Nicotine as a Neuromodulator of the Gastric-Mill Rhythm

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ABSTRACT

Neuromodulators can have long-lasting effects on neural circuits by changing the intrinsic membrane properties of neurons as well as the synaptic connections between them. Over recent years, many neuromodulatory substances have been identified in the stomatogastric ganglion (STG) of different crustacean species. The majority of them are now known to be released onto the STG as either descending or ascending inputs from projection neurons, and some have been shown to circulate as hormones.

One neurotransmitter agonist that has not been characterized thoroughly in the STG is nicotine. Previous studies have found however, that some neurons in the STG do exhibit nicotinic-like responses when their receptors are bound by other cholinergic agonists. Considering that nicotine's modulatory actions are mostly unknown, the exogenous application of nicotine to the STG can be an insightful method for the detection of large-scale changes to motor rhythm output. Results from nicotine-application experiments show that nicotine can activate or terminate the gastric-mill rhythm in *Cancer borealis*, and increase the rate of action potentials fired by the lateral gastric neuron (LG). Wider implications of nicotine's effects are that there are nicotinic acetylcholine receptors in the STG network, and that nicotine can be a state-dependent modulator of the gastric-mill rhythm.

I. INTRODUCTION

The past few decades have allowed for the revelation that many behaviors are driven by the concerted actions of a large distributed network as opposed to simple, dedicated pathways[1]. Simply put, a single network of neurons can switch between activity patterns under various conditions to produce multiple outputs[13]. For example, certain neurons that are involved in tadpole swimming are also recruited for the tadpole's response of struggling[1]. Or, a single network of neurons has been found to oscillate in phase with both crawling and swimming in the leech[1]. The ability of anatomically defined neural circuits to be multi-functional greatly contributes to how central nervous systems can produce and modify behaviors.

The mechanisms that drive multifunctional circuits have mostly been studied in invertebrate crustaceans because of their relatively simple nervous systems. Furthermore, central pattern generators (CPG) - neural circuits that produce rhythmic motor patterns- have been investigated in detail because they can continue to operate *in vitro*. It is now known that neuronal activity patterns are dependent upon the intrinsic membrane properties of each neuron as well as the synaptic connections between them[8]. Change in these properties, therefore, is one mechanism that causes neurons to switch between behaviors and exhibit multi-functionality. The agents that cause this change, also known as neuromodulators, have been studied extensively. The effects of many neuromodulatory substances have now been characterized in the stomatogastric ganglion (STG) of crustaceans.

One substance that has not yet been studied in depth for its modulatory effects is nicotine. Nicotine is of particular interest because of its widespread implications for humans and role in cigarettes as a public health concern. It is also a nicotinic acetylcholine receptor agonist, and acetylcholine is one of the primary neurotransmitters used between neurons in the STG. Previous

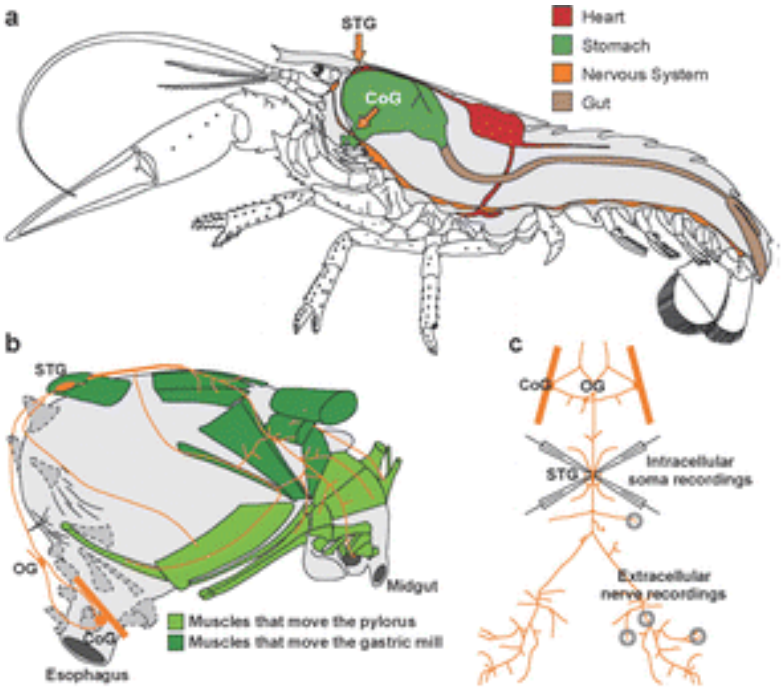
studies show that the cholinergic receptors within the STG are mostly muscarinic. Through bath-application of nicotine to isolated preparations of *Cancer borealis*, the effects of nicotine on STG neurons will be examined, and its modulatory actions will be characterized.

i. An overview of the stomatogastric ganglion (STG)

The stomatogastric nervous system (STNS) is a network of neurons and axons that extends throughout the crab's stomach. The main function of the STNS is to control the movement of four crustacean stomach regions with four different rhythmic patterns; the pyloric, gastric-mill, esophageal, and cardiac sac rhythms. The neurons that drive each rhythmic pattern are dispersed amongst several ganglia within this network, but the two most understood rhythms- the pyloric and gastric-mill rhythms- are driven by neurons that are located in the stomatogastric ganglion (STG).

The STG consists of approximately 30 neurons that surround a neuropil. These neurons are either linked by classic chemical synapses and/or electrically coupled. At chemical synapses, presynaptic neurons release vesicles of neurotransmitters to either excite or inhibit the post-synaptic neuron. At electrical synapses however, the path between coupled neurons is thought to be through gap junctions[6]. Gap junctions mediate communication between neurons by directly linking the cytoplasm of adjacent cells[34]. Current injected into one of the members of a pair of electrically coupled cells is reflected in the other cell. In many cases in the STG, the coupling is bidirectional and injection of current into the second cell results in a voltage deflection in the first[6].

Figure 1. The stomatogastric nervous system



a) Side view of a lobster showing the position of the STNS (*b*) Side view of the lobster stomach showing the muscles that move the pylorus and gastric mill, and the major motor nerves innervating the stomach muscles. (*c*) The STNS *in vitro.* The nerves and ganglia are dissected off free of the stomach. Extracellular recordings are made with pin electrodes placed in Vaseline wells around the motor nerves. Intracellular recordings are made with glass microelectrodes. (*Adapted from Marder, E., and Bucher, D. 2007)*

Most of the STG neurons are motor neurons that innervate striated muscles located in the foregut of the crab (**Fig. 1b).** The cell bodies of these neurons are relatively large in size, allowing for sharp-electrode recordings and reliable intracellular neuron identification. One advantage of this system is that it is routinely possible to have 6-8 extracellular nerve recordings and 4 intracellular soma recordings simultaneously. Another advantage in studying the STG is that the synaptic connections that are important for the production of rhythmic motor patterns are actually found amongst the motor neurons themselves, as opposed to between interneurons of other systems[2, 12]. In this way, the neurons of the STG often participate in motor output as well as in central pattern generation. Before delving deeper into central pattern generation however, it is necessary to first define some of the intrinsic membrane properties that significantly shape the activity patterns of neurons.

ii. The building blocks of network function: intrinsic cell and synaptic properties

All networks depend on both the intrinsic properties of neurons and the synaptic connections between them[4, 9, 13]. The intrinsic properties of neurons result from the sum of all ion channels and conductances found in their membranes[13]. Detailed recordings of STG neurons show that they can exhibit a wide range of intrinsic behaviors that include but are not limited to remaining silent, repetitive firing, rectification, and adaptation [4,5,6, 13, 15]. Rather than describe all of the possible behaviors that neurons show, it is more relevant for my discussion to focus upon a few that play dominant roles in STG central pattern generation. Endogenous bursting, plateau potentials and post-inhibitory rebound are described in more depth below **(Figure 2)** [6, 13].

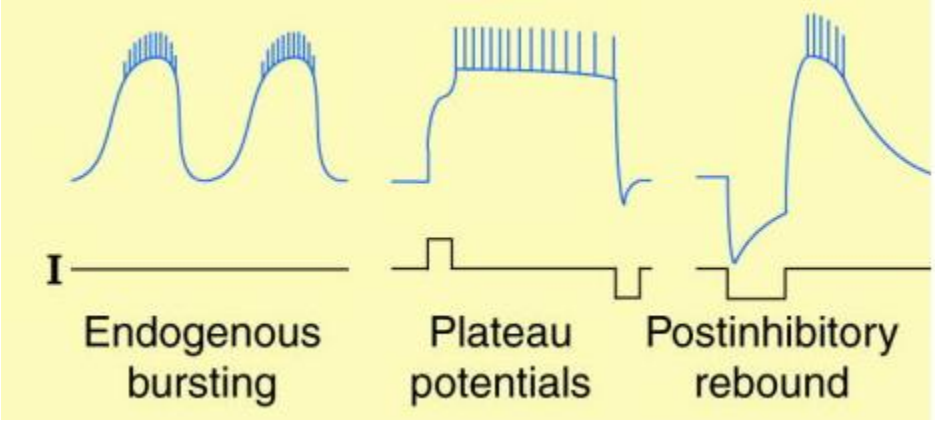


Figure 2. Intrinsic membrane properties

Neurons have different intrinsic properties. Some neurons fire bursts of action potentials endogenously, some neurons can have plateau potentials that outlast the duration of the depolarization, and some some neurons respond to inhibition with rebound firing
(Figure adapted from Marder, E., and Bucher, D. 2001)

Many neurons have endogenous oscillatory properties and can produce rhythmic bursts of action potentials[13, 15]. These neurons often also act as pacemakers in central pattern generating circuits[13, 15]. There are two types of bursting neurons: constitutive and conditional bursters. Constitutive bursters continue to fire rhythmic bursts of impulses when completely isolated from all synaptic input. Conversely, conditional bursters must be activated by modulatory input and lose their ability to burst when isolated[33]. The pacemaker neuron that drives the pyloric rhythm is an example of a conditional burster[33].

Some neurons can display plateau potentials, which means that they are capable of existing at two different stable membrane potentials: a hyperpolarized state with little or no activity, and a depolarized "plateau" state that is tonically active[6,13, 15]. Brief excitation can cause a neuron to transition between each state[8, 13, 15]. Plateau properties are often crucial to pattern generation because they permit rapid transitions between inactive and active periods[5]. The on/off nature of neurons with plateau potentials can act like memories of the neurons' last synaptic input, and produce a discharge pattern that long outlasts their excitatory drive[6].

Finally, there are neurons that respond to strong hyperpolarization with a rebound excitation, which can trigger action potentials[13, 15]. This process is known as post-inhibitory rebound, and results from the activation of a sag-current, or hyperpolarization-activated inward current[13]. When a neuron is under inhibition, the sag-current brings the neuron closer to its threshold even as the inhibition is still maintained. Once the inhibition is released, the neuron reaches threshold rapidly and can fire by rebound[6]. Post-inhibitory rebound is especially important in the STG because most of the synaptic connections within the STG are inhibitory[3,4,5, 6].

The active membrane properties of STG neurons have mostly been identified, and many of them have similar properties[6]. For example, all of the gastric neurons except the gastric medial (GM) neuron can produce plateau potentials. However, this does not mean that neurons which have the same intrinsic properties are identical. Besides their active membrane properties, each neuron differs in at least two other general ways[6]. First, the expression of membrane properties is conditional upon the influence of extrinsic inputs to the circuits. This can come from neuromodulation, sensory input, or a number of other sources that are outside of the network itself. For example, the anterior burster (AB) neuron is suited to serve as a pacemaker neuron because it is the least dependent on extrinsic activation, and so under conditions in which other neuron types are passive, the AB neuron continues to oscillate and drive the pyloric rhythm[6]. Secondly, each neuron's synaptic inputs are unique, and their different activities in network output patterns can stem from the specifics of their synaptic connectivity[6].

Synaptic connectivity takes into account a number of different factors. Of obvious importance are the sign (excitation or inhibition) and strength of a synapse[15]. In the STG, as aforementioned, most of the synaptic connections are inhibitory. Another important measure is the amount of voltage dependence that each neuron has at a synapse, as neurotransmitter release is often a function of pre-synaptic voltages. Temporal considerations also play a large role in synaptic communication, as every neuron has a characteristic post-synaptic potential (PSP) that may vary in time course, often creating differences in the speed of depolarization or hyperpolarization in the post-synaptic cell[15].

The combination of intrinsic cell properties and synaptic connections between neurons influences the dynamics of all neural networks in the STG. One type of neural network that has been frequently studied is the central pattern generator. As in other networks, the motor patterns

produced by central pattern generators are also dependent on the intrinsic cell and synaptic properties mentioned above.

iii. The production of rhythmic movements: central pattern generators (CPG)

Central pattern generators (CPG) are neural circuits that produce rhythmic movements. The kinds of patterns produced by CPGs can fall into two categories—those which are on continuously (respiration, cardiac contraction) and those which are produced intermittently (locomotion, chewing) [45]. Under appropriate experimental conditions, CPGs can produce fictive motor patterns, *in vitro*, even in the absence of sensory or descending inputs that carry specific timing information[4]. Thus, these rhythmic patterns indicate that much of the neural mechanisms required for the production of rhythmic motor patterns reside in the actual CPG neural networks themselves [6].

In general, there are two recognized mechanisms for rhythm production: some networks are driven by pacemaker neurons and some rhythms emerge from synaptic connections between neurons that are not themselves intrinsically rhythmic[4]. A closer look at the pyloric rhythm gives a more detailed description of how pacemakers can shape a rhythm. The pyloric rhythm is characterized by a tri-phasic sequence of activity in the pyloric dilator (PD), lateral pyloric (LP) and pyloric (PY) neurons which alternately dilate and constrict the pylorus[3] **(Fig. 3a)**. It has a cycle frequency between 0.5 and 2 Hz, and is spontaneously active[7, 9]. The dilator phase is generated by the electrical coupling of the pacemaker AB and two PDs- collectively called the pacemaker kernel. Together they inhibit the other neurons of the pyloric rhythm. The LP and PY neurons in turn fire from post-inhibitory rebound and act to constrict the pyloric chamber.

The pyloric rhythm of the STG is driven by a pacemaker, the anterior burster (AB) interneuron, which bursts endogenously and acts as a core oscillator. AB in turn drives other neurons that are not endogenous bursters into a rhythmic firing pattern- such is the case for PD, LP, and PY[4]. On the other hand, the gastric-mill rhythm can be partially explained by the half-center model, which is one of the simplest emergent rhythms[32]. In this case, reciprocal inhibition causes two neurons that fire non-rhythmically in isolation to fire in alternating bursts when coupled[4].

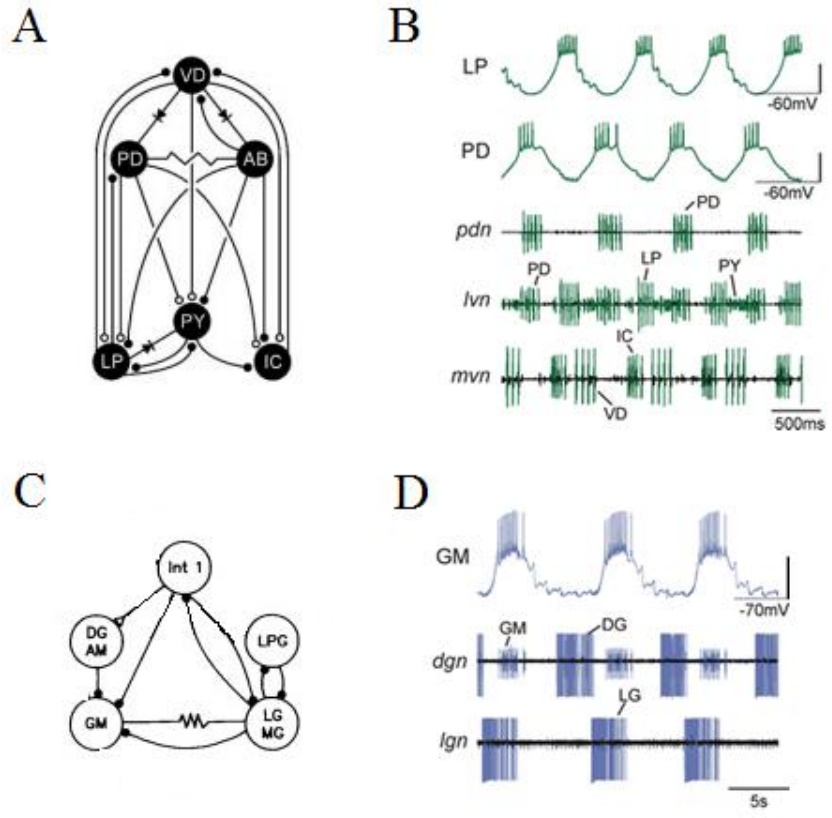


Figure 3. The pyloric and gastric mill circuits

Connectivity diagram showing the synaptic connections between (a) pyloric cells and (b) gastric cells. (c) Intracellular recordings (LP and PD) and extracellular recordings (pdn, lvn, and mvn) of pyloric neurons (d) Intracellular recording of GM and extracellular recordings (dgn and lgn) showing activity of gastric DG, and LG *(A from Weaver, A. L., and Hooper, S.L. 2002) (C adapted from Marder, E. 2012) (B and D from Stein, W. 2009)*

There is much less known about the gastric rhythm than the pyloric rhythm, and there is still much to be studied in terms of how the gastric neurons interact. The gastric mill rhythm is not spontaneously active, but rather episodic and only observed in about half of isolated preparations[7]. The heart of its rhythm consists of a mutually reinforcing endogenous oscillator- the dorsal gastric neuron (DG)- and a half center oscillator between the lateral gastric/medial gastric neurons (LG/MG) and interneuron 1 (Int1) [6, 31]. In the absence of DG oscillation, LG/MG remain silent because there is no mechanism that allows them to escape from inhibition. However, once DG starts to endogenously burst, the resulting inhibition of LG/MG actually triggers a post-inhibitory rebound and causes them to fire in alternation with Int1[6]. The gastric mill (GM) and lateral posterior gastric (LPG) neurons fire in anti-phase with LG/MG[6].

In this way, the gastric pattern uses the reciprocally-inhibited connections between LG/MG and Int1 to shape a two-phase rhythm[7] **(Fig. 3c)**. The first is the "squeeze" mode, and the two lateral teeth are initially spread apart while the medial tooth is held back[6]. MG and LG close the lateral teeth and the four GM neurons move the medial tooth downward and forward[6, 9]. Then, the second "cut and grind" phase involves the retraction of all three teeth, which is caused by the actions of LPG and DG[6, 7]. The gastric rhythm is much slower than the pyloric and has a frequency between 0.05 and 0.2 Hz[7, 9].

The pyloric and gastric central pattern generators have been characterized very well; their neuron's membrane properties have been studied and in some species all of the synaptic interactions within the networks are known. Also, the motor patterns that each network produces *in vitro* are fairly stereotypical, and closely resemble the ones presented above. However, *in vivo*, each motor pattern can show considerable behavioral variation due to extrinsic control. The bath application of many exogenous neuromodulatory substances can often be used to mimic the *in*

vivo conditions, and as a result much recent research has focused on using bath-application of modulatory substances to identify modulatory inputs and the neurons that release them.

iv. Neuromodulation in the STG

Neuromodulation allows nervous systems to adapt their control of physiological functions to a continually changing environment, and it also serves as the basis for many long-lasting changes in animal behavior[16]. STG neurons are the targets of many neurons that synapse on to and influence their network behavior. Some of these inputs may use "classical" neurotransmitters with rapid actions that can change rhythmic motor patterns on a cycle-to-cycle basis[6]. Most chemical synapses within the STG involve neurons that use either acetylcholine or glutamate as classical fast-acting neurotransmitters [6, 20, 21].

On the other hand, many inputs to the STG are modulatory neurons that use slowly acting transmitters, or neuromodulators. It is important to note that many neurotransmitters can also act as neuromodulators if they can modify or alter the intrinsic properties and synaptic connections of neurons for longer periods of time[6]. Sometimes these effects can last for minutes, as opposed to the immediate actions of substances that strictly act as classical neurotransmitters. A variety of mechanisms may explain how neuromodulators work, but the best understood mechanisms are those in which the properties of specific ion channels are modified by the activation of different second messenger systems. Second messenger systems use molecular cascades that transduce information from the binding of a ligand to its receptor to intracellular protein targets[25]. The result of this modulatory input is to change the activity patterns produced by the STG, thereby allowing distinct anatomical neural networks to be multifunctional.

STG neurons receive neuromodulation from three sources; descending axons from cell bodies in higher centers such as the esophageal ganglion (OG) and commissural ganglion (COG); axons ascending from peripheral sensory neurons; and hormones liberated from neurosecretory structures[5]. **Figure 4** summarizes a partial list of the neuromodulatory molecules that have been identified in the input axons that project to the STG as well as by hormone-releasing structures such as the pericardial organ and the sinus gland[22]. With the exception of acetycholine, these substances are not found within the cell bodies of STG neurons themselves[6]. These data were accumulated over the years by many laboratories using a combination of immunocytochemistry and biochemical techniques[5, 10].

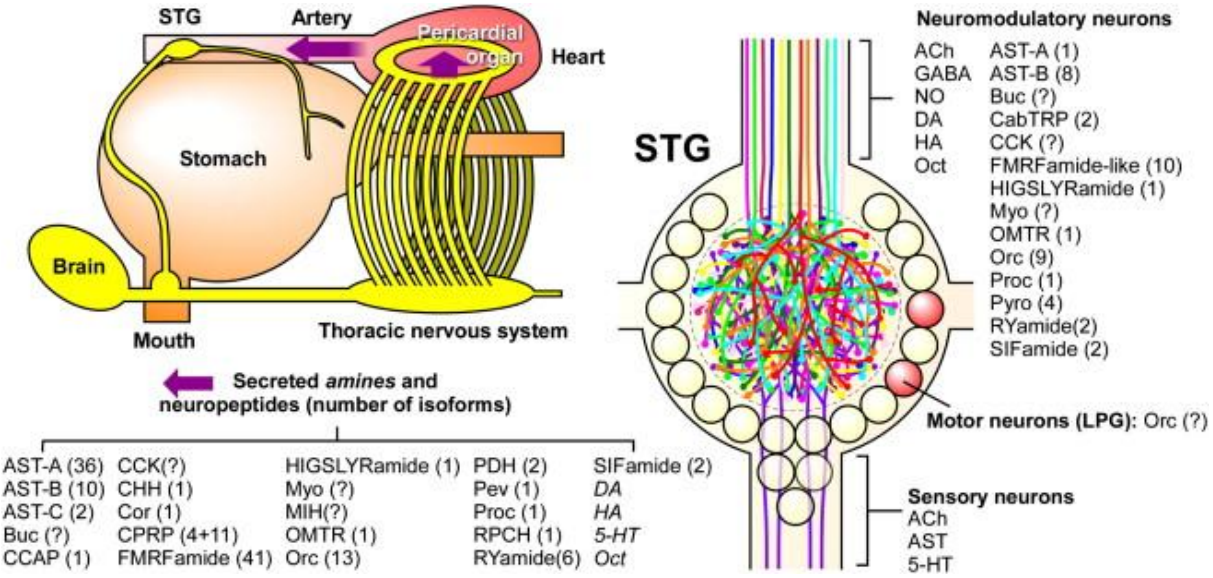


Figure 4. Partial summary of neuromodulatory substances affecting the STG

(Bottom list) Amines and neuropeptides that are secreted by the pericardial organ and circulated to the STG via an artery anterior to the heart. The number of related family members is in parentheses for each of the listed substances. (Right list) Substances that are brought down to the STG by 25 pairs of descending modulatory inputs from neuromodulatory neurons *(Figure from Marder, E. 2012)*

Studies have used the exogenous application of neuropeptides, neuroamines, and neurotransmitters to STG preparations as a first step in discovering the modulatory actions of

these substances. Many of them can modulate ongoing motor patterns, and some can serve as triggers to initiate the motor pattern itself[6]. Modulatory input to the STG can determine the cycle frequency, firing frequency, duty cycle, and phase relationships of the participating neurons[6]. Peptides such as proctolin, red pigment concentrating hormone (RPCH) and cholecystokinin-like (CCK-like) have all been shown to modulate both the pyloric and gastric mill rhythms [6, 23,] . Dopamine, octopamine and serotonin are examples of aminergic substances that can do so as well[6]. Two classical neurotransmitters- GABA and acetylcholine- have also had various modulatory effects on the STG[6]. Most of the identified modulators thus far are released from nerve terminals, but some of them are circulated as hormones. For example, Turrigiano and Selverston found that CCK-like peptides are found within the neuropil and within hormone-releasing organs of crustaceans[26]. They also showed that CCK-like peptide levels rise in the hemolymph of lobsters after they feed, and that injection of CCK can induce the gastric-mill rhythm[38].

Although applying neuromodulators exogenously to the STG is a useful method for determining how neuromodulators influence their targets, there are a couple of reasons why it is also necessary to study the actual neurons that release these substances. For one, a bath applied modulator might have access to more receptors than when it is released from within the network, and the effective concentrations may differ[6]. Another issue is that neurons often release more than one neurotransmitter at a time in a process known as co-transmission, and so modulation in the STG may actually differ quite significantly compared with that resulting from application of single exogenous neuromodulators[17].

Proctolin, one of the best characterized modulators, has been used to study the role of co-transmission in neural networks. Different proctolin-releasing neurons have been found to elicit

different motor patterns when impaled and stimulated[24]. There are three pairs of proctolin containing neurons that innervate the STG: they are the modulatory proctolin neurons (MPN), modulatory commissural neuron 1 (MCN1), and modulatory commissural neuron 7 (MCN7)[17]. MPN contain proctolin and GABA, and strongly activate the pyloric rhythm. MCN7 contains only proctolin, and elicits a pyloric rhythm. MCN1 contains proctolin, GABA, and C. borealis tachykinin-related peptide (CabTRP), and activates a gastric mill rhythm[17].

The study of neuromodulator-releasing neurons and co-transmission is ultimately necessary for further characterization of modulatory substances, but in this study nicotine was applied exogenously as a preliminary observation of its modulatory effects. Although nicotine had not been thoroughly studied in this context before, its role as a nicotinic acetylcholine receptor agonist made it a particularly interesting substance to investigate. Considering that acetylcholine is the primary neurotransmitter that STG neurons use in their central synapses, the neuromodulatory effects of nicotine could be important for further understanding of the activity patterns in the STG.

v. Nicotine as a potential neuromodulator in the STG

Nicotine addiction continues to be a problem for many cigarette smokers- contributing to one of the most significant public health problems in the United States and the rest of the world. Accumulated evidence indicates that nicotinic acetylcholine receptors are a heterogeneous family of ligand-gated ion channels, and that they are expressed in various parts of the brain[30]. By binding to nicotinic acetylcholine (ACh) receptors, nicotine increases the levels of several neurotransmitters, including dopamine. It is thought that the increased levels

of dopamine in the reward circuits of the brain are what cause nicotine to be so addictive for humans[36].

There are two main types of cholinergic receptors: nicotinic and muscarinic. Both types, by definition, respond to acetylcholine. Although nicotinic cholinergic receptors are very common at neuromuscular junctions, their role in synapses between STG neurons themselves is less understood. In general, it seems as though muscarinic responses have been better classified in the STG, and it is thought that the metabotropic currents elicited by muscarinic receptors predominate. Muscarine, oxotremorine, and pilocarpine- all muscarinic cholinergic agonists- have been shown to trigger rhythmic patterns from neurons in both the pyloric and the gastric mill networks[27, 28, 29].

In one experiment by Marder and Paupardin-Tritsch, it was found that a nicotinic depolarization- similar to the type that would be found on STG muscles- could be elicited on PD, VD, and AB by the iontophoretic application of ACh[37]. This response was mimicked by the application of nicotine and blocked by curare and other nicotinic blocking agents[37]. There have also been a couple of modulatory neurons found in the STNS that have nicotinic actions on STG neurons.

In a study by Russell and Hartline, stimulation of two input neurons (termed "IV neurons") appeared to act on curare-sensitive (a nicotinic ACh receptor blocker) receptors on PD, generating brief conventional nicotinic EPSPs[51]. Another study by Katz and Harris-Warrick showed that stimulation of gastropyloric receptor cells (GPR) evoked excitatory postsynaptic potentials (EPSPs) in both LG and DG. The EPSPs exhibited nicotinic pharmacology, indicating that they may be due to the release of acetylcholine from the GPR cells[31,50]. Fast nicotinic cholinergic EPSPs, therefore, may be important for reflex functions in the gastric mill[31].

II. RESULTS

i. Both the gastric rhythm and nicotine-application obscure lvn recordings

Initially, I applied nicotine to STG preparations with the front-end intact. Lvn recordings were taken from preparations in control saline when the gastric rhythm was both on and off, and also after the application of nicotine. In control conditions when the gastric rhythm was off, three cells fired in the expected tri-phasic sequence. The first burst of spikes per pyloric cycle was LP, which was followed by PY and then PD. Every preparation (N=7) had these units when sitting in control saline with the gastric rhythm off.

In preparations where the gastric-mill rhythm spontaneously turned on, however (N=3), the lvn also showed periods of spiking from another cell, in addition to the spikes from LP, PY, and PD. When active, the spikes fired by the other cell obscured the underlying pyloric rhythm, but after the termination of the extra spikes the pyloric rhythm became clearly visible on the lvn again.

Interestingly, bath-application of nicotine also excited a similar spiking pattern on the lvn, as shown in **Figure 6**. At first, it was thought that the application of nicotine may have caused the pyloric rhythm to crash by altering the activity of the pyloric neurons, but similarly to the gastric rhythm recordings it appeared that the pyloric rhythm was only being obscured. Based on previous literature, it was assumed that this extra component was the lateral gastric (LG) neuron[32]. This was later determined to be the case as the spikes were shown to fire simultaneously with extracellular lgn spikes and out-of-phase with extracellular dgn spikes. This behavior was observed in 3 out of 7 preparations that nicotine was applied to.

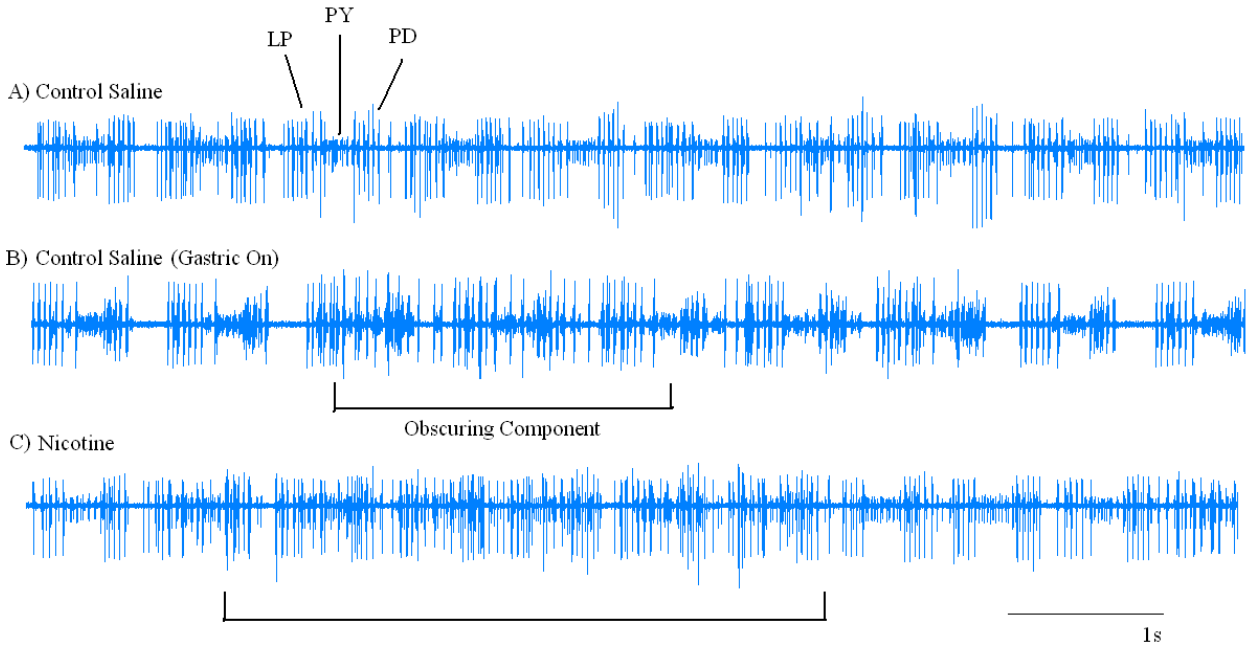


Figure 6. Extra lvn component in the presence of nicotine and gastric rhythm

(a) Extracellular lvn recording showing triphasic pyloric firing of LP, PY, and PD spikes in control saline when the gastric is off (b) With the gastric rhythm spontaneously turned on, there are periods where an extra spiking component on the lvn partially obscures the underlying pyloric rhythm. After the spikes stop the pyloric rhythm can be seen again (c) In the presence of nicotine, lvn recordings closely resemble that of conditions in which the gastric rhythm is on

The similarities between lvn recordings under gastric activity and during nicotine application led to the early hypothesis that nicotine was activating the gastric rhythm. Thus, experiments were focused towards the effects of nicotine on gastric cells. MG, IC, GM, LG, and DG are all STG neurons that had been shown to participate in the gastric rhythm in previous literature[9]. The first step was to determine which of these gastric cells, if any, would respond to nicotine. DG was expected to be the most sensitive neuron since it is often used as a marker for the gastric rhythm, but after performing intracellular recordings on DG (N=2), GM (N=2), MG (N=1) and IC (N=1), I found that none of these cells showed much of a response to nicotine that differed from their control behaviors. LG, on the other hand, increased its spiking frequency after nicotine was applied in 16 out of 16 preparations.

ii. LG increases firing-frequency when STG is bathed in nicotine

When first impaled by a sharp electrode, LG fired spikes rapidly for about 10 seconds in response to the physical perturbation before returning to its resting potential. It then usually remained silent, but in a few preparations it also fired tonically. Under control conditions when the gastric was off, LG had membrane oscillations of about 8mV in amplitude. In the trace shown in **Figure 7A,** LG's resting potential varied between -65mV and -58mV, but no spikes were fired. In other preparations, LG remained silent for the most part but was occasionally able to fire spikes in control saline. The average frequency with which LG spiked in control saline was 0.30 ± 0.10 spikes per second **(Table 1)**.

300 seconds after 10^{-5}M nicotine was applied to the preparation, LG showed the same waveform, but the frequency with which it fired action potentials increased to 1.37 ± 0.23 spikes per second. The threshold for LG to spike varied in each preparation, but it was usually within 1mV of LG's peak membrane oscillation. For example in **Figure 7B,** LG repeatedly depolarized to about -58mV without firing a spike, but then rapidly fired spikes once it reached -57mV. Nicotine seemed to allow LG to reach its threshold voltage more frequently, suggesting that it made the cell more excitable. The increase in LG spike-frequency was seen in all 16 preparations performed, and thus implies a strong correlation between nicotine-application and increased LG activity. Furthermore, in 5 of these preparations, LG also transitioned from tonic firing to periodically bursting after the application of nicotine.

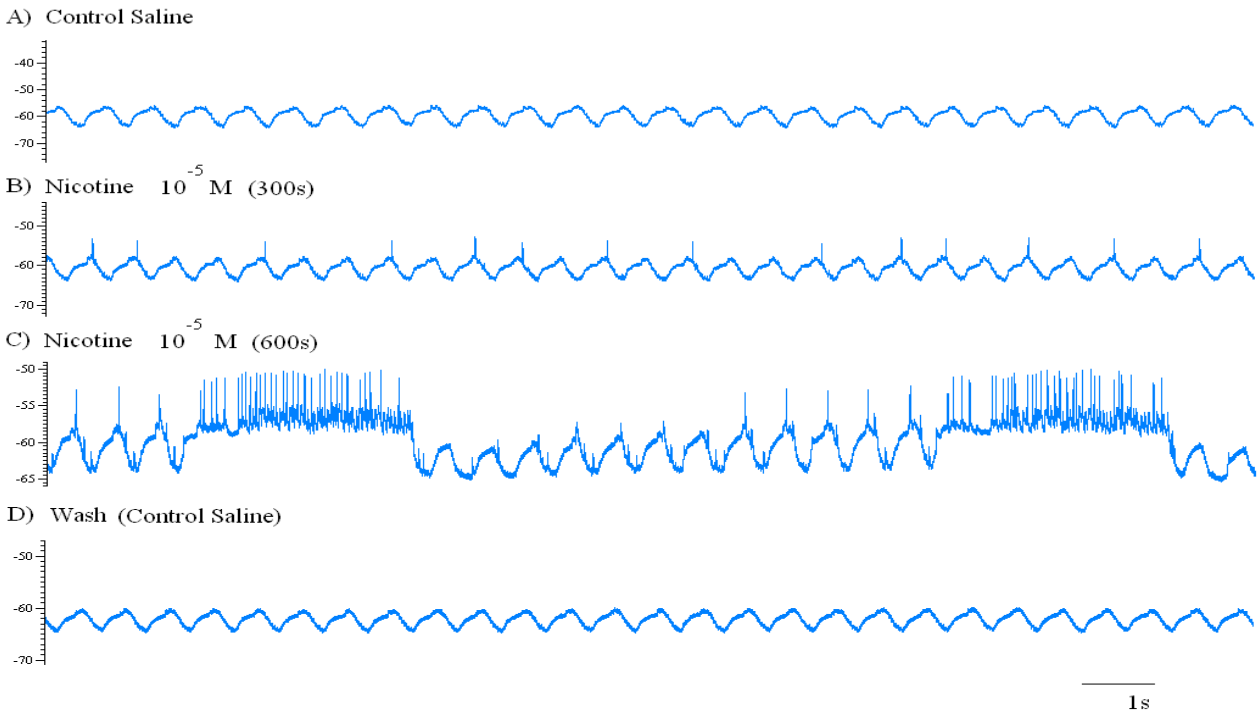


Figure 7. Nicotine inducing spiking and bursting in LG

(a) In control conditions LG remained silent (b) After the application of 10^{-5}M nicotine LG periodically fired spikes in a un-patterned fashion (c) When nicotine was applied for longer periods of time, LG switched from un-patterned tonic firing into a bursting mode, firing many spikes before returning to its resting membrane potential (d) Half an hour of washing with control saline removed the effects of nicotine

iii. Nicotine can also trigger LG to burst

As shown in **Figure 7,** LG switched from tonic un-patterned firing into a bursting mode 600 seconds after 10^{-5}M nicotine was applied (N= 5). During a burst, LG fired many spikes in rapid succession without returning to its resting potential. Instead, LG stayed depolarized above its threshold level for spiking until the burst ended. In preparations where bursting was triggered, the average frequency of spiking was 6.40 ± 4.05 spikes per second, as compared to 1.37 ± 0.23 spikes per second when LG was not bursting. Also, before each burst, LG fired 4-5 tonic spikes

that seemed to lead into the burst. After the termination of each burst, LG returned to its resting potential and oscillated silently before starting the cycle again.

The cumulative results suggest that nicotine's most significant influence on LG is to increase the frequency by which it fires action potentials, often changing its intrinsic membrane property from remaining silent to firing tonically. In some preparations nicotine also switched LG from tonic firing to bursting. There were also other aspects that changed in each preparation with the application of nicotine- including pyloric frequency, spike amplitude, and voltage fluctuations- but no pattern emerged as clearly as the increased rate of spiking in LG. The spike-frequency data is summarized in **Table 1** below.

	Pre-Nicotine		Post-Nicotine		t-test for spike frequency (p)
N=16	Total Spikes per 300s	Spike Frequency (s^{-1})	Total Spikes Per 300s	Spike Frequency (s^{-1})	
Average During Tonic Activity (N=11)	92 (SEM=31)	0.30 (SEM=0.10)	411 (SEM=69)	1.37 (SEM=0.23)	0.00043
Average During Bursting (N=5)	416 (SEM=120)	1.39 (SEM=0.40)	1919 (SEM=544)	6.40 (SEM=1.81)	0.025

Table 1. Total spike and spike-frequency data

Values are expressed as mean (standard error). Total spikes were counted 300 seconds prior to and after nicotine application. Spike-frequency values were calculated by dividing total spikes by duration of application. Results were counted during tonic firing and during bursting. Spike-frequency t-test data show that the difference is significant. $p < 0.05$

iv. T-test results indicate that the changes in spike-frequency are significant

Spike-frequency data for LG, **Table 1**, was separated into two categories: during tonic firing and during bursting. The purpose of grouping the data in this manner was to limit the variability between preparations. The large number of spikes fired during each burst could have potentially been misleading when combined with the relatively smaller changes in spiking during tonic firing.

For preparations in which LG remained in a tonic firing mode, the average number of spikes fired per second before nicotine application was 0.30 (SEM=0.10). After nicotine application, the average was 1.37 (SEM=0.23) spikes per second. In preparations where LG bursted the average frequency of spiking before nicotine application was 1.39 (SEM=0.40) spikes per second and 6.40 (SEM=1.81) spikes per second after nicotine application. The initial data seem to show that firing-frequency does in fact increase in response to nicotine-application, but the standard error for each value was very high, suggesting that there was much uncertainty in terms of how representative the study sample was.

A paired t-test was performed in order to determine whether or not the spiking frequency increased after the application of nicotine. The p-values for both cases ($p= 0.00043$ when tonic firing and $p= 0.025$ when bursting) were below the threshold value of $\alpha= 0.05$, indicating that the results were statistically significant and different. Therefore, it appears as though spiking-frequency in LG does increase after the application of nicotine.

As another method of showing that LG increases its spiking in response to nicotine, 10^{-4} M tetrodotoxin (TTX) was first used to block all ascending modulatory input to the STG.

v. Nicotine affects LG even when descending input is blocked

When TTX, a sodium channel blocker, was placed in a vaseline well around the stn, all modulatory input to gastric cells from projection neurons in the front-end was cut off and the activity of LG stopped immediately in control saline (N=1). The pyloric rhythm frequency slowed down, but LP, PY, and PD continued to fire as shown from lvn trace in **Figure 8.** After 10^{-5}M Nicotine was applied to the preparation, the pyloric frequency increased and LG began to fire tonically.

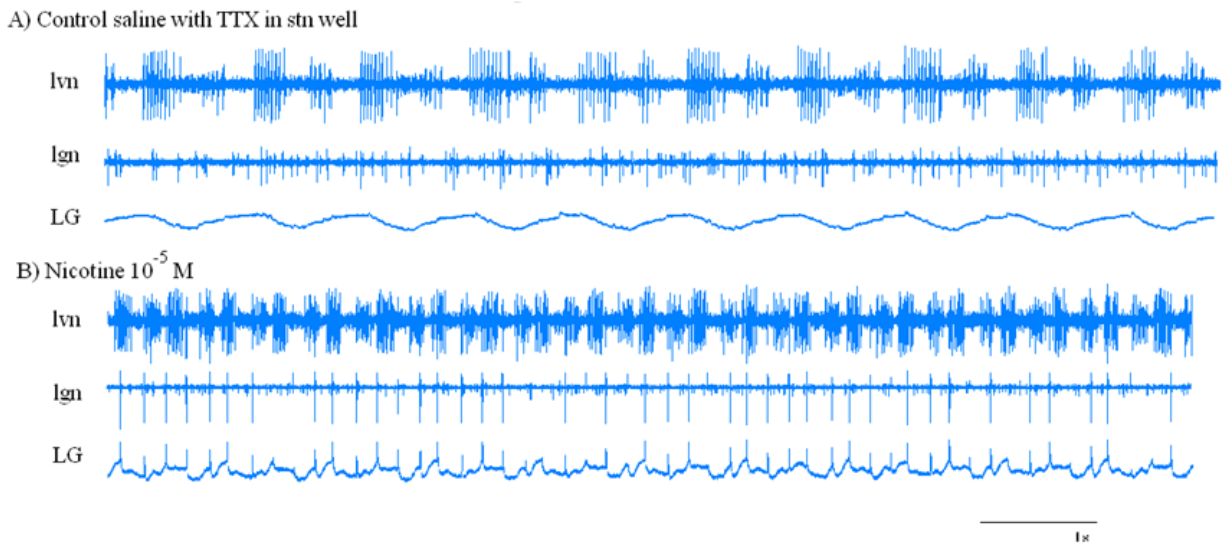


Figure 8. Nicotine increasing

(a) In control saline, 10^{-4}M TTX and 750mM sucrose were placed in a well around the stn, immediately slowing down the pyloric frequency and making LG silent (b) After the application of 10^{-5}M nicotine, LG began to spike tonically

This experiment shows that LG's activity is due to modulatory input from nicotine, and not from other descending modulatory inputs. It appears as though nicotine either acts on neurons within the STG directly, or on projection neurons that provide ascending inputs. The implications of these results will be discussed later, but they may be evidence for identifying the location of nicotinic cholinergic receptors in the STG network.

vi. The effects of nicotine are dosage-dependent

One question that surfaced from previous results was whether or not the concentration of nicotine was important for LG's behavior. To further investigate the mechanisms triggering LG's switch from being silent to tonically firing or bursting, four different concentrations of nicotine (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) were applied to each preparation (N=3) in order from least concentrated to most concentrated. Saline was perfused onto the preparations to wash out nicotine in between each concentration, and LG's frequency of spiking was measured after the application of each concentration. LG traces during the experiment are shown in **Figure 9**.

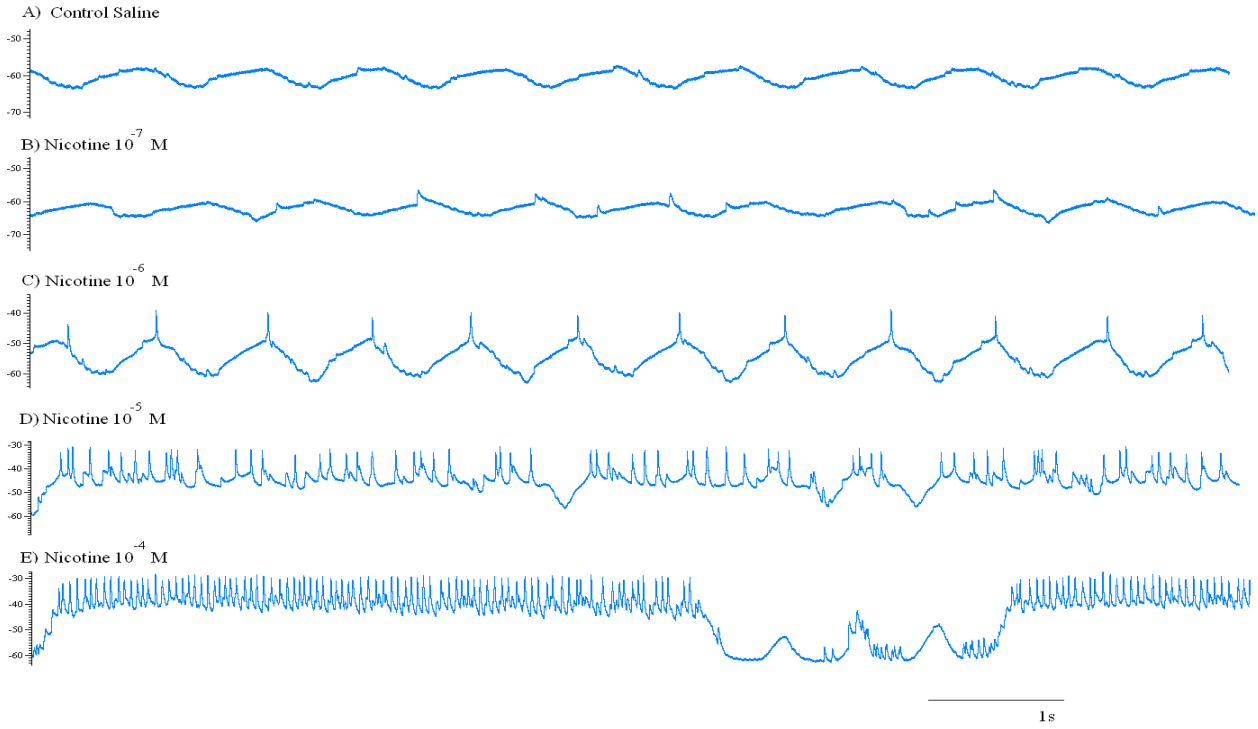


Figure 9. Dosage-dependent spike frequency of LG in response to nicotine

Dose-response of the LG neuron to bath application of nicotine. (a) In control saline, LG remains silent (b) nicotine at 10^{-7}M does not induce spikes but does change the shape of its EPSP (c) 10^{-6}M nicotine caused LG to spike tonically (d) 10^{-5}M nicotine increased the spike frequency and caused LG to weakly burst (e) 10^{-4}M nicotine fully switched LG into a bursting mode. Half an hour saline washes were performed in between each concentration

In all 3 preparations, LG remained completely silent in control saline and did not fire a single action potential. 10^{-7} M nicotine increased the spike frequency (N=2/3) of LG to an average value of 0.12 spikes per second. All concentrations of nicotine above 10^{-7} M (N=3/3) also increased the frequency of spiking in LG. After 10^{-6} M nicotine, LG fired 1.05 spikes per second. After 10^{-5} M nicotine, LG fired 4.02 spikes per second. Lastly, after application of 10^{-4}M nicotine, LG fired 5.63 spikes per second.

These results show a correlation between concentration of nicotine and spike-frequency in LG **(Figure 10)**. There is a trend that the spike-frequency in LG increases as the concentration of nicotine applied to the preparation increases. Furthermore, it appears as though the threshold concentration for nicotine's actions on LG was lower than 10^{-7} M, since 10^{-7} M nicotine affected LG's behavior in 2 out of 3 preparations. The big discrepancy in spike-frequency (1.05 spikes per second at 10^{-6} M and 4.02 spikes per second at 10^{-5} M) is partially due to the activation of bursting in LG between these concentrations. This may show that the threshold concentration for nicotine to induce bursting in LG lies between 10^{-6} M and 10^{-5} M.

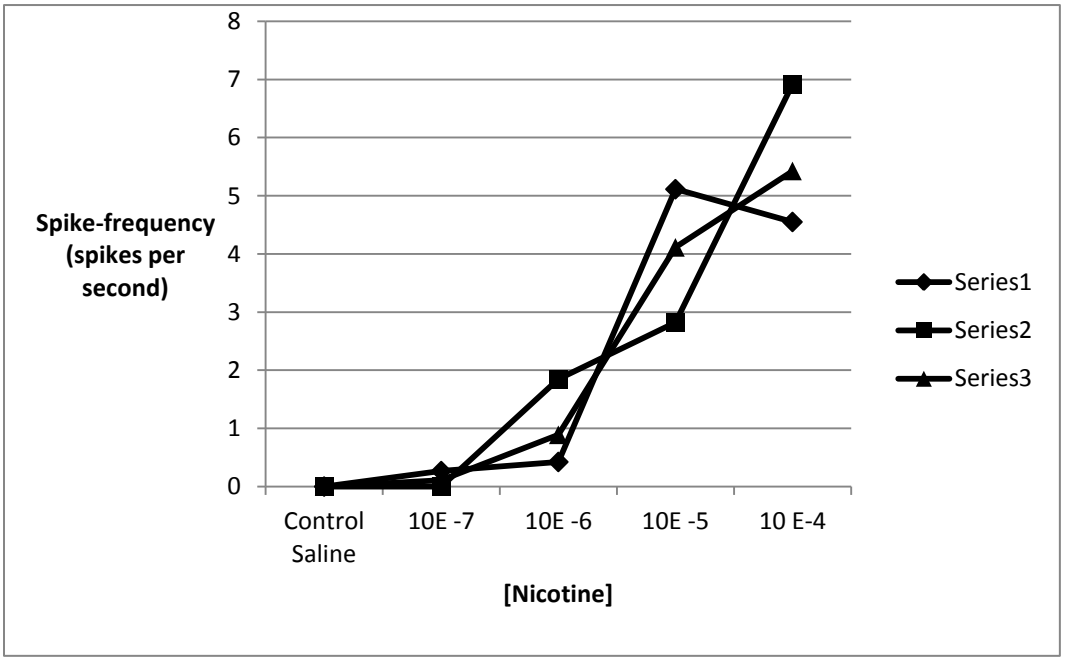


Figure 10. Plot of nicotine's dosage-dependent actions

For 3 preparations, the spike-frequency of LG was plotted against different concentrations of nicotine. There seems to be a correlation between increased spike frequency and increased concentration

Despite the trend that spike-frequency increased with increased nicotine concentration, a confounding variable is the timing of nicotine's effects. It is important to note that nicotine's effects also became more pronounced as each experiment went on. This presents the possibility that nicotine's effects were additive over time and not due to increased concentration alone. Another problem is that at concentrations above 10^{-6}M, it often became difficult to wash out the effects of nicotine in between experiments. Although control saline was perfused for up to two hours, it is possible that longer periods of wash were required to return the preparation to control conditions. On the other hand, the persistence of nicotine's effects on LG even after nicotine was completely removed from the bath may be a representation its long-lasting modulatory effects.

vii. Nicotine can activate or terminate the gastric rhythm

The dosage-dependent experiments show that LG can have a progression of behaviors. It seemingly switched from being silent to tonic firing and then to bursting. Spike-frequency data, see **Table 1**, also showed that bursting could be triggered in LG. To determine how LG's activity state before nicotine application influences its activity state after nicotine-application, results from earlier experiments were examined for activation of the gastric rhythm. In 5 out of 16 total preparations, the gastric rhythm turned on within ten minutes of 10^{-5} M nicotine application.

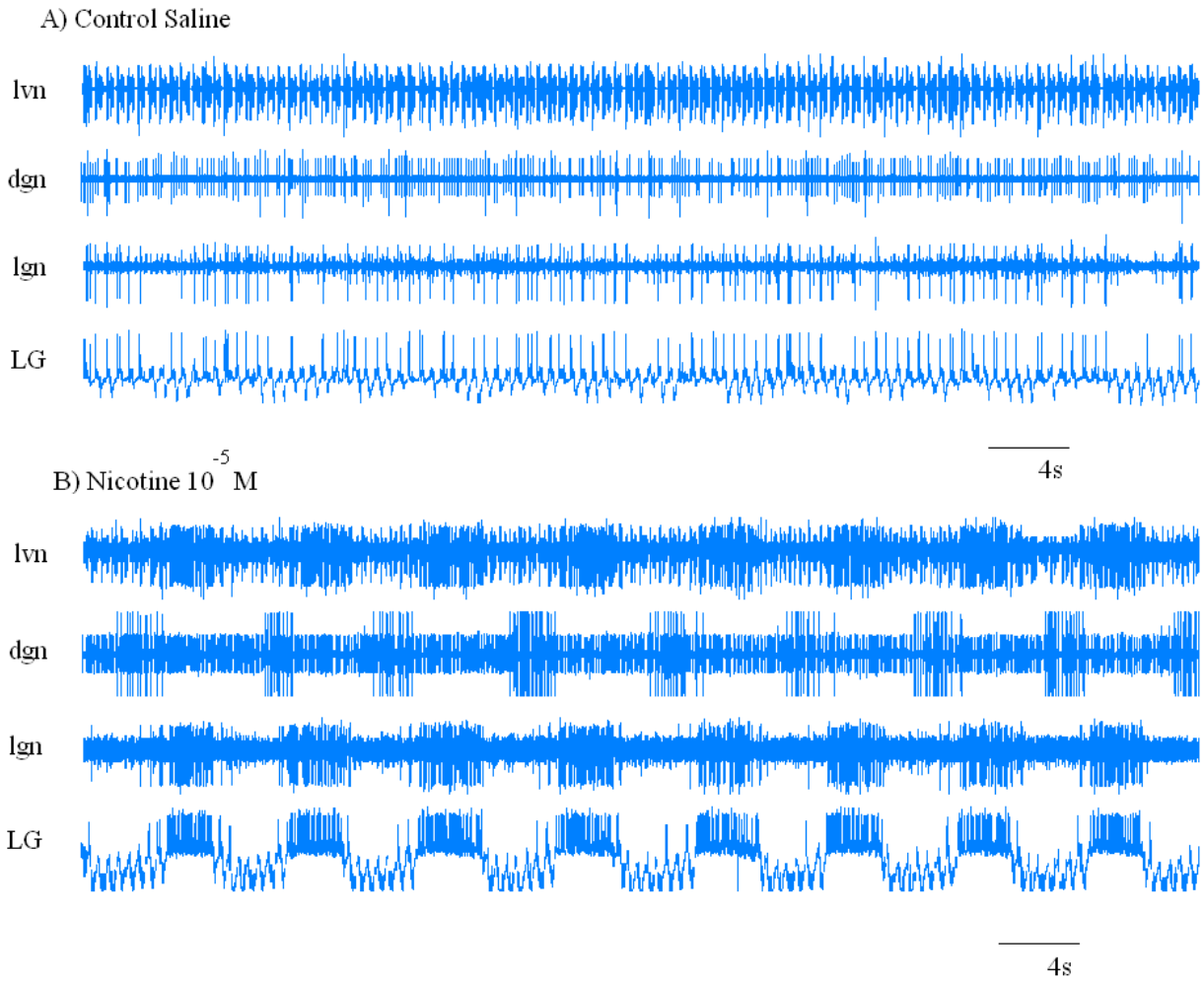


Figure 11. Activation of the gastric-mill rhythm by nicotine

(a) In control saline, extracellular and intracellular traces of LG show that it is weakly bursting. Extracellular dgn traces show that DG is relatively silent. Only the largest spikes on the dgn are from DG. (b) After the application of 10^{-5}M nicotine, both LG and DG fired strong bursts of spikes in alternation. This pattern of firing resembles the gastric rhythm.

In **Figure 11**, LG was weakly bursting and firing only a few spikes before returning to its resting potential when the preparation was in control saline. DG, on the other hand, was relatively silent and only fired the occasional spike. In a previous study Marder and Weimann found that the activation of bursting and plateau properties in DG appeared to play a significant

role in the activation of the gastric rhythm[23]. Here, however, the endogenous bursting of DG was not necessarily a requirement for the activation of the gastric rhythm. After nicotine was perfused onto the preparation, LG fired very robust bursts that also coincided with alternating DG bursts. In the single preparation shown above, the two neurons fired out-of-phase with each other; LG fired for 4 seconds at a time while DG remained silent, and then DG fired for 3 seconds while LG remained silent. This pattern of firing closely resembles the gastric rhythms that were activated by the exogenous application of other substances in previous works[23, 38, 46].

In all preparations, the gastric rhythm was only activated if LG was already in an active state- either spiking frequently or bursting. From **Table 1** the average spiking frequency of LG in control saline was 1.39 (SEM=0.40) spikes per second when LG ended up bursting. This was much higher than the spike-frequency, 0.30 (SEM=0.10) spikes per second, seen in control conditions when nicotine-application did not induce bursting. There were no cases in which LG changed from being in a silent state to directly participating in the gastric rhythm. This suggests that increased excitability of LG is an important factor for nicotine when turning the gastric mill rhythm on. On the other hand, when the gastric rhythm turned on spontaneously in several preparations, the activity of LG just prior to activation of the rhythm was variable. In some preparations, LG was silent just before the gastric rhythm turned on and DG and LG immediately began to alternately burst.

In 2 preparations in which the gastric rhythm did not naturally turn off after a long period of time, 10^{-5} M nicotine application turned the gastric rhythm off and caused DG and LG to become silent. One of the preparations is shown below in **Figure 12**. Although this result was only seen in 2 preparations in all of the experiments that were performed, the change suggests that nicotine may modulate the gastric circuit in a state-dependent manner.

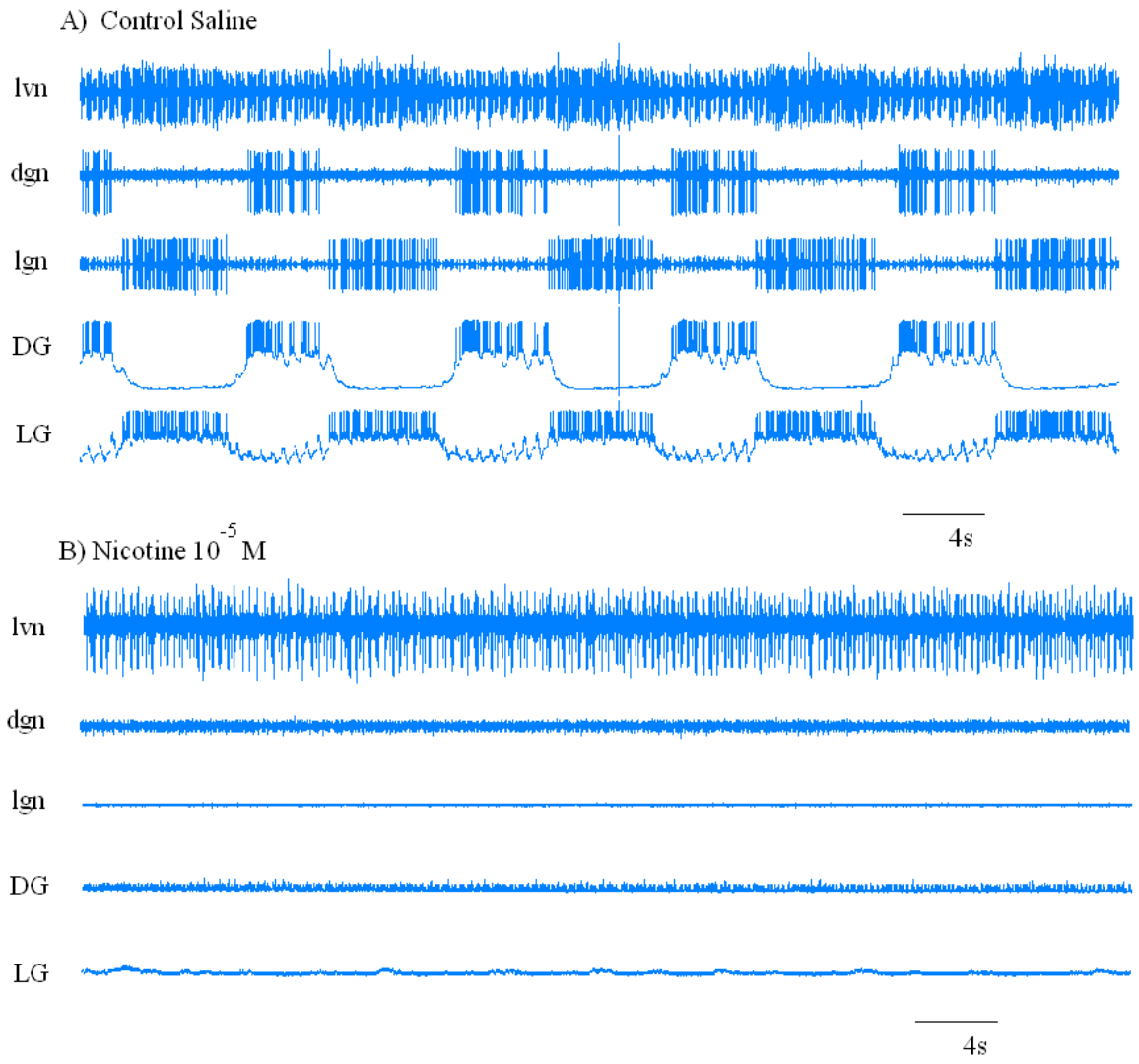


Figure 12. Termination of the gastric-mill rhythm by nicotine

(a) In control saline, the gastric mill rhythm was sometimes spontaneously turned on. Extracellular and intracellular traces show the alternating bursts of behavior from LG and DG. (b) After the application of 10^{-5}M nicotine, however, the gastric mill rhythm turned off and both DG and LG became silent

III. DISCUSSION

i. Ionic currents that lead to neuron spiking and bursting

At the cellular level, the distribution of voltage-dependent and calcium-dependent channels across the membrane of a neuron collectively determine the properties of the neuron. LG's increased firing of action potentials in response to nicotine may be due to changes in the conductance of a number of channels. In the simplest case, an increase in LG's membrane potential could activate voltage-dependent sodium or calcium channels that act to further depolarize its membrane potential. It is during this rapid inflow of ions that a spike forms. Following the spike, activation of outward potassium channels eventually reduces the membrane potential back to rest conditions. It is more likely however, that LG fires action potentials through mechanisms that are mediated by a variety of other ion channels. Harris-Warrick's review of voltage-dependent ion channels presents some of the important currents that increase firing rates in STG neurons[44].

The currents that voltage-dependent sodium channels produce, I_{Na}, are usually divided into two categories. The first, as just mentioned, is a rapidly inactivating current that drives the rising phase of an action potential. The second is a low-threshold persistent current, I_{NaP}, that can initiate and maintain tonic firing[44]. Another current, I_h, is a hyperpolarization-activated inward current that deactivates relatively slowly at subthreshold voltages, suggesting that it could contribute to the sub-threshold inward currents that initiate tonic or burst firing[44]. I_h is also known as the sag-current because it acts as a depolarizing leak current that can tonically depolarize neurons, thereby causing the onset of increased spike frequency[44]. I_A, the transient potassium current, is an outward current that is activated by depolarization. Once activated, it

can retard spike initiation in response to depolarized potentials. When this current is reduced by 4-aminopyridine, the cells involved in the pyloric circuit fire at higher frequencies[44].

Besides increased tonic spiking, there were also preparations in which LG switched into a bursting mode as shown in **Figure 7 and 8.** One known mechanism for bursting in several STG neurons starts with a decrease in the potassium leak current, which depolarizes the neuron until voltage-gated sodium and voltage-gated calcium currents are activated[45]. This further depolarizes the neuron and activates the fast-spiking and plateau currents, which lead to the firing of many spikes. Inactivation of the sodium and calcium currents, combined with the actions of the calcium-activated potassium current, I_{KCa}, brings the cell back to its resting potential[45]. It is possible that the bursting seen in LG is caused by the mechanism just described, but the results shown here do not provide any evidence in support for or against it. Further work with specific channel blockers must be done to identify the exact channels that are affected by the application of nicotine.

ii. Intrinsic properties that can drive LG to burst

The intrinsic properties of each neuron that generate bursts have been studied in the STG, but other systems have also been helpful. In molluscan pacemaker neurons, for example, it has been shown that depolarizing spike after-potentials are a major factor in sustaining normal bursts[52]. Or, in the leech heart, postinhibitory rebound has been shown to help initiate and sustain bursts[6]. An especially dominant burst-generating mechanism in STG neurons is plateau potentials. Plateaus result from a sufficient depolarization that initiates inward current and leads to a self-sustained depolarized state. This depolarized state, as seen in **Figure 7C**, is an

underlying component of bursting. Although plateau potentials were not specifically tested for in my studies, LG is known to be capable of having plateau potentials[6].

iii. Synapses within STG neurons or modulatory input from projection neurons

All of the changes seen in LG after the application of nicotine imply, at the most basic level, that there are in fact nicotinic acetylcholine receptors somewhere in the STNS. These receptors could be directly on LG itself, or on other projection neurons that are outside of the STG. Knowing that nicotinic receptors produce fast EPSPs when bound by nicotine, it is reasonable to argue that exogenous nicotine may just directly bind to LG receptors to cause increased spiking. This model could explain some of the results seen, as nicotine-produced EPSPs could periodically cause LG to reach its threshold potential and fire action potentials. Acetylcholine, another neurotransmitter and cholinergic agonist, serves as a good analogy because it has been shown to bind to muscarinic receptors directly on neurons within the STG and exhibit its modulatory actions through metabotropic pathways. One problem that exists with the above explanation however, is that nicotinic acetylcholine receptors are ionotropic receptors, meaning they are directly linked to ion channels and do not use secondary messenger pathways. Ionotropic receptors typically do not have modulatory actions, and only act on the scale of milliseconds[6]. This would contradict the results seen as the effects that nicotine had on LG often lasted for minutes.

It seems more likely that nicotine binds to receptors on projection neurons that in turn release other neuromodulatory substances onto the STG. There are many identified projection neurons, most of which have cell bodies in the OG or CoG, and many of them have been shown to release substances that modulate the gastric rhythm. However, as **Figure 8** shows, nicotine

still appears to be effective even when descending modulatory inputs from the OG and CoG are blocked. This leads to two hypotheses: either that the STG receives its modulation from projection neurons located inside the STG itself, or from projection neurons that provide ascending input to the STG. There have currently been no identified projection neurons within the STG itself, but there are a couple of identified projection neurons that are located below the STG (GPR on the lvn and AGR on the dvn)[6, 53] . Both of these neurons have also been shown to modulate the gastric rhythm when stimulated.

iv. LG bursting into the gastric-mill rhythm

The gastric CPG was originally proposed to be a network oscillator, whose rhythms arose mainly from synaptic connections[29]. When they are recorded, gastric rhythms correlate with the presence of active and bursting membrane potentials in at least some gastric neurons[29]. Thus far, DG is the only known endogenously bursting neuron in the gastric circuit that can burst even in the absence of modulatory input. As a result, DG assumes a critical role in the gastric rhythm. Marder and Weimann have suggested that the activation of bursting and plateau properties in DG by TNRNFLRFamide and SDRNFLRFamide appears to play a significant role in gastric rhythm activation[29].

An interesting result seen in the nicotine experiments is that the gastric rhythm only turned on when LG was already in an excited state. The activation of the gastric rhythm seemed to be preceded by tonic firing or weak bursting in LG, as indicated by the higher number of LG spikes fired per second in control conditions **(Table 1).** This seems to suggest that the underlying ability of LG to burst is also important in the gastric rhythm-generating mechanism. Evidence supporting the role of LG in the gastric rhythm can be drawn from experiments in which Elson

and Selverston applied pilocarpine to modulate the gastric rhythm[29]. After blocking descending modulatory input, they found that LG became a conditional burster in the presence of pilocarpine, and that it was able to burst repetitively via intrinsic oscillations. However, when bursting in LG was suppressed with hyperpolarizing current injection, the gastric rhythm was strongly perturbed and the activity of all other recorded cells was altered.

v. State-dependency of nicotine's modulation

In addition to turning on the gastric rhythm, the application of nicotine was also involved in the termination of the gastric rhythm in a few cases, as seen in **Figure 12**. Although the termination of motor patterns is an important feature of neuromodulatory control, its mechanisms are less understood than those for activation. For one, termination could result from the cessation of firing in projection neurons that sustain motor patterns. Alternatively, there could be projection neurons that have the specific action of turning components of CPG networks off. In the lobster *H. gammarus*, a brief discharge of the pyloric suppressor neuron (PS) has been shown to halt pyloric activity for prolonged periods of time[54]. PS appears to suppress oscillatory properties in PD and LP when activated.

The only cases (N=2) that had nicotine turning off the gastric rhythm were observed when the gastric rhythm was already spontaneously on. The termination of the gastric rhythm, therefore, may be a state-dependent response to nicotine. The physiological response of each neuron to modulatory input is not absolutely fixed, and each neuron can respond differently to a given input depending on the previous history of the CPG. For example, proctolin can turn on the gastric rhythm when it is silent, or it can also slow down the pattern if the gastric mill is already highly active[6]. The intrinsic properties of a neuron also depend on its recent history of

activation[55]. Thus, state-dependent mechanisms are likely to be important for CPGs to provide communicate with their modulatory inputs, further emphasizing the role of ascending sensory feedback in STG systems.

vi. Future insights

Nicotine appears to exert modulatory effects on the gastric circuit in the STG, showing the ability to turn the gastric rhythm on or off. Furthermore, nicotine-application to STG preparations resulted in repeated increases in the spiking-frequency of LG, indicating that LG may be the target of nicotine's actions. There are questions that cannot be answered at this time- such as 'what are the ionic mechanisms that lead to nicotine's effects?' or 'why does the gastric rhythm only turn on in select preparations'- but the characterization of nicotine in this work contributes to a growing list of known neuromodulatory substances that affect the STG. Future studies should further investigate the presence of nicotinic acetylcholine receptors in the STG, as well as the influence that bursting in LG may have for the gastric rhythm.

IV. MATERIALS AND METHODS

Animals

Cancer borealis were purchased from Commercial Lobster (Boston, MA). The tanks were kept at 11ºC and crabs were used within a week of purchase.

Solutions

C. borealis physiological saline was composed of 440mM NaCl, 11mM KCl, 13mM $CaCl_2$, 26mM $MgCl_2$, 11mM Trizma Base, and 5mM Maleic acid in distilled water. The pH was between 7.4-7.5. 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4}M nicotine were prepared by serial dilutions of nicotine (purchased from Sigma) in saline. A stock solution of 10^{-2}M was frozen and separated into aliquots that were thawed before each experiment. 10^{-4} tetrodotoxin and 750mM sucrose were used to block stn input.

Electrophysiology

The stomatogastric nervous system was dissected out of the animals and pinned out in a Sylgard-coated plastic petri dish containing chilled saline (11-12ºC). The stomatogastric ganglion was de-sheathed to expose the underlying neurons and vaseline wells were made around motor nerves for extracellular recording. During all experiments, temperatures were controlled by a peltier device (Warner Instruments) and held between 11-12ºC. Extracellular recordings were taken by placing steel pins inside of the vaseline wells and the signals were monitored on pCLAMP10 (Axon Instruments). The signals were amplified by amplifiers purchased from A-M Systems. Intracellular recordings were obtained by impaling neurons with 10-20 MΩ glass microelectrodes, and neurons were identified using procedures from Weimann, J., Meyrand, P., and Marder, E. 1991. The microelectrodes were pulled with a Flaming/Brown micropipette puller (Sutter Instrument, Co.), and the microelectrode solution contained 0.6M K2SO4 and 20mM KCl.

Exogenous Nicotine Experiments

Saline was constantly perfused onto the preparation. Controls were taken before intracellular identification and after intracellular identification. After reaching a steady state, the saline perfusion was replaced by different concentrations of nicotine in saline. For dosage-dependent experiments, saline was used to wash out the effects of nicotine before continuing on to the next experiment.

Data Analysis

Recordings were analyzed using ClampFit 9.0 (Axon Instruments) and Spike2 (Cambridge Electronic Design). Figures were made with Adobe Illustrator (Adobe Systems). Spike counts were measured using Spike2. T-test data and dosage-dependent plot were calculated with Microsoft Excel.

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