Translation and transcription are required for endogenous bursting after long term removal of neuromodulators

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ABSTRACT

TRANSLATION AND TRANSCRIPTION ARE REQUIRED FOR ENDOGENOUS BURSTING AFTER LONG TERM REMOVAL OF NEUROMODULATORS

by

Stefanie Eisenbach

Motor pattern-generating networks depend on neuromodulatory inputs to regulate the network activity. The pyloric network of the Cancer borealis stomatogastric ganglion (STG), a rhythmic motor pattern-generating network, requires modulatory inputs to generate this activity. When neuromodulatory inputs are removed, the pyloric network falls silent. However, patterned pyloric activity recovers spontaneously in about 24 hours in organ culture. To determine if synthesis of new proteins are involved in the recovery of pyloric activity after prolonged elimination of neuromodulators, translation inhibitors are tested on the recovery process of pyloric activity in C. borealis. In vitro experiments are conducted; the STG is exposed to the protein synthesis inhibitor Cycloheximide (CHX) and the transcription inhibitor Actinomycin D (ACD). The results suggest that early transcription and late protein synthesis play an important role in the recovery process of the rhythmic activity in the pyloric network of the crab STG. Late protein synthesis probably involves ion channels, since it is known that ionic conductance changes are affected by decentralization, which compensate for changes in the neuromodulatory environment of the pyloric network.
TRANSLATION AND TRANSCRIPTION ARE REQUIRED FOR ENDOGENOUS BURSTING AFTER LONG TERM REMOVAL OF NEUROMODULATORS

by Stefanie Eisenbach

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TABLE OF CONTENTS

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>INTRODUCTION.............................................................................. 1</td>
<td></td>
</tr>
<tr>
<td>1.1</td>
<td>Objective................................................................................. 1</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>Central Pattern Generators and Control of Movements................. 2</td>
<td></td>
</tr>
<tr>
<td>1.3</td>
<td>Stomatogastric Nervous System: Pyloric Network........................ 3</td>
<td></td>
</tr>
<tr>
<td>1.4</td>
<td>Neuromodulation in the Stomatogastric Nervous System............... 6</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>Long Term Effects of Removal of Neuromodulators on the Pyloric Network........................................................................... 9</td>
<td></td>
</tr>
<tr>
<td>1.6</td>
<td>Purpose of This Study.............................................................. 10</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>MATERIALS AND METHODS............................................................ 12</td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td>Electrophysiology......................................................................... 12</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>Isolation of the STG from Descending Inputs and Inhibition of Translation and Transcription................................................. 13</td>
<td></td>
</tr>
<tr>
<td>2.3</td>
<td>Data Analysis............................................................................... 14</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>RESULTS........................................................................................ 15</td>
<td></td>
</tr>
<tr>
<td>3.1</td>
<td>Transcription Inhibition has no Effect on Pyloric Rhythm in the Intact STNS................................................................. 15</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>Transcription During a Critical Time Window is Required for the Recovery of the Pyloric Rhythm.................................................... 17</td>
<td></td>
</tr>
<tr>
<td>3.3</td>
<td>Protein Synthesis Inhibition has no Effect on Pyloric Rhythm in the Intact STNS................................................................. 18</td>
<td></td>
</tr>
<tr>
<td>3.4</td>
<td>Translation is Required for Endogenous Bursting After Long-Term Decentralization.............................................................. 20</td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>Translation Occurs During a Critical Time Window to Allow for Recovery................................................................................. 21</td>
<td></td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS
(Continued)

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6 Translation Possibly Occurs During a Critical time Window to Allow for Recovery of the Pyloric Rhythm</td>
<td>22</td>
</tr>
<tr>
<td>4 DISCUSSION</td>
<td>27</td>
</tr>
<tr>
<td>4.1 Synaptic Plasticity is Inhibited by Modulatory Regulation of Ionic Currents</td>
<td>27</td>
</tr>
<tr>
<td>4.2 Transcription is Required in a Critical Time Window for Recovery</td>
<td>28</td>
</tr>
<tr>
<td>4.3 Protein Synthesis is Required for Recovery of the Pyloric Activity and During a Critical Period</td>
<td>31</td>
</tr>
<tr>
<td>4.4 Future Directions</td>
<td>32</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>34</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>The Pyloric Network of <em>C. Borealis</em></td>
<td>5</td>
</tr>
<tr>
<td>1.2</td>
<td>Neuromodulatory control of the stomatogastric ganglion (STG)</td>
<td>7</td>
</tr>
<tr>
<td>3.1</td>
<td>Recovery of the pyloric rhythm after decentralization</td>
<td>15</td>
</tr>
<tr>
<td>3.2</td>
<td>ACD has no effect on pyloric frequency in the intact STNS</td>
<td>17</td>
</tr>
<tr>
<td>3.3</td>
<td>ACD critical time window prevents recovery of pyloric activity</td>
<td>19</td>
</tr>
<tr>
<td>3.4</td>
<td>CHX effect on intact pyloric frequency over time</td>
<td>21</td>
</tr>
<tr>
<td>3.5</td>
<td>Extracellular recording on the <em>lvm</em> at different times during the experiment where CHX was applied during 0-4 hours, 4-24 hours, and 16-20 hours</td>
<td>23</td>
</tr>
<tr>
<td>3.6</td>
<td>CHX incubation intervals affect recovery</td>
<td>24</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Effect of Incubation Times in ACD on Pyloric Rhythm Recovery</td>
<td>20</td>
</tr>
<tr>
<td>3.2 Controlling Frequency Variance Between Preparations Shows Critical Time Window</td>
<td>26</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Objective

The objective of this thesis is to examine the contribution of transcription and translation to the stabilization and recovery of endogenous rhythmic bursting activity following activity changes induced by long-term deprivation of neuromodulatory input. Networks responsible for rhythmic motor activity called central pattern generators (CPGs) have shaped much of our intuition about the mechanisms underlying the generation and maintenance of oscillatory activity. Research using CPG networks in the stomatogastric ganglion (STG) of crabs and lobsters have yielded many important findings with regard to neuronal excitability, network oscillations, neuromodulation and homeostatic plasticity.

The pyloric CPG generates stable rhythmic activity that depends on the presence of neuromodulatory input from the central nervous system and may interact with activity-dependent homeostatic plasticity in hitherto unknown ways. In the absence of neuromodulators, all rhythmic activity ceases; however, after continued absence of neuromodulation the network can recover stable rhythmic activity that is similar to that expressed in the presence of neuromodulatory input (Thoby-Brisson and Simmers, 2000).
It is hypothesized that recovery is controlled by the synthesis of new protein to compensate for the loss of neuromodulatory input (Thoby-Brisson and Simmer, 1998). These changes in neuronal responsiveness are hypothesized to be dependent on critical periods of gene expression and protein synthesis as has previously been shown in many different model systems (Kaczmarek and Chaudhuri, 1997).

1.2 Central Pattern Generators and Control of Movements

All animals have the ability to generate movements such as walking, breathing, flying and swimming. These movements are controlled by oscillatory neuronal circuits known as CPGs (Marder and Bucher, 2001). CPGs do not require patterned input to produce patterned rhythmic output. Moreover, neurons and networks can generate different versions of the same output depending on environmental cues as well as their internal state. This flexibility is partly due to neuromodulation, which affects intrinsic excitability and properties of synaptic connections. Since the pyloric CPG can recover its activity in the absence of neuromodulators, it is thought that the circuit is subject to not only neuromodulator-dependent, but also activity-dependent changes to ensure stable and robust function in the face of perturbation. Findings from the Stomatogastric Nervous System suggest that there are complex rules governing the homeostatic regulation of ion channels and synapses and that these rules depend on the presence of neuromodulators (Khorkova and Golowasch, 2007). Indeed, with the appropriate balancing of levels of ionic channels, similar activity can be produced despite heterogeneous biophysical properties. Understanding how these CPGs function is a vital part of understanding stable oscillatory activity underlying different behaviors, such as different sensory, motor, as
well as cognitive tasks: olfaction (Laurent and Davidowitz, 1994), visual processing (Gray, 1994), memory formation (Lisman and Idiart, 1995, Yuste et al., 2005).

Regulation of complex movements by neuromodulatory input can occur by altering synaptic and voltage-dependent currents, which shape the network activity (Golowasch et al., 1999a, Marder and Bucher, 2001, Harris-Warrick and Johnson, 2010). These biophysical properties stabilize function of the network and allow for plasticity, which is required for the nervous system to perform effectively. The mechanisms that allow CPG’s to change their biophysical properties, allowing the system to adapt to changes in neuromodulatory input, still needs to be investigated at many levels.

Although CPGs are generally characterized as not requiring sensory input, neuromodulatory input on rhythmic oscillating networks that control muscle movements allow the animal to respond to sensory input from its environment. For example, neurons that innervate muscles that are required for an escape response are controlled by neuromodulatory inputs in crayfish (Herberholz et al., 2004). All animals respond to their environment or to injury through neuroplasticity. Therefore, it is important to understand the mechanisms that underlie long-term modifications in neuronal properties after changes in modulatory input, and how they can compensate for loss of neuromodulatory input after perturbations and injury (Thoby-Brisson and Simmers, 1998, Golowasch et al., 1999b)
1.3 Stomatogastric Nervous System: Pyloric Network

One of the best-studied CPGs that are controlled by neuromodulatory input is the pyloric network of crustaceans such as the crab *C. borealis*. The pyloric network is found in the Stomatogastric Nervous System (STNS) and generates a rhythmic motor pattern driven by a peacemaker neuron (Marder, 1996, Marder and Bucher, 2007). The *C. borealis* STNS controls several feeding functions, such as the animal's ability to chew, filter, swallow, and store food. The STNS consists of four ganglia, the paired of commissural ganglia (CoGs), the unpaired esophageal ganglion (OG), and the stomatogastric ganglion (STG) (Figure 1.1 A). The CoGs and OG contain modulating neurons that control the STG neural networks. The STG is made up of 25-30 neurons that all can reliably identified in each animal. This ganglion contain two CPG circuits, the pyloric and gastric network, whose combined output control coordinated muscle movements of the foregut. The STG primary neurite and dendritic trees form a structure called the neuropile (Marder and Bucher, 2007).

This system has significantly contributed to the understanding of how motor circuits operate at the cellular level, and has provided insight into the mechanisms of network homeostasis and plasticity (Thoby-Brisson and Simmers, 2002, Temporal et al., 2012).
Figure 1.1 The pyloric network of *C. borealis* (A) Schematic of the stomatogastric nervous system of the crab *C. borealis*. The neurons in the commissural (CoG) and oesophageal (OG) ganglia represent modulatory projection neurons that affect the networks in the stomatogastric ganglion (STG). These neurons connect to the STG via the stomatogastric nerve (stn). Most STG neurons are motor neurons that project axons posteriorly through nerves such as the lateral (lvn) or medial (mvn) ventricular nerves. (B) Circuit diagram showing connectivity among pyloric neurons: AB: anterior burster; PD: pyloric dilator, LP: lateral pyloric; IC: inferior cardiac; PY: pyloric constrictor; VD: ventricular dilator. Each color of the cells show neurons that burst in phase with each other (C) Top: an example of intracellular recordings of individual cells in the STG. Bottom: example of extracellular recordings from four different nerves (lvn, mvn, pyn, pdn). Recordings show the activity of each of the indicated STG motor neurons during the ongoing pyloric rhythm. Figure courtesy of Dr. Farzan Nadim.

The pyloric network is made of the lateral pyloric (LP), two pyloric dilator (PD), several pyloric constrictor (PY), the anterior buster (AB), the ventricular dilator (VD), and the inferior cardiac neurons (IC) (Figure 1.1B) (Kilman and Marder, 1996). All of the cells produce a continuous rhythmic output pattern similar to what is seen *in vivo* when recoded from *in vitro* (Figure 1.1C) (Rezer and Moulins, 1992). These neurons and their connections create a neuronal circuit that produces an oscillatory pattern controlling the movements of the pylorus. For example, AB and PD are electrically coupled and form a
pacemaker kernel, while the other neurons and their connections are follower neurons that fire on rebound from inhibition, producing the triphasic pyloric rhythm. Across preparations the pyloric triphasic rhythm cycle frequency can vary, but the phase relationship between the different neurons is well maintained (Goaillard et al., 2009).

The pyloric network can also regain stability in response to changes in modulatory input and other perturbations and stimuli. The network is able to adapt to these changes by altering intrinsic properties of its neurons and reconfigure synapses within the network to maintain the same motor output (Thoby-Brisson and Simmers, 2002, Luther et al., 2003, Khorkova and Golowasch, 2007). This plastic nature of the pyloric network is thought to be stabilized by the constant maintenance of physiological properties such as ion channels and receptors numbers on the membrane, which can be regulated by neuromodulators (Khorkova and Golowasch, 2007). There are neuromodulators that appear to stabilize the pyloric network by altering ion channel conductances (Turrigiano et al., 1995, Khorkova and Golowasch, 2007, Marder and Bucher, 2007), and activity has been shown to allow the network to adapt to perturbations and maintain a homeostatic state (Khorkova and Golowasch, 2007).

1.4 Neuromodulation in the Stomatogastric Nervous System

Neuromodulators have been found to be essential for network function. In humans, the spinal cord is believed to include many CPGs, which are used to generate locomotion and other behaviors and require different neuromodulators (MacKay-Lyons, 2002).
Neuromodulators allow CPG networks to alter their basic motor patterns controlling specific behaviors in response to environmental or internal changes (Harris-Warrick and Marder, 1991).

In the STNS there are many different substances, including amines, neuropeptides, and gases (Figure 1.2) that modulate the pyloric circuit activity. These substances can be released from the pericardial organs into the circulatory system as hormones (Figure 1.2A) (Marder and Bucher, 2007). Neuropeptidergic and aminergic projection neurons located in the CoGs and OG release neuromodulators into the STG
(Coleman et al., 1992). Some projection neurons receive synaptic connections from their target neurons in the STG, creating local feedback circuits to maintain stable pyloric activity (Blitz and Nusbaum, 2012). Most of the modulatory neurons contain multiple cotransmitters, allowing for a variety of post synaptic actions on different target neurons (Beltz et al., 1984, Coleman and Nusbaum, 1994, Skiebe and Schneider, 1994, Swensen and Marder, 2000, Marder and Thirumalai, 2002, Szabo et al., 2011). Understanding how the release of these substances affect the target neurons, is important to understand their function in the pyloric network and to understand how multiple substances affect a network.

It was previously shown that these neurotransmitters play an important role in modulating the pyloric motor patterns by altering the circuit frequency and phase of different cells. Exogenous applications of different neuromodulators have given insight to the role that each neuromodulator plays in the pyloric network. One of the most studied neuromodulators in this system is proctolin. There is a set of protolin releasing neurons in the STNS located in the CoGs and OG: modulatory commissural neuron 1 (MCN1), modulatory commissural neuron 7 (MCN7), and modulatory proctolin neuron (MPN). These neurons also contain other neuromodulators such as GABA, CabTRP and Allatostatins, but strongly activate the pyloric network (Blitz et al., 1999, Szabo et al., 2011). Co-transmission provides projection neurons with strategies for eliciting multiple outputs from the network. When proctolin is applied to the isolated STG, the pyloric rhythm increases in cycle frequency and many pyloric circuit neurons show increased activity levels. Also, it has been shown that proctolin elicits activity in a state-dependent manner, i.e. pyloric rhythm activation is highest in preparations that have very slow or no
rhythm and minimal at ≥1Hz. This neuropeptide activates voltage-dependent channels that conduct a current named modulator-activated inward current (I_{MI}) (Swensen and Marder, 2000, 2001).

1.5 Long Term Effects of Removal of Neuromodulators on the Pyloric Network

One of the most interesting qualities of the pyloric network is its ability to recover its rhythmic activity after it has been eliminated by prolonged removal of neuromodulators (Golowasch et al., 1999b, Thoby-Brisson and Simmers, 2000, Luther et al., 2003). Neuromodulatory input from the CoGs and OG is necessary for normal pyloric activity, and when removed the network typically falls silent over time. The removal of neuromodulatory input to the STG can be experimentally achieved by blocking action potential transmission along the stomatogastric nerve (stn). This technique is referred to as decentralization (Thoby-Brisson and Simmers, 1998, Luther et al., 2003). It has been shown in both *Jasus lalandii* (Spiny Lobster) (Thoby-Brisson and Simmers, 1998) and *C. borealis* (Thoby-Brisson and Simmers, 1998, Golowasch et al., 1999b, Luther et al., 2003) that over 24 hours after removal of neuromodulatory input from the STG, the pyloric activity will gradually recover. The phase relationships of the bursting neurons also gradually returns close to control levels (Luther et al., 2003). This phenomenon will be referred to as recovery, and is thought to be due to the ability of pyloric neurons to modify their intrinsic properties. Previous research in *C. borealis* has shown that recovery is due to change in ionic current levels and perhaps co-regulation of currents (Khorkova and Golowasch, 2007). The change in ionic current levels suggests that intrinsic
properties of the cells are altered in order to achieve recovery. (Colvis et al., 2005, Ingolia, 2014).

It was reported that changes in transcription and mRNA levels might be responsible for the long-term modifications induced by decentralization and the consequent removal of neuromodulators (Thoby-Brisson and Simmers, 2000). This was seen using the transcription blocker actinomycin D (ACD) applied at different times after decentralization of the pyloric network in J. lalandii. In particular, the transcription blockade showed that transcription is required soon after decentralization. The result from these experiments suggest that recovery in J. lalandii is controlled by changes in the intrinsic excitability of individual pyloric neurons, requiring an early critical period of transcription (0-4 hours) during the time modulatory inputs are eliminated (Thoby-Brisson and Simmers, 2000).

1.6 Purpose of This Study

The aim of the present study is to understand the mechanism underlying the ability of the pyloric rhythm in C. borealis to recover after long-term neuromodulator removal. The mechanism that was studied is the combined action of transcription and translation, and to examine the temporal epochs during which these processes occur after decentralization. This was achieved by reexamining the results from previous work on J. lalandii in C. borealis. Translation was examined by using Cycloheximide (3-[2-(3, 5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl] glutaramide) (CHX), which is an antibiotic derived from a microbial source that blocks translation of mRNA on the
cytosolic 80s ribosomes but does not inhibit organelle protein synthesis, allowing cells to continue to function.

The importance of transcription and translation can be established using these different blockers during small time windows to determine if translation, like transcription (e.g., (Thoby-Brisson and Simmers, 2000)), is required during a short critical window. If both translation and transcription are required during a critical period it is expected that their respective critical time windows are shifted relative to each other, with transcription leading translation. Another possibility to explain recovery could be that neither transcription nor translation could be required for recovery of the pyloric rhythm. Although, it has been shown that transcription is required for recovery in *J. lalandii* it may not play an important role in recovery in *C. borealis*. Other possible processes controlling recovery are channel phosphorylation (Lee, 2006), which is classically involved in other forms of plasticity, or the transport of ion channels in or out of the cell membrane (Carroll et al., 2001).
CHAPTER 2

MATERIALS AND METHODS

2.1 Electrophysiology

All experiments were performed on adult male *C. borealis* obtained from local fish markets in Newark NJ. The STNS was dissected out from the animal, and the STG was desheathed (Selverston, 1987). The dissected STNS was placed on a sylgard (Dow-Corning)–lined Petri dish filled with physiological saline (440mM NaCl, 11mM KCl, 26mM MgCl$_2$, 13mM CaCl$_2$, 12mM Trizma base, and 5mM malic acid; pH 7.4-7.5). Electrophysiological recordings were made at 11-13°C. Preparations were maintained in a low temperature incubator at 4-12°C.

Extracellular recordings were performed using monopolar stainless steel electrodes placed into Vaseline wells made around the lvn and mvn. A reference electrode was placed in the bath and the signal was amplified with a differential AC amplifier (1700 A-M systems). Recordings were made throughout the experiment, excluding the incubation times. The signals were recorded using pClamp 10.1 software and Digidata 1322A digitizer board (Molecular Devices, CA).

2.2 Isolation of the STG from Descending Inputs and Inhibition of Translation and Transcription

Measurements were taken with saline circulating continuously and once the preparations had reached a stable temperature of 11°C. Normal triphasic pyloric rhythm in the control
intact STNS was recorded for 20 minutes at the beginning of the experiments when STNS was intact (Figure 2.1A). To isolate the STG from modulatory inputs the stn was cut using scissors approximately halfway along the stn (Figure 2.1A). The CoGs were completely removed to ensure no neuromodulators were able to be released into the extracellular solution.

Forty minutes after the STG was disconnected from the descending modulatory inputs, recordings were obtained (henceforth labeled “decentralized” or time = 0). After that, the different drugs were applied. Transcription was inhibited using Actinomycin D (50 μM) (ACD; Sigma Aldrich, (Thoby-Brisson and Simmers, 2000). Stock solutions were made at 1mg/mL and diluted immediately before use in C. borealis saline. ACD was applied in a Vaseline well constructed around the desheathed STG, and renewed hourly because ACD is absorbed by plastic and glass in standing solution (Zenser et al., 1984). Also, ACD is very sensitive to light so incubations with ACD where conducted in the dark. In other experiments translation was inhibited using 60μM Cycloheximide (CHX; Sigma) (Beaumont et al., 2001), made by dissolving lyophilized powder at 10mg/ml in DMSO, and diluted in normal saline. CHX was applied similarly to ACD, but was not kept in the dark and not replaced during incubation because it has been reported that CHX at this concentration remains active for 0-24 hours (Mimnaugh et al., 2004). CHX and ACD were both applied at different intervals and different conditions including intact and decentralized preparations for 24 hours, 20 hours, and various 4 hour intervals.
2.3 Data Analysis and Statistics

Analysis of the pyloric rhythm frequency was performed using DataMaster 2.2. Statistical analysis was performed using the Sigmaplot version 11.0 software package (SPSS, Chicago IL), and Origin version 9.1 was used for graphing. Statistical significance is reported if p<0.05. Experiments in normal saline were compared over time using a one-way repeated measures ANOVA. A t-test was used to compare the effects of both ACD and CHX in intact preparations in normal saline. Comparisons of the pyloric frequency of preparations subject to different treatments with either ACD or CHX were performed on the frequencies recorded at 24 hours after decentralization after the frequency of the preparation at time = 0 (decentralized) was subtracted. This was done in order to remove the variability observed in the frequency of the rhythm after decentralization. χ² test (Fisher’s Exact Test) was used to compare the effect of treatments on the frequency of occurrence of recovery of pyloric activity at 24 hours after decentralization. Recovery was defined as an increase in pyloric frequency of at least 40% above the frequency recorded after decentralization (i.e., time = 0). At least 15 cycles were used to calculate pyloric frequency for each condition and then averaged across experiments. Average values are reported as means ± standard error (SE) of the mean.
CHAPTER 3
RESULTS

3.1 Recovery of the Pyloric Activity After Long-Term Removal of Neuromodulators

To confirm that the pyloric rhythm recovers after slowing down or ceasing as a result of decentralization the pyloric rhythm was recorded for 20 minutes at the beginning of the experiments with the STNS intact. The mean frequency thus measured was $1.07 \pm 0.10$ Hz ($n = 10$) (Figure 3.1 A and D). When the anterior ganglia containing the neuromodulator inputs were removed by cutting the $sm$, the pyloric rhythm fell silent or slowed down to an average frequency of $0.35 \pm 0.12$ ($n = 10$) (Figure 3.1 B and D).

Figure 3.1 Recovery of the pyloric rhythm after decentralization. A) The intact preparation. B) The rhythm ceases after modulatory input is removed. C) The rhythmic activity resumes after 24 hours in the absence of modulatory input. In A-C, Left: Schematic diagram of the preparation. The arrows indicate where the stn was cut for removal of neuromodulatory input; Right: extracellular recoding from the lvn showing the pyloric activity. D) Each bar represents the average ± SE of the pyloric frequency. Preparations were recorded while intact and in normal saline for 20 minutes and then decentralized. Measurements were taken 40 minutes after decentralization and then after 24 hours ($n=10$).
Consistent with what has previously been reported (Golowasch et al., 1999b, Thoby-Brisson and Simmers, 2000, Luther et al., 2003), over 24 hours in organ culture, the pyloric rhythm eventually resumed to a mean frequency of $0.34 \pm 0.08$ Hz ($n = 10$) (Figure 3.1C and D). This shows that there is an essential alteration in intrinsic properties allowing the rhythm to resume in the absence of neuromodulatory inputs, which are otherwise normally necessary. In control decentralization experiments a significant number of preparations (7 out of 10, $\chi^2$ test, $p=0.001$) recovered rhythmic activity 24 hours after decentralization.

### 3.2 Transcription Inhibition has no Effect on Pyloric Rhythm in the Intact STNS

First, the conditions that were previously tested in *J. lalandii* where tested in *C. borealis*, to determine if transcription plays an important role in recovery in this animal. ACD (50μM) was tested in an intact preparation for 24 hours ($n=4$). During this time there was no significant difference in pyloric frequency compared to preparations incubated in normal saline ($n=4$) compared to those incubated in ACD ($p=0.735$) (Figure 3.2) suggesting that the pyloric network neurons maintain minimal transcription activity in the presence of neuromodulatory input.
Transcription During a Critical Time Window is Required for the Recovery of the Pyloric Rhythm

Two incubation intervals with ACD during the recovery period were compared: 0 to 4 hours after decentralization, 20 to 24 after decentralization. The pyloric rhythm was recorded over a 10 minute period after the STNS was removed from the animal and before decentralization (Intact), then 40 minutes after decentralization (time 0) and then 24 hours later for each treatment. The mean frequency (±SE) of the pyloric activity before decentralization in the 0-4 hrs treatment group was $1.3 \pm 0.1$ Hz (n=10), and that of the 20-24 hrs treatment group was $1.1 \pm 0.05$ Hz (n=8) (Figure 3.3, Intact). At time

Figure 3.2. ACD has no effect on pyloric frequency in intact STNS over time. The mean pyloric frequency was measured in preparation first in normal saline (control), immediately after ACD is added to the STG (time = 0), and 24 hours later. A two way ANOVA was performed to compare the data. This test showed that there is no significant difference between the normal saline groups and ACD groups at any time point ($p = 0.735$, n= 4).
zero (decentralized) the pyloric rhythm dropped to 0.21 ± 0.08 Hz for the 0-4 hrs group, and 0.3 ± 0.2 Hz for the 20-24 hrs group. The preparations were stored in an incubator at 3°C at all times after decentralization and then taken out for recoding at 12°C, except during the ACD treatment period, which was performed while continuously perfusing and ACD was replaced every hour for 4 hours, and preparations were kept in the dark.

The mean cycle frequency measured 24 hours after decentralization of the 0-4 hrs treatment group was 0.21 ± 0.09 Hz (n = 10) and for the 20-24 hrs group was 0.50 ± 0.17 Hz.(n = 8) (Figure 3.3).

**Figure 3.3** ACD in a critical time window prevents recovery of pyloric activity. Preparations were recorded while intact and in normal saline for ten minutes before decentralization and the 40 minutes after the stn was cut. Preparations were incubated with ACD during 0-4 hour (Black) (n=10) and during hours 20-24 (Red) (n=8) after decentralization.
To test if there is a significant difference between these two groups, and to control for variation in frequency when decentralized, the frequency at 24 hours was corrected by subtracting the frequency at decentralization in each experiment. The differences were: 0-4 hrs: 0.02 ± 0.13 Hz; 20-24 hrs: 0.21 ± 0.19). The corrected frequencies were tested using a t-test. The differences were not significant (p=0.681) (Table 3.1). Thus, according to this criterion, transcription block during the initial 4 hours after decentralization has a slightly stronger effect than at the end of the 24 hour period, but this is not statistically significant.

**Table 3.1. Effect of Incubation Times in ACD on Pyloric Rhythm Recovery**

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4 hrs</td>
<td>10</td>
<td>0.02</td>
<td>0.13</td>
</tr>
<tr>
<td>20-24 hrs</td>
<td>8</td>
<td>0.21</td>
<td>0.19</td>
</tr>
<tr>
<td>0 hrs</td>
<td>10</td>
<td>0.99</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Thoby-Brisson & Simmers (2000) used a different measurement for recovery, namely the fraction of preparations that recovered at the time point tested. Thus, we performed a χ2 test for a similar comparison. The number of preparations incubated in ACD for 0-4 hrs whose pyloric rhythm recovered at 24 hours was significantly different from those incubated between 20-24 hrs (p=0.02). Thus, according to this criterion ACD blocked transcription required for recovery of the triphasic pyloric rhythm
3.4 Protein Synthesis Inhibition has no Effect on Pyloric Rhythm in the Intact STNS

To determine whether pyloric activity requires new protein synthesis, long-term exposure to CHX was tested in intact preparations. The mean pyloric frequency was recorded immediately after dissection (1.20 ± 0.04 Hz, n = 4) and that pyloric frequency did not change over 24 hours (1.22 ± 0.11 Hz) in normal saline (p > 0.05). When incubated in CHX pyloric frequency started after dissection with a mean frequency of 1.17 ± 0.03 Hz) and did not significantly change over 24 hours (1.17 ± 0.04 Hz) (p = 0.489). A two-way ANOVA test was performed to compare the data (Figure 3.4).

![Figure 3.4 CHX effect on intact pyloric frequency over time.](image)

**Figure 3.4** CHX effect on intact pyloric frequency over time. The mean pyloric frequency can be seen first in normal saline (control, 30 minutes later – time 0, and after 24 hours) (n = 3). For the CHX-treated preparations (n = 4), control is before superfusion of CHX, time 0 is ~30 minutes after CHX is added, and 24 hours later. A two-way ANOVA was performed to compare the data (p=0.489).
3.5 Translation is Possibly Required for Endogenous Bursting After Long-Term Decentralization

After confirming, that CHX had no adverse effects on the pyloric rhythm in the intact system it was determined whether translation is necessary for recovery after decentralization. Control pyloric frequency was initially determined as before. The stn was then cut, after 40 minutes a new recording was made (decentralized) and CHX (60μM) was then added. As before, the mean cycle frequency of decentralized preparations decreased. None of the 11 preparations incubated in CHX recovered after 24 hours.

A χ² test was performed to compare the effect of CHX on recovery at 24 hours. The number of recovered preparations incubated in CHX for 24 hours was significantly smaller from those incubated in normal saline, which is the control group (p<0.001). Thus, it appears that 60μM of CHX blocked protein synthesis, and is required for recovery of the pyloric rhythm.

3.6 Translation Possibly Occurs During a Critical Time Window to Allow for Recovery of the Pyloric Rhythm

CHX was applied during different intervals after decentralization to determine if there was a specific time window for protein synthesis requirement during the time of recovery. Extracellular recordings of one representative preparation are shown for the incubation times 0-4, 4-24, and 16-20 and 20-24 hours in Figure 3.5. For all of the preparations frequency was measured when the preparations where first dissected and
temperature was stable (0-4 hours: $1.13 \pm 0.08$ Hz (n = 6), 4-24 hours: $1.17 \pm 0.09$ Hz (n = 8), 16-20 hours: $1.41 \pm 0.08$ Hz (n=7), 20-24 hours: $1.13 \pm 0.08$ Hz (n=10) then 40 minutes after decentralization and finally 24 hours later. Approximately half of the preparations went completely silent after decentralization; the others showed a clear drop in frequency (0-4 hours: $0.12 \pm 0.11$ Hz, 4-24 hours: $0.37 \pm 0.17$ Hz, 16-20 hours: $0.25 \pm 0.12$ Hz, 20-24 hours $0.35 \pm 0.09$).

**Figure 3.5** Representative extracellular recordings from lvn at different times during CHX incubation. Here CHX was applied during 0-4 hours, 4-24 hours, and 16-20 hours and 20-24 hours. Before decentralization all of the preparations are intact and in normal saline. Once the pyloric rhythm and temperature were stable, the preparations were decentralized and recorded 40 minutes later (Decentralized). All the preparations were incubated with CHX at the different times as indicated, and the pyloric activity was recorded at 24 hours.

The preparations were kept in an incubator after 4 hours of CHX incubation. At 24 hours 4 out of the 8 of the decentralized preparations that were treated with CHX between 0-4 hours recovered, only one of 8 recovered in the 4-24 hours group, 4 out of
the 7 in the 16-20 hours group recovered, and 3 out of the 10 preparations in the 20-24 hours group recovered.

A $\chi^2$ test was performed to compare the effect of CHX at these different intervals on the number of preparations that recovered. The number of experiments incubated in CHX for 0-4, 4-24, 16-20, and 20-24 were significantly different from each other ($p < 0.001$), with those preparations incubated in CHX at later times showing recovery a significantly fewer number of times. This shows that there is a difference between

![Figure 3.6 CHX incubation intervals affect recovery. Each bar represents the average ± SE of the pyloric frequency. Preparations were recorded while intact and in normal saline for ten minutes and then decentralized. Measurements were taken 40 minutes after decentralization and then again 24 hours after decentralization. different incubation times on recovery.](image)

From these data, it can be concluded that a critical time window exists for translation, and that it occurs later than the critical time window for transcription, i.e.
after the 4 hour time window. However, a more exact time window within the 4-24 hours interval cannot be identified with this analysis.

To further identify a more precise critical time window for translation, a one-way RM ANOVA was performed using the 24-hour frequencies after subtraction of the frequencies measured at the time of decentralization. The difference in the mean values among the treatment groups is statistically significantly difference (p<0.001) (Fig. 3.6 and Table 3.2). To isolate the groups that showed the most significant differences. The pairwise Holm-Sidak post-hoc test for multiple comparison was used. Statistically significant differences were observed between the following treatment groups: 0-4 vs. 4-24 hrs, 0-4 vs. 0-24 hrs, 16-20 vs. 4-24 hrs, 16-20 vs. 0-24 hrs, 0 vs. 4-24 hrs, 0-4 vs. 20-24 hrs, and 0 vs. 0-24. Although all other differences were not statistically significant (see significance levels in Table 3.2; e.g., surprisingly, the 0 vs 20-24 hrs comparison showed a difference that was relatively weak), some of the largest differences of the means were those between intervals separated by a boundary near the 20-hour incubation time, suggesting that late transcription in the 24 hour interval is critical for pyloric rhythm recovery.
Table 3.2 Controlling for Frequency Variance Between Preparations Shows Critical Time Window.

<table>
<thead>
<tr>
<th>Group Comparison</th>
<th>Difference of Means</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4 vs. 4-24 hrs</td>
<td>0.741</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>Yes</td>
</tr>
<tr>
<td>0-4 vs. 0-24 hrs</td>
<td>0.630</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>Yes</td>
</tr>
<tr>
<td>16-20 vs. 4-24 hrs</td>
<td>0.561</td>
<td>0.003</td>
<td>0.004</td>
<td>Yes</td>
</tr>
<tr>
<td>16-20 vs. 0-24 hrs</td>
<td>0.508</td>
<td>0.003</td>
<td>0.005</td>
<td>Yes</td>
</tr>
<tr>
<td>0 vs. 4-24 hrs</td>
<td>0.532</td>
<td>0.003</td>
<td>0.005</td>
<td>Yes</td>
</tr>
<tr>
<td>0-4 vs. 20-24 hrs</td>
<td>0.529</td>
<td>0.004</td>
<td>0.005</td>
<td>Yes</td>
</tr>
<tr>
<td>0 vs. 0-24 hrs</td>
<td>0.480</td>
<td>0.004</td>
<td>0.006</td>
<td>Yes</td>
</tr>
<tr>
<td>16-20 vs. 20-24 hrs</td>
<td>0.348</td>
<td>0.042</td>
<td>0.006</td>
<td>No</td>
</tr>
<tr>
<td>0 vs. 20-24 hrs</td>
<td>0.320</td>
<td>0.061</td>
<td>0.007</td>
<td>No</td>
</tr>
<tr>
<td>20-24 vs. 4-24 hrs</td>
<td>0.212</td>
<td>0.236</td>
<td>0.009</td>
<td>No</td>
</tr>
<tr>
<td>0-4 vs. 16-20 hrs</td>
<td>0.181</td>
<td>0.312</td>
<td>0.013</td>
<td>No</td>
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<tr>
<td>20-24 vs. 0-24 hrs</td>
<td>0.160</td>
<td>0.331</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>0-24 vs. 4-24 hrs</td>
<td>0.0525</td>
<td>0.525</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>16-20 vs. 0 hrs</td>
<td>0.0280</td>
<td>0.867</td>
<td>0.050</td>
<td>No</td>
</tr>
</tbody>
</table>
4.1 Plasticity is Inhibited by Modulatory Regulation of Ionic Currents

Our results show that the mechanism underlying the ability of the pyloric rhythm in *C. borealis* to recover after long-term neuromodulator removal is due to the synthesis of mRNA and new proteins. In addition, it was determined that transcription and translation are possibly required during a short critical window. The critical time window of translation appears to occur much later than the critical time window for transcription. An unexpected observation was that both these processes appear to be normally inhibited in the intact STNS, suggesting that neuromodulatory input is somehow responsible for this inhibition. As soon as neuromodulators are removed after decentralization, both transcription and translation become important resulting in the recovery of the pyloric network activity. The newly synthesized proteins are likely new ion channels used by the cells to compensate for the lack of neuromodulatory input, consistent with the observed changes in ionic current densities in PD neurons (Thoby-Brisson and Simmers, 2002, Sutton and Schuman, 2006, Khorkova and Golowasch, 2007). Without these new intrinsic modifications, the pyloric activity is unable to recover.

It is thought that the cellular changes that underlie various forms of short-term plasticity involve structural changes in the membrane and are protein-synthesis dependent (Klann et al., 2004, Sutton and Schuman, 2006). The STNS exhibits plasticity to balance
the pyloric network activity after perturbations, but when the STNS is intact it has been hypothesized that ionic currents and their co-regulation contribute to stabilizing activity of the network controlled by neuromodulators (Khorkova and Golowasch, 2007). It is possible that the modulatory regulations of ionic currents are independent of protein synthesis. This hypothesis is supported by the results obtained by applying ACD to an intact preparation, which verifies that the new genes are not expressed and not necessary for 24 hours of activity and might be inhibited when the STNS is intact. It is possible that modulatory regulation not only regulates ion channels directly, but also inhibits gene expression.

These results were further confirmed when the translation inhibitor CHX was applied to the intact STNS, also causing no change in the pyloric frequency. The overall neuromodulation places a constraint on the level of gene expression and level of mRNA, inhibiting the production of new proteins. Perhaps the mRNA production is regulated by activity-dependent mechanisms (Haedo and Golowasch, 2006, Temporal et al., 2014), and allows the system and its outputs to be dynamically regulated through a balance of activity and modulation. It can be concluded from these results that transcription or translation in the intact STNS is strongly reduced.

4.2 Transcription is Required in a Critical Time Window for Recovery

Transcription is a process by which neurons can intrinsically regulate activity and maintain a homoeostatic state when there is a change in the environment. This homeostatic plasticity is an adjustment of the neural network mechanisms to adapt its function to changes in environmental conditions such as injury or other perturbations.
During development, the brain has the ability to undergo structural changes in response to external cues. There are different cellular elements that underlie plasticity, but one key mediators of plasticity in the developing brain is transcription (Wong and Ghosh, 2002). Similarly, in the STNS after long-term removal of neuromodulatory input, transcription regulates plasticity to allow for recovery of the pyloric network. Once the neuromodulators are eliminated, the system seems to shift into a plastic state that requires expression of specific genes needed to create new ion channels. The genes expressed during this time period could be immediate early genes, which generally encode for transcription factors that regulate gene expression (Klann et al., 2004). Ultimately, this change in gene expression allows the pyloric network return to its homeostatic output after a major change in the neuromodulatory environment.

In an attempt to confirm previous observations of an early transcription time window by Thoby-Brisson and Simmers (2000) two time points where tested 0-4 and 20-24 hours here with ACD. Our results were not conclusive, with one method producing a non-significant effect and the other a clear effect on recovery. Interestingly, the method that supported the existence of a time window is similar to that used by Thoby-Brisson and Simmer (2000) . It is possible that a large sample size will be required to confirm this with both methods. Tentatively, it can be concluded that early (0-4 hrs) transcription is critical for recovery after decentralization. This initial sensitivity to ACD at the time of decentralization suggests that, once triggered, transcription is short lasting, and is followed by a cascade of cellular events that requires 24 hours to complete.

Immediate early gene expression is correlated with various types of stimulation and characteristically only happens during an early critical period after stimulation
(Montarolo et al., 1988, Ribera and Spitzer, 1990). Modulatory input could be repressing these genes from being expressed, and transcription be reactivated when modulators are absent, thus allowing them to play a role in plasticity during recovery. In terms of molecular mechanisms that enable this to happen, one possibility is that, the loss of neuromodulatory input could cause an opening of calcium channels generating a Ca\(^{2+}\) influx, which initiates a cascade of events that causes the activation of calcium/calmodulin-dependent protein kinase II (CaMKII). CaMKII then activates cyclic-AMP-responsive-element-binding protein and other transcription factors, initiating transcription, and regulating the expression of genes leading to ion channel synthesis (Kaminska et al., 1997). It has been shown that many of the ionic currents that regulate pyloric network in an intact STNS change after decentralization. One of the currents that are most drastically altered is the I\(_{Ca}\) current. Another possibility is that Ca\(^{2+}\) is released from the ER. There are several proteins housed in the ER that control movements of Ca\(^{2+}\) across its membrane in normal conditions and in response to environmental stimuli. The ER controls levels of cytoplasmic free Ca\(^{2+}\) locally, and regulates functional and structural changes in nerve cell circuits in different model systems in both the developing and adult nervous systems (Mattson et al., 2000). From our results, we can conclude that transcription appears to be an important factor in recovery of the pyloric activity, but how transcription is activated is unknown.

### 4.3 Protein Synthesis is Required for Recovery of the Pyloric Activity During a Late Critical Period

Protein synthesis, is required for several forms of plasticity. Therefore, regulation of translation initiation is likely to be intimately involved in modulating recovery of the
pyloric activity. The translation-initiation process, which is possibly initiated by perturbations such as the removal of neuromodulators, allows for signaling pathways to be activated (Klann et al., 2004).

When transcription is inhibited during the early (0-4 hrs) time window, the preparations recover their pyloric activity like control preparations do. This is possibly the time in which the mRNA is transported out of the nucleus to the cytoplasm but is not yet ready for translation, or the long distance transport to the dendrites takes a long time. In our hands, CHX incubation periods that included later time windows (4-24 hrs, 20-24 hrs, etc), all blocked recovery of the pyloric activity. These results show that the critical period for translation is late and follows the critical window for transcription by close to 20 hours.

Recovery is possibly a reflection of long-term potentiation and depression of intrinsic properties (Daoudal and Debanne, 2003), which requires changes in gene expression and production of new proteins. Translation is critical for establishing recovery of the pyloric activity. Local protein synthesis enables the STG to control pyloric activity via rapid protein production allowing the network to regain function. The delay between transcription and translation could be the time in which Ca\textsuperscript{2+} signals transcription factors are activated (Kloppenburg et al., 2007, Ebert and Greenberg, 2013) plus the time it takes for mRNA to be transported out of the nucleus to the cytoplasm (and perhaps to the dendrites) where translation takes place (Kaczmarek and Chaudhuri, 1997). After new proteins are produced, they can be used to help recover the pyloric rhythm by adding new ion channels to compensate for the loss of neuromodulatory input.
Neuromodulatory inputs to the pyloric network play a role in the regulation of ionic current expression, and once removed ionic channels are no longer regulated. Most ionic currents in PD neurons after decentralization show transient current density changes that are maximal during the first 24 hours after decentralization (Khorkova and Golowasch, 2007). Neuromodulatory inputs to the pyloric network play a role in the direct and fast regulation of select ionic currents, and once removed ionic channels are no longer regulated. After decentralization, however, most ionic currents in PD neurons show transient but slow current density changes that are maximal during the first 24 hours after decentralization (Khorkova and Golowasch, 2007). These new ionic channels could compensate for the loss of neuromodulatory input.

Overall we found that transcription takes place immediately after decentralization (0-4 hrs) leading to translation closer to the time when recovery of activity typically begins (i.e. 24 hrs). Although we cannot conclude that only translation occurs, or even mainly, during the 20-24 hour time window because there is no significant data, it seems as though translation occurs around that period.

4.4 Future Directions

There are still many unknown aspects of the mechanisms underlying recovery of the pyloric activity. Interpretations of this data can be confirmed by looking closer at the processes that are happening before and after transcription and translation. First, the critical time window of translation can be identified by looking past the 24-hour marker. Translation could possibly be constantly needed to produces a pyloric rhythm in the absence of modulatory input, because the system is no longer stabilized by modulators. In
addition, the initiation of the process of recovery could be more closely examined by looking at the role of Ca\(^{2+}\) in recovery. This can be achieved by using low Ca\(^{2+}\) saline to determine its effect on recovery. Another aspect of recovery that remains unclear is whether there are reserve pools of mRNA or protein before decentralization, which could play a role in recovery. This can be tested by labeling mRNA and proteins using antibodies to see the change in mRNA concentrations when neuromodulators are present and when they are not. More importantly, future experiments could be done to determine what type and where these newly synthesized proteins are being used to allow for the change in intrinsic properties of the neurons. This could be done by labeling specific proteins with immunohistochemistry to look at the location of proteins synthesized and inserted. Another possible mechanism that could play a role in recovery could be endocytosis from, and exocytosis to the plasma membrane of existing ion channels and receptors causing the recycling or destruction of their proteins. Overall, from this study we can conclude that transcription and translation play an important role in recovery of the pyloric network after long-term removal of neuromodulatory input, but these processes are not necessary when the system has neuromodulatory input.
REFERENCES


