Restoration of descending inputs fails to rescue activity following deafferentation of a motor network

Jebun Nahar,* Kawasaki M. Lett,* and David J. Schulz
Department of Biological Sciences, University of Missouri, Columbia, Missouri

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Nahar J, Lett KM, Schulz DJ. Restoration of descending inputs fails to rescue activity following deafferentation of a motor network. J Neurophysiol 108: 871–881, 2012. First published May 2, 2012; doi:10.1152/jn.00183.2012.—Motor networks such as the pyloric network of the stomatogastric ganglion often require descending neuromodulatory inputs to initiate, regulate, and modulate their activity and their synaptic connectivity to manifest physiologically appropriate output. Prolonged removal of these descending inputs often results in a compensatory response that alters the inputs themselves, their targets, or both. Using the pyloric network of the crab, Cancer borealis, we investigated whether isolation of motor networks would result in alterations that change the responses of these networks to restored modulatory input. We used a reversible block with isotonic sucrose to transiently alter descending inputs into the pyloric network of the crab stomatogastric ganglion. Using this method, we found that blocking neuromodulatory inputs caused a reduced ability for subsequently restored modulatory projections to appropriately generate network output. Our results suggest that this could be due to changes in activity of descending projection neurons as well as changes in sensitivity to neuromodulators of the target neurons that develop over the time course of the blockade. These findings suggest that although homeostatic plasticity may play a critical role in recovery of functional output in a deafferented motor network, the results of these compensatory changes may alter the network such that restored inputs no longer function appropriately.

neuromodulation; homeostatic plasticity; stomatogastric ganglion; deafferentation

MOTOR NETWORKS OFTEN CONSIST OF central pattern generator circuits (CPGs) that endogenously encode network output features as a result of the intrinsic properties of the constituent neurons as well as their pattern of synaptic connectivity (Grillner 2006; Selverston 2010). Moreover, the ability to generate this endogenous activity often requires both excitatory drive as well as neuromodulatory inputs that tune synaptic and intrinsic properties of the constituent neurons to enable pattern generation (Harris-Warrick 2011), such that loss of these inputs often results in a loss of function, even though the motor networks themselves remain intact.

More recently, a substantial amount of plasticity in this dependence on descending drive and motor output has been observed. For example, in the crustacean stomatogastric ganglion (STG), the pyloric motor network is a CPG that is dependent on ascending neuromodulatory inputs to produce a rhythmic motor output (Moulins and Cournil 1982). Yet although the pyloric motor output ceases soon after the modulatory inputs are removed, over a period of 3–7 days (depending on species studied) the pyloric rhythm can recover its functional output in the absence of all descending neuromodulation (Golowasch et al. 1999; Thoby-Brisson and Simmers 1998, 2002). These results suggest that in the absence of the neuromodulatory inputs that produce ongoing activity, the intrinsic motor circuits “retune” themselves so that they are able to produce rhythmic activity by input-independent mechanisms. These mechanisms in the STG include at least in part altering membrane conductances and ion channel expression patterns of individual neurons (Khorkova and Golowasch 2007; Mizrahi et al. 2001; Thoby-Brisson and Simmers 2002). This kind of functional recovery following removal of command inputs is not unique to the STG. Similar phenomena have been reported in recovery of stepping function in spinal cord transected cats (Barbeau and Rossignol 1987; Rossignol et al. 2004a,b), as well as recovery of function in the vestibular system following deafferentation (Darlington and Smith 2000).

Although some properties of these networks have been studied subsequent to recovery, little is known about the functional state of these networks during the process of recovery. In the pyloric network of the STG, loss of neuromodulation is sufficient to change relationships in individual neurons at both the level of ionic conductances and expression of ion channel mRNA prior to recovery taking place, detectable even after 24 h (Khorkova and Golowasch 2007; Temporal et al. 2012). Yet it is unclear whether these changes prior to recovery have an immediate impact on the ability of the network to generate neuromodulation-induced activity. Our focus in this study is to better understand whether the changes that are initiated as a result of removal of modulatory inputs influence the ability of the system to respond appropriately if descending inputs are ultimately restored. This has particular implication for nerve injury and disease, because if potentially compensatory changes are initiated in networks deprived of afferent input, then the effects of restoring input may be unpredictable. In this study, we investigate the impact of removal and restoration of descending inputs on functional output of the pyloric network of the STG over a 3- to 4-day time frame of deafferentation. Recovery of rhythmic activity in the STG can occur over a time course between 1 and 7 days (Luther et al. 2003; Mizrahi et al. 2001), although the most common that we have observed is on the order of 3–5 days, similar to other reports in the literature (Thoby-Brisson and Simmers 1998, 2002). The time frame of our experiments therefore likely coincides with the initiation of cellular mechanisms involved in the recovery process. We then determine whether changes in the output of
these restored networks result from changes in presynaptic inputs, target neurons of the pyloric network, or both.

MATERIALS AND METHODS

Animals and the stomatogastric preparation. Adult crabs, Cancer borealis, were obtained from The Fresh Lobster Company (Gloucester, MA) and maintained in artificial seawater at 12°C until used. Crabs were anesthetized by keeping them on ice for 30 min before dissection. The complete stomatogastric nervous system (STNS) was dissected out of the animal and pinned out in a Sylgard-coated (Dow Corning) dish containing chilled (12–13°C) physiological saline.

The stomatogastric nervous system consists of the STG motor networks and their associated inputs (Fig. 1). The output of the pyloric network of the STG can be most directly monitored via projections of motor neurons through two nerve types, the paired lvn and mvens. The STG receives central inputs by only a single nerve, the stomatogastric nerve (stn), connecting the STG with the rest of the crustacean central nervous system (Fig. 1), including in this preparation the paired commissural ganglia (CoGs) and the oesophageal ganglion (OG). The stn of the crab, Cancer borealis, contains approximately 60 large axons, as well as a bundle of smaller fibers (Coleman et al. 1992). Of the 60 large fibers, about 40 descend from the paired CoGs, 10 from the OG, and 10 represent ascending interneuron axons from the STG (Coleman et al. 1992; Goldberg et al. 1988). These stn inputs are responsible for initiating, maintaining, and altering the activity of the pyloric network (Stein 2009).

Solutions. The physiological saline solution consisted of the following (in mM): 440 NaCl, 11 KCl, 13 CaCl₂, 26 MgCl₂, and 10 HEPES buffer, pH 7.45. In most experiments, the stomatogastric nerve (stn; see Fig. 1) was blocked with a solution composed of isotonic sucrose (750 mM). In some experiments, neuromodulators were superfused to the STG to determine their effects on preparations that previously had stn activity blocked with sucrose. These modulators include octopamine (Sigma Chemical, St. Louis, MO), pilocarpine (Acros Organics, Geel, Belgium), and proctolin (American Peptide Company, Sunnyvale, CA). Unless otherwise specified, chemicals were obtained from Fisher Chemical. When the STG was superfused with modulators, the stn was additionally blocked with 10⁻⁵ M tetrodotoxin (Alomone Labs, Jerusalem, Israel) in keeping with common practice in STG preparations, as insurance that no propagation of signals up and/or down the stn could occur.

Electrophysiology. For electrophysiological recordings, petroleum jelly wells were placed on the nerves from which recordings would be made (lvn, lateral ventricular nerve; mvn, medial ventricular nerve; dgn, dorsal gastric nerve; stn, stomatogastric nerve; son, superior oesophageal nerve; ion, inferior oesophageal nerve). Extracellular recordings from the nerves were made by placing stainless steel pin electrodes in the wells. Signals were amplified and filtered using a differential AC amplifier (A-M Systems, Sequim, WA). Throughout the experiments, both during recordings and incubation times between the preparations, were maintained in chilled (12–13°C) physiological saline. During incubation periods, penicillin and streptomycin (Sigma) were added to the saline to prevent bacterial infection of the culture (50 U/mL: penicillin and 50 μg/mL streptomycin). Data were acquired using a Digidata 1322 data acquisition board (Axon Instruments, Sunnyvale, CA). In some preparations, the descending projection neurons were stimulated extracellularly via the stomatogastric nerve (10 Hz, 90 s). The stn was stimulated using a Grass S88 stimulator (Grass Instruments, West Warwick, RI). Extracellular nerve stimulation was achieved by placing the wires used to record nerve activity into a stimulus isolation unit (Grass Instruments) that was connected to an S88 stimulator.

The pyloric rhythm was monitored by recording the activities of the lateral pyloric (LP), pyloric constrictor (PY), pyloric dilator (PD), ventricular dilator (VD), and inferior cardiac (IC) neurons on the lvn and mvn (Fig. 1). Pyloric burst frequencies were derived from the cycle period as determined by the time between two consecutive bursts of PD neurons. The cycle period of the VD neuron was also
determined as the time between VD bursts measured on the mvn. We also used the spiking activity of the anterior gastric receptor (AGR) neuron to monitor the propagation ability of the stn across a time course of pharmacologic blockade. AGR is a sensory neuron that has a cell body in close proximity to the STG and projects axon collaterals both centrally, through the stn to both CoGs (see Fig. 1, inset), and peripherally, through the dgn toward the stomach musculature. AGR action potentials can be monitored often as the largest spike on the stn, and can also be measured from the paired sons and the dgn (Smarandache and Stein 2007). Multisweep recordings (Spike2 v6.0, Cambridge Electronic Design, Cambridge, UK) were used to detect AGR spikes at all of these recording sites. If AGR was detectable on the dgn, stn (both in the blocking well and the other; see Fig. 1), and the son after removal of the sucore blockade, the block was considered as likely not having damaged the stn signaling capability. Any preparations for which we could not use AGR to confirm the propagation ability of the stn were omitted. Finally, we measured the overall spiking frequency of the units contained within the sons. For son recordings, we measured the instantaneous firing frequency. However, in some of our recordings, bursting activity of the esophageal OD1 neuron was detected on the son (Nagy et al. 1981). When bursting was present, we used average firing frequency of the son spikes between bursts of OD1 for a minimum of 10 interburst intervals as the data for son spiking frequency.

Data analysis. Pyloric activity was measured as the average burst frequency of consecutive cycles for 10 min in which the range of values did not change visibly and were assumed to represent the steady state. Spiking activity of the AGR and son were measured as instantaneous frequency or average firing frequency of a section of the recording (duratons ranged from 30 to 240 s). For experiments where the block was removed and pyloric frequency measured (seen in Figs. 3 and 4), relative frequency was plotted as the ratio (for a given preparation) of the frequency at time x/control frequency. We chose to express the data this way, when possible, to ensure that differences across groups after restored modulation were not simply due to differences in starting frequency (Spitzer et al. 2008). Differences in mean firing rates or bursting frequencies were compared using unpaired t-tests. Correlations were analyzed using Pearson’s Product Moment tests. All statistical tests were performed using SigmaPlot v11.0 (Systat Software, Inc., San Jose, CA).

RESULTS

We used a simple blocking regime with isotonic sucrose to examine reversible neuromodulatory blockade effects on the output of the pyloric rhythm. Our data show that sucrose blockade virtually eliminates all pyloric activity on both the lvn (LP, PY, and PD activity) and the mvn (VD and IC activity) usually within 1 h of application (Fig. 2). Simultaneous recorded activity made from the blocking well (stn-a) and posterior to the blocking well (stn-b) show a dissociation of spiking activity with the sucrose block in place (Fig. 2, bottom traces). stn-a recordings with the sucrose block in place show activity for units that largely originate above the block and descend through the stn, whereas stn-b recordings show units that largely originate below the block that ascend the stn. Multisweep analyses of AGR before and after the placement of the block show the inability of AGR spiking to propagate past the sucrose block and into the son; AGR spiking is absent on the son when the sucrose block is in place (Fig. 3C). Taken together, these data indicate that isotonic sucrose is sufficient to block propagation of action potentials through the stn, likely resulting in a loss of modulatory drive to the STG during the course of the blockade.

Our hypothesis was that removal of descending neuromodulatory inputs to the STG, and the subsequent change in activity, would alter the properties of the STG neurons, or the descending inputs, or both and result in impaired ability of the descending projections to appropriately drive rhythmic activity following subsequent reconnection of the modulatory drive. We tested this hypothesis using the reversible sucrose block-
Fig. 3. Effects of stn blockade over time on the ability of restored connectivity (when the block is removed) to generate pyloric activity. A: representative recordings of stn (ascending and descending projections) and lvn (pyloric activity) for two preparations over the course of 4 days in culture. All recordings shown have the stn unblocked. CONTROL preparations were maintained in physiological saline for the entirety of the experiment. SUCROSE preparations had the stn blocked with isotonic sucrose solution. The block was removed once every 24 h, and a series of recordings (as shown here) made to determine whether rhythmic pyloric activity was restored. Following these recordings, the block was put back into place for the next 24 h. B: relative frequency of lvn bursting and AGR spiking over time with and without sucrose blockade. Data for the sucrose group were collected while the block was removed. Relative frequency for each preparation was calculated as a proportion of the intact (control, day 0) activity of that preparation, and expressed here as mean ± SD. * represents a significant difference relative to control for a given day (P < 0.05; t-test). C: multisweep recordings of AGR activity showing the reversibility of the sucrose block over time in culture. AGR propagation into the son is blocked in sucrose, but restored when sucrose is removed from the blocking well, here shown at 24 and 48 h following the initial sucrose block. stn-a was the blocking well in this experiment. During sucrose blockade, AGR action potentials (initiated in the stn and propagated both anteriorly and posteriorly) sometimes can propagate into, but not beyond, the blocking well (see 24-h blocked recording).
ade. Descending inputs were blocked over the course of 4 days. Once per 24-h period we removed the stn block for 1 h and recorded the activity of the pyloric network in response to this reestablished modulatory connectivity. Representative recordings of stn (descending and ascending inputs) and lvn ( pyloric output) for a single preparation in each treatment group (control and sucrose) across the time course of an experiment are provided in Fig. 3A. All of the recordings in Fig. 3A and measurements summarized in Fig. 3B were made during the time when the sucrose blockade had been removed for that day; thus, these preparations all feature potentially intact connections from the descending projections to the STG. None of the preparations used in these experiments, or any subsequent experiments, had yet undergone full recovery of the pyloric rhythm in the absence of descending projections. Thus, in most cases, there was no pyloric activity in the blocked preparations prior to removal of the block.

Other than an initial slight decrease in pyloric burst frequency, control preparations showed consistent and robust pyloric output across the time course of the experiment (Fig. 3B, left). Conversely, as the preparations spend more time in a decentralized state via sucrose block, they are subsequently less likely to reestablish pyloric output when the block is removed (Fig. 3B, left). After 24 h, sucrose-blocked preparations were still capable of generating a pyloric rhythm that was not significantly different from the control when the blockade was removed, but after 48 h of blockade there was a significant decrease in pyloric frequency. After 4 days, virtually all pyloric activity was eliminated even with the sucrose block removed (Fig. 3B). This effect was not the result of damage to the stn by the sucrose blockade. Using the AGR neuron as one indicator of the propagation ability of the stn, there was no significant difference in AGR spiking frequency on the stn across the time course of the experiment between control and sucrose blocked preparations (see Fig. 3, A and B, right). Furthermore, using AGR as an indicator of stn propagation ability, we determined that sucrose likely was not resulting in the death of axons in the stn over the time course of the experiment. Figure 3C shows a representative multisweep analysis demonstrating the efficacy of the blockade at 24 h, as well as the reestablishment of AGR action potential propagation into the son following removal of the blockade at 24 and 48 h. By 48 h, we already saw a substantial decrease in pyloric frequency with the blockade removed (Fig. 3B), yet stn propagation ability appears to have remained intact.

Our results demonstrate that there is a decrease in the ability of restoring connectivity in the stn and the ability of the STG to generate pyloric activity following a period of removal of the inputs. One possible mechanism for the decrease in functional output in reconnected stomatogastric networks is a change in the activity of the descending projections influencing pyloric activity. Therefore, we measured changes in activity of two projection neurons, MCN1 and MCN5. Our analysis of the ion revealed no consistent relationships in the firing of these three units with blockade (data not shown). We did not obtain all of the appropriate recordings to unambiguously identify MCN1 and MCN5 spikes on the ion. However, our analysis showed there was substantial variability in the presence or absence of MCN1 and MCN5 activity from preparation to preparation in both control and sucrose-blocked conditions. In other words, we sometimes saw only OMN activity, whereas in some preparations we saw either one or two smaller units on the ion that could correspond to MCN1 and/or MCN5. When these units were present in our recordings, they also varied between simply spiking or bursting. Overall, although we could not definitively quantify the MCN1 and MCN5 activity, we detected no consistent qualitative relationships between ion unit activity (present or absence) or spiking pattern and pyloric frequency in this study (data not shown), although previously such a relationship was reported (Hedrich et al. 2011). However, we did see a relationship with son spiking activity across the time course of our blockade experiments. The son contains many more fibers than the ion, including both ascending and descending units, making identification of individual units far more challenging. To examine whether more global patterns of activity in the son may relate to the activity of the pyloric rhythm, we measured the overall spike frequency in the son as it related to the STG activity. The spike frequency of the son significantly decreased over the time course of the experiment with sucrose blockade (Fig. 4A), in a pattern reminiscent of that seen for the pyloric burst frequency in our earlier experiments (see Fig. 3B). We therefore determined whether son spiking and pyloric bursting activity were correlated. Our results show that overall pyloric burst frequency and son spiking frequency were significantly correlated across blocking conditions in this study (Fig. 4B). However, these analyses do not fully explain the relationships seen in our data. When we plotted the relationships among son spike frequency, AGR spike frequency, and the pyloric burst frequency in individual preparations, we saw a surprising shift in these relationships. In control preparations maintained in physiological saline for 3 days, we see a significant correlation between son spike frequency and pyloric burst frequency (Fig. 4C, top), but no relationships between AGR and the pyloric output, or son and AGR activity (Fig. 4C, middle and bottom). Conversely, preparations in which the stn has been blocked with sucrose over the course of 3 days have no relationship between son and the pyloric output (Fig. 4C, top and middle), but a significant correlation between AGR spiking and the pyloric burst frequency (Fig. 4C, bottom). These results show that there is not simply a scaling of all activity in the preparation over time, but rather that there are distinct alterations to spiking activities of projection neurons that correspond to changes in pyloric network output in the STG.

The overall incremental effects of sucrose blockade and removal are summarized in Fig. 5. All of the recordings in Fig. 5 have been made while the sucrose block has been removed and replaced with physiological saline, effectively restoring communication between the descending and ascending neurons in the stn. The control recording (Fig. 5, control) shows the activity of an intact preparation freshly dissected from the animal. When projections are restored following 24 h of sucrose blockade of the stn, the pyloric network generates rhythmic activity that is reduced in bursting frequency from controls (Fig. 5, 24-h block). At this time point we see con-

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served AGR spike propagation via multisweep analyses across the son, dgn, and both stn recording sites (Fig. 5, 24-h block, right), ensuring the stn remains capable of spike propagation. At 24 h we can also see a reduction in activity on the son as well. After 48 h deprived of inputs, removal of the blockade results in further weakened activity on the lvn and mvn, as well as reduced activity of the son. After 72 h deprived of inputs, removal of the blockade is insufficient to restore activity to the pyloric network, although the individual pyloric units can still be seen to be present on the lvn recording. Again, spikes are seen propagating through the blocking site (Fig. 5, 72-h block, right) confirming the viability of the projections, and son activity is further decreased (Fig. 5, 72-h block). However, in this preparation we also saw a substantive change in the activity of the AGR neuron. It begins to fire two independent spikes (instead of a single, tonic spike), one of which propagates through the son, stn, and dgn that appears to originate in the stn, whereas a second independent spike propagates only in the dgn in our recordings (Fig. 5, 72-h block, far right; see also Daur et al. 2009).

Although our measurements of son spiking suggest that the activity of modulatory and interneuron projections may be altered by the sucrose blockade, another possible explanation is that decreased pyloric activity could be the result of a loss in the ability of modulatory projections to release neuromodulator. We tested whether the descending modulatory projections are still capable of releasing neuromodulator after sucrose blockade by stimulating the stn both above and below the site of the blockade after 3 days of blockade with sucrose. If sucrose blockade has eliminated the ability of the modulatory projections to influence the pyloric rhythm, then we predict stimulation will be ineffective in producing output changes in the STG. This was not the case; after 3 days of sucrose blockade, electrical stimulation either below the blocking well

Fig. 4. Effects of stn blockade over time on bursting activity of the pyloric rhythm as well as son and AGR spiking activity. A: relative frequency of son spiking over time with blockade. Data were collected while the block was removed. Relative frequency for each preparation was calculated relative to that preparation’s control, intact (day 0) activity, and expressed here as mean ± SD. * represents a significant difference relative to control for a given day (P < 0.05; t-test). B: correlation between mean relative pyloric burst frequency and mean son spike frequency in sucrose and control preparations over time. Day 0 (control) is omitted because this is the reference point for relative frequency calculation. C: correlation among pyloric bursting, AGR, and son spike frequencies in control and sucrose blocked preparations for up to 72 h blocked. All data collected while the sucrose block was removed. Statistics reported reflect the outcome of Pearson’s correlation tests. Different point markers refer to measurements taken at 24 h, 48 h, and 72 h as shown in the legend of B.
or above the blockade (with the block still intact; Fig. 6A) or above the blockade (with the block removed; Fig. 6B) resulted in similar ability to drive the pyloric frequency. This stimulation effect is reversible, as 20 min after stimulation ceased, the pyloric rhythm had largely returned to its previous baseline levels (Fig. 6, A and B). These results demonstrate that the stn fibers are still capable of releasing neuromodulator to influence pyloric activity. Furthermore, these results provide strong support that we are not damaging stn axons via sucrose blockade, because we get the same stimulation effect whether we stimulate above or below where the sucrose blockade was placed.

A third mechanism by which reconnected projections might fail to appropriately generate network output is through altered sensitivity to neuromodulation of the target neurons of the pyloric network. We tested this by analyzing the responses of isolated pyloric networks (subsequent to sucrose blockade) to exogenously applied neuromodulators. After 3 days either with blockade via sucrose, or as an unblocked control preparation, the stn of each preparation was blocked with tetrodotoxin to ensure no interaction of the superfused modulator with descending neuromodulation. We then perfused a range of increasing concentrations of the neuropeptide proctolin, the biogenic amine octopamine, or the muscarinic agonist pilocarpine, all of which are known to exogenously activate pyloric activity (Goaillard et al. 2004; Hooper and Marder 1987; Swensen and Marder 2001), on to the isolated STG preparation and recorded pyloric burst output. We found a significant increase in response to proctolin in preparations that had previously been blocked with sucrose for 3 days relative to control (Fig. 7, top). At three of four concentrations tested (10⁻⁸ M, 10⁻⁷ M, and 10⁻⁶ M) there was a significantly higher frequency of pyloric burst output in the sucrose-blocked preparations relative to controls. Conversely, there were no significant differences in response to octopamine between sucrose and control preparations (Fig. 7, middle). Finally, we found a significant decrease in response to pilocarpine in sucrose-blocked preparations (Fig. 7, bottom). Although burst frequency responses of the pyloric rhythm were altered in response to different modulators, there appeared to be no substantial difference in the pattern of the pyloric rhythm elicited by modulators between control and sucrose-blocked preparations. Representative recordings time-scaled to compare one complete pyloric burst.
pattern suggest that although proctolin, octopamine, and pilocarpine elicit somewhat distinct patterns (e.g., LP activity levels), no differences were apparent between control and sucrose rhythms for a given modulator (Fig. 7). Together, these results suggest that the sucrose blockade does indeed result in a change in neuromodulator sensitivity of pyloric neurons, perhaps in particular for those neurons involved in the generation and frequency of the bursting of the pyloric rhythm, specifically AB and/or PD.

DISCUSSION

It is only somewhat recently that the occurrence of, and mechanisms underlying, homeostatic compensation in neural circuits have been more widely recognized and studied (Marder and Tang 2010; Turrigiano 2008). Such studies include examples of homeostatic plasticity as compensation for gene knockouts (Bergquist et al. 2010), synaptic scaling (Turrigiano 2008), sensory-modality specific compensation (Deeg and Aizenman 2011), and recovery of function in networks deprived of sensory input (Cullen et al. 2009) and descending command fibers (Golowasch et al. 1999; Tillakaratne et al. 2010). Yet despite the substantial amount that we have learned about homeostatic plasticity in these systems, very few, if any, studies have examined the impact of these homeostatic processes on the subsequent ability of the network to appropriately respond to further perturbation or even reversal of conditions that initially triggered compensation. Our study is one of the first to examine effects of removal and restoration of inputs to a motor network over the time course of homeostatic compensation. Our data demonstrate that following blockade of modulatory inputs, the system is altered such that removal of the block is insufficient to restore network activity. These effects do not seem to simply be due to damage of the descending stn fibers. Using AGR activity as a monitor of stn propagation ability (Daur et al. 2009), we found no significant differences in AGR spike propagation ability over time in both control and sucrose preparations, indicating the propagation ability of the stn is likely maintained throughout these experiments.

We investigated whether these effects are due to changes in the descending projections themselves, responses of the pyloric network, or both. To determine whether blockade causes a loss of modulator release from descending fibers, we electrically stimulated the stn both above and below the blocking well after sucrose blockade. Stimulation was equally effective in eliciting pyloric output regardless of whether the stimulation was below the block (with the block intact) or above the block (with the block removed). Thus, although we are unable to determine whether quantitative changes occur in modulator release as a result of our blocking manipulation, at least qualitatively we determined that the ability to release neuromodulator is not lost from presynaptic terminals of descending projections, nor is the ability of the STG to respond to released

Fig. 6. Effects of stn stimulation after 3 days of blockade on pyloric burst frequency. In these experiments, three wells were made on the stn; the middle was used for blockade, whereas those above (upper stim) and below (lower stim) were sites of extracellular stimulation. Preps either were maintained with the block intact and the stn stimulated below the blocking well (filled bars; A) or unblocked and stimulated above the blocking well (open bars; B). A: pyloric frequency (mean ± SD) and lvn representative recordings of stn-stimulated sucrose-blocked preparations with stimulation below the blocking well. Baseline recordings were made immediately prior to stimulation. Stimulated data were acquired immediately following stimulation of the stn. POST-STIM data were acquired 20 min after the end of the stimulation. B: pyloric frequency and lvn representative recordings of preparations with the block removed and stimulation done above the blocking well. Recordings were made in parallel fashion to sucrose block experiments. * represents a significant difference relative to control for a given group (P < 0.05; t-test). Sample size indicated in each bar. Individual pyloric units (LP, PY, and PD) are labeled as recorded on the lvn for reference.

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modulator lacking. These results also provide support for the maintenance of stn propagation ability throughout the study, because stimulation above a removed block was able to trigger effects that traversed the area of blockade.

In addition to blocking descending fibers, the blockade may also influence any ascending feedback from the STG to the commissural ganglia, as represented in our studies by AGR activity (Daur et al. 2009). In this study we consider both possibilities, but do not distinguish between the two. We analyzed the activity of the son nerve, which predominantly contains descending projections from the CoGs (Coleman et al. 1992), and found a significant decrease in firing rate on the son with increasing duration of blockade that mirrored changes seen in pyloric frequency. Indeed, there was a strong quantitative correlation overall between son activity to the pyloric output over the time course of the blockade. In control preparations, son spiking and the output of the pyloric network are coupled. Yet as a result of blocking the stn projections, this relationship completely disappears, and pyloric output becomes strongly correlated with

Fig. 7. Effects of exogenously applied neuromodulators on pyloric burst frequency (mean ± SD) after 3 days of stn blockade. After 3 days of sucrose, or time in culture control, the stn was completely blocked with tetrodotoxin (TTX) to ensure elimination of any endogenous modulator release. Increasing concentrations of proctolin, octopamine, and pilocarpine were superfused on preparations and pyloric burst frequency measured at steady state for a given concentration. * represents a significant difference relative to control preparations for a given concentration (P < 0.05; t-test). Representative lvn recordings for control and sucrose blocked preparations are shown during the perfusion of 10⁻⁶ M proctolin (top), 10⁻⁴ M octopamine (middle), and 10⁻⁵ M pilocarpine (bottom). A single pyloric burst is scaled for each recording to compare the pattern elicited by modulator application in each treatment group. Individual pyloric units (LP, PY, and PD) are labeled for reference.
the spiking activity of AGR. We have even seen dramatic qualitative changes in the activity of neurons like AGR in this system (Fig. 5, 72-h block) that may be further indicative of alterations in projections neurons that influence STG activity. We cannot determine from these experiments whether these changes in son, AGR, and pyloric network activity are causative or correlative. When combined with the fact that stimulation experiments reveal these projections have not lost the capacity to release modulators, the data overall suggest that disruption of interactions between the STG and the higher ganglia (CoG and OG) may lead to a change in activity underlying modulation, but not the capacity for modulator release, over time.

Finally, we determined whether alteration of descending projections affects response to exogenously applied neuromodulators. Proctolin is a neuropeptide that is a major constituent of releasate of presynaptic fibers of the STN (Billimoria et al. 2005) and is able to exogenously restore network activity to deafferented preparations (Golowasch and Marder 1992; Hooper and Marder 1987). There is a significant increase in the effect of proctolin on pyloric frequency in preparations that have been blocked with sucrose relative to control preparations. It is not possible to determine from our data whether this is a result of increased receptor signaling activity, or downstream targets of modulation such as voltage-gated conductances, or both. However, there was not an increase in pyloric cycle frequency sensitivity for all neuromodulators; octopamine application (Goaillard et al. 2004) did not result in significant differences in pyloric cycle frequency between control and sucrose-blocked preparations, whereas response to pilocarpine (Swensen and Marder 2001) was significantly lower in sucrose-blocked preparations than in controls. These data demonstrate that there is not a uniform change in response to all modulators in the system, but rather distinct responses to different classes of modulatory compounds. This could be the result of changes in modulator receptors, or perhaps intracellular pathways involved in mediating modulator response. However, these data suggest that the convergent target of several modulators in this system, a mixed-inward current elicited by modulator exposure (Golowasch and Marder 1992), may not be the source of the differing responses. Both proctolin and pilocarpine influence this current (Swensen and Marder 2000) in similar fashion, yet our preparations show divergent alterations in the responses to these two modulators.

The changes we see in the ability of restored projections to elicit output following different blockade regimes must represent a change at the cellular level, both in the ability of the STG neurons to respond to neuromodulatory substances, and in presynaptic activity and subsequent release of modulator. It is already established that pyloric neurons alter some intrinsic properties in response to decentralization. These include changes in the magnitude of ionic currents such as $I_{\text{ET}}$ (Thoby-Brisson and Simmers 2002), $I_A$ (Khorkova and Golowasch 2007; Mizrahi et al. 2001), $I_{\text{Ba}}$, and $I_{\text{KCa}}$ (Khorkova and Golowasch 2007), and disruption of correlations among ionic conductances as well as channel mRNA levels (Khorkova and Golowasch 2007; Temporal et al. 2012). Our results suggest that one possible impact of these changes is that subsequently restored neuromodulation may be ineffective at generating typical output because the downstream targets of modulators have been altered by decentralization. Indeed, due to the fact that these changes have been documented previously, it is possible that such changes in intrinsic excitability alone are sufficient to account for our results.

Less is known about changes in receptor expression following decentralization in the STG. Our data demonstrate changes in responses to proctolin and pilocarpine in blocked preparations, but at this time we cannot determine whether this is an effect of receptor expression, activity, or signaling, or due to changes in downstream targets. In other systems, changes in receptor expression following deafferentation have been reported, including 5HT-2C receptor expression in mammalian spinal cord (Murray et al. 2010). However, not all systems that undergo homeostatic compensation have corresponding changes in receptor expression. For example, vestibular deafferentation leads to plasticity and compensation, but changes in expression and distribution of $\gamma$-aminobutyric acid (GABA) (Gliddon et al. 2005), glutamate (King et al. 2002), glucocorticoid (Lindsay et al. 2005), and cannabinoid (Ashton et al. 2004) receptors have not been detected, despite changes in responses to these compounds.

The interaction of neuromodulation and neural network output is a complex interplay that occurs over multiple time scales. A good deal is known of how neuromodulators affect plasticity in these networks over shorter time scales, as well as influence properties of cells and synapses over more organizational time scales (Harris-Warrick 2011). However, as progress is made toward understanding regenerative processes in nerve injury, it becomes more imperative that potential impacts of changes in isolated or deafferented neural networks as a result of loss of modulatory inputs be more completely understood as well.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
J.N., K.M.L., and D.J.S. conception and design of research; J.N. and K.M.L. performed experiments; J.N., K.M.L., and D.J.S. analyzed data; J.N., K.M.L., and D.J.S. interpreted results of experiments; J.N., K.M.L., and D.J.S. edited and revised manuscript; J.N., K.M.L., and D.J.S. approved final version of manuscript; D.J.S. prepared figures; D.J.S. drafted the manuscript.

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