but they returned towards control when the drug was washed off.

DISCUSSION

Adult central pattern generating networks must maintain the ability to produce stable neuronal outputs over long time periods, in some cases over many years. Nonetheless, they must also be responsive to the animal's behavioral needs in the short-term. Sensory and neuromodulatory control systems enable the animal to adapt the output of its circuits to the specific behavioral context in which the animal is found. At the same time there must be a stable "baseline state" to which central pattern generating networks return after short-term perturbations. In this study we use the pyloric rhythm of the adult crab as a model system in which to study the return to a "baseline state" in response to long-term removal of descending neuromodulatory inputs.

Adult crabs live many years, and the animals that we used in this study were several years old. *In vivo* recordings from behaving crabs showed ongoing pyloric rhythms in the 0.2-0.4 Hz frequency range in unfed animals, while rhythms of 1.0-1.5 Hz were routinely seen subsequent to feeding (R. Zarum and E. Marder, unpublished results). Thus, in the behaving animal the unfed "baseline state" has frequencies similar to those that we obtain subsequent to recovery after removal of descending modulatory inputs (Golowasch, Casey et al. 1999).

Bout properties. Bouts of activity occurred in all preparations that showed recovery in this study, but the temporal pattern of bouting and overall number of bouts was highly variable between and within preparations. Although bouts had not been previously reported in decentralized STG preparations (Thoby-Brisson and Simmers 1998; Golowasch, Casey et al. 1999; Mizrahi, Dickinson et al. 2001), in the earlier studies recordings were not made continuously and the low frequency of bouting events, (on average, one ~3 min bout per hour), would make them easily overlooked without continuous recordings.

The episodic bouts of activity characterized in this study in some ways resemble spontaneous activity bouts seen in developing neural networks in many systems (Katz and Shatz 1996; O'Donovan 1999) (Kamioka, Maeda et al. 1996) (Murphy, Blatter et al. 1992) (Ben-Ari 2001). Similar spontaneous bursts are also observed in epileptiform bursting in adult hippocampal CA3 networks (Staley, Longacher et al. 1998). Bouting in these other systems is thought to be important for guiding the appropriate construction of the developing networks (Katz and Shatz 1996; O'Donovan 1999) (Ben-Ari 2001). An attractive possibility is that bouting in the recovering pyloric network represents a similar underlying mechanism that guides the restructuring of the pyloric network to allow a physiologically meaningful output. Nonetheless, there are some important differences between bouts seen in developing motor systems and the bouts we see in the recovering pyloric network. There does not seem to be a refractory period or activity-dependent depression following bouts in the pyloric network because we observed no correlation between bout duration and the preceding or subsequent interbout interval. In contrast, a refractory period is thought to be a common factor contributing to episodic bouts in a variety of developing neural networks (O'Donovan 1999). Additionally, bouting was not tightly correlated to percentage recovery of phase or frequency of the pyloric rhythm. The overall number of bouting events, number of bouts per hour, overall time spent in a bouting state or average bout duration in a given preparation was not correlated to the time to recovery or the percentage recovery of phase or frequency in our study (data not shown). This is in contrast to developmental changes in the delayed rectifier potassium

current kinetics seen in amphibian spinal neurons, which are dependent on spontaneous activity bouts in a particular frequency range (Gu and Spitzer 1995). In the future it will be important to determine if bouting is strictly a necessary process that regulates pyloric rhythm recovery or if it is an epiphenomenon that occurs due to another underlying homeostatic process.

What processes might cause the bouting events that we have observed? In developing retina and spinal cord it is believed that spontaneous bouts of activity are caused by excitatory GABAergic, glutamatergic or cholinergic synaptic contacts through network-dependent mechanisms, while the duration of the interbout is determined by the recovery from synaptic depression or depletion (Feller, Wellis et al. 1996; Chub and O'Donovan 1998; Feller 1999). As all chemical synapses among the pyloric network neurons are inhibitory it is possible that an endogenous burst in any pyloric network neuron could trigger a bout by a postinhibitory rebound-like mechanism. This seems unlikely, because we observed that blockade of glutamatergic synaptic transmission between STG neurons did not block bouting or recovery. Additionally, we saw no evidence for a refractory period following bouts. We showed that both the recovery process and bouting persist after descending modulatory inputs were killed. This indicates that activity in the presynaptic terminals of the descending modulatory neurons is not solely responsible for the production of bouts. The recovery process subsequent to loss of activity and neuromodulation seems likely to involve changes in the intrinsic properties of neurons (Golowasch, Casey et al. 1999; Mizrahi, Dickinson et al. 2001) as individual neurons and synapses retune themselves in response to their altered environment and level of activity (Golowasch, Abbott et al. 1999; Soto-Trevino, Thoroughman et al. 2001). Thoby-Brisson and Simmers (2002) reported that in the lobster, *Jasus lalandii*, the delayed rectifier and calcium-dependent potassium currents were downregulated and the hyperpolarization-activated cation current was upregulated in PD neurons following rhythm recovery. This alteration of membrane currents favors endogenous, regenerative bursting behavior, which PD neurons do not posses immediately after stn transection. These data support our hypothesis that recovery of pyloric activity is due to changes in the intrinsic properties of neurons. They also found that chemical synapse efficacy was generally decreased between pyloric neurons after recovery. If channel densities are functionally linked to neuronal activity, as suggested theoretically (LeMasson, Marder et al. 1993; Liu, Golowasch et al. 1998) it can be imagined that activity bouts arise as channel densities are altered. An incremental change in one or more membrane conductance may allow a temporary resumption of the pyloric rhythm, but further changes in membrane conductances could result in the network falling silent again. The rhythm could switch on and off in this manner until the processes that link activity and membrane conductances reach equilibrium. If this were true then the pattern of bouting over time and the time to recovery will depend on the initial conditions of the network elements. This is likely to be different for each preparation, explaining the variability we see in the recovery process.

We are not aware of other descriptions of spontaneous bouts of activity that accompany recovery of output in an adult neural network after disruption of input that results in altered activity patterns. Epileptiform bursting in adult CA3 hippocampal networks shares certain features of spontaneous bouting activity in developing networks

(Staley, Longacher et al. 1998), but lacks the homeostatic nature of bouting described here.

Crabs must benefit greatly from having a high degree of stability in a system that is essential to life such as the pyloric network. Indeed, a highly plastic or unstable feeding pattern generating network would be seriously detrimental to any animal. The processes that maintain a stable pyloric network may be common to many rhythmic networks, including essential systems such as those that control breathing, circulatory or digestive processes. The mechanisms that stabilize the pyloric network seem to be highly versatile, as they can compensate for a change as dramatic as complete removal of all neuromodulatory input. Understanding these processes in the pyloric network will help elucidate mechanisms that maintain stability in neural networks in general during learning, physical growth, development and in disease.

Acknowledgments: We thank Dr. William Miller for help with data analysis in the early stages of this work and Dirk Bucher, Ph. D. for invaluable help with Spike 2 analysis. We especially thank Dr. Farzan Nadim for his generous support of JG, JY and CR during part of this project. Research supported by R01 MH 64711 (JG), R37 MH 46742 (EM), T32 NS07292 (JL) and an undergraduate summer IGERT 9972756 NSF (AR).

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