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Responses of central pattern generators in the American lobster STNS to multiple members of a novel neuropeptide family

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RESPONSES OF CENTRAL PATTERN GENERATORS IN THE AMERICAN LOBSTER STNS TO MULTIPLE MEMBERS OF A NOVEL NEUROPEPTIDE FAMILY

AN HONORS PROJECT FOR THE PROGRAM OF NEUROSCIENCE

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ABSTRACT

The American lobster, *Homarus americanus*, has been widely studied due to the simple model circuits present in its nervous system. The stomatogastric nervous system (STNS) is a network of neurons that control digestion, commonly found in arthropods such as insects and crustaceans. Located in the lobster's foregut, the STNS contains four central pattern generating circuits (CPGs), two of which were considered here. CPGs can dictate rhythmic motions (e.g., locomotion, respiration) in both invertebrates and vertebrates alike, and are unique in their intrinsic control of motor systems in the absence of external timing inputs; however, this intrinsic activity can be flexibly modulated by several types of endogenous chemicals, including neuropeptides. This project focused on a novel family of endogenous *H. americanus* neuropeptides, the GSEFLamides. First identified in the lobster in 2017, the GSEFLamide family contains six isoforms: I-, M-, AL-, AM-, AV-, and VM-GSEFLamide. Recent work in the Dickinson lab identified AMGSEFLamide as the most abundant isoform in this family, and found that it increased activity in both the cardiac ganglion and STNS central pattern generators. There is also previous evidence from the Dickinson lab showing that individual isoforms of a single neuropeptide family can have differing functional effects on a pattern generating system. However, the effects of the other five GSEFLamide isoforms have not yet been considered in the STNS – this project thus explored whether the GSEFLamide family would act together or individually to modulate two CPGs in the STNS.

The stomatogastric ganglion (STG) houses ~30 neurons that comprise the two STNS central pattern generators studied here. Each CPG controls a distinct part of the lobster's stomach: the intermittently active gastric mill pattern controls three teeth used to break down food, and the constantly active pyloric pattern controls a filter that leads to the lobster's midgut.

In recent unpublished work from the Dickinson lab that examined the full GSEFLamide family in the isolated lobster heart, five of the six isoforms elicited similar increases in contraction amplitude when perfused through the system, while one (AVGSEFLamide) had virtually no effect. Here, we found the pattern of GSEFLamide effects on the STNS gastric mill to be similar to the pattern observed in the lobster cardiac system; the intermittently active gastric mill circuit was fairly consistently activated by all isoforms except AVGSEFLamide. The constantly active pyloric pattern was significantly enhanced by three out of five peptide isoforms, and nearly significantly enhanced by two more, but was likewise non-responsive to AVGSEFLamide. While the reason AVGSEFLamide had no effect on either pattern is unknown, the similar phenomenon noted in the isolated whole heart potentially indicates that this isoform lacks any function in the lobster. The mechanisms that made the gastric pattern sensitive to several GSEFLamide isoforms but the pyloric pattern less sensitive to this family are also not yet understood; however, these differences might be explained in part by the pyloric filter's inherent constant activity as compared to the gastric mill's intermittent activation. Modulatory activity in these CPGs is constantly influenced by inputs from the STNS commissural ganglia, among other inputs, so future work to elucidate the isolated GSEFLamide effects on the gastric mill and pyloric patterns could consider cutting or blocking those inputs.

INTRODUCTION

1.1 The American lobster

The American lobster, *Homarus americanus* (Milne-Edwards, 1837), is a species of decapod crustacean commonly found on the North American Atlantic coast, where it serves as a staple of the Maine economy. The commercial lobster supply chain produced more than $950 million in economic output in 2016, supporting over 5,500 local jobs (Donihue, 2018). These invertebrates are useful for more than their market value, though; *H. americanus* have also been studied for decades because of the simple model circuits present in their nervous system.

The American lobster is found throughout the northwest Atlantic Ocean, where they feed opportunistically on any available seafloor prey, from mollusks to worms (NOAA, 2019). Unlike many species, lobsters do not chew food before it passes through the esophagus; rather, they utilize a set of teeth in the foregut known as the gastric mill to mechanically masticate. Broken down food then moves to the pyloric stomach, where the pylorus acts as a selective fine particle filter into the midgut. Crustaceans first develop foregut teeth in their larval stage, during which they are primarily carnivorous. These teeth begin as “hard pads” that grow into to the full gastric mill at a later larval stage, before metamorphosis occurs (Factor, 1981). After metamorphosis, young lobsters descend to the ocean’s benthic surface, where they proceed to normal adult opportunistic feeding. While many details of the lobster diet and their specific breakdown of food are not well understood, the small neural circuits that control the stomach are excellent models for complex pattern generators across a wide range of species.

Three model circuits located in the lobster’s heart and stomach, known as central pattern generators (CPGs), are commonly investigated due to the small number of relatively large

diameter neurons present in each circuit. The cardiac ganglion is one such CPG, and drives neurogenic heart contractions. The two foregut CPGs are located in the stomatogastric nervous system (STNS), and control digestion via the gastric mill teeth and selective pyloric filter. The simplicity of many CPGs allows researchers to identify and study them using electrophysiological techniques, among others. This project used electrophysiological recordings to study the gastric mill and pyloric filter CPGs in the STNS.

1.2 Central Pattern Generators

Central pattern generators are small neural circuits capable of causing rhythmic muscular sequences without cues from outside the central nervous system (Bucher, 2009; Dickinson, 2006; Marder & Bucher, 2007; Selverston, 2005). These circuits are unique in their ability to direct motor function like walking, breathing, or chewing in the absence of timing cues, in both vertebrate and invertebrate systems. Such intrinsic activity has been identified across several species. Neural circuits that have their external inputs removed can still exhibit fictive activity, defined by bursts of rhythmic firing that would lead to motor function *in vivo* if the organism were intact (Marder & Bucher, 2001). Fictive motor patterns from isolated preparations originate from physically invariable nerve circuits; however, such rhythmic activity can be functionally variable when influenced by neuromodulators (Marder & Bucher, 2007). Neuromodulators are small molecules that can affect fixed neural circuits through a variety of pathways – in addition to intrinsic communication at synapses within each circuit, extrinsic modulation can come from projection neurons that directly impact a circuit, and from circulating neurohormones (Marder, 2012). Neuromodulation can therefore drastically affect behavior by flexibly changing the patterned outputs of a neural circuit. Investigating how neuromodulators affect CPGs has been the foundation of decades of neuroscience research.

1.3 Stomatogastric Nervous System

The specific organization of the STNS was first identified in the 1970s (Maynard et al., 1974), and research on the system has continued over several decades. Physiologically, the stomatogastric nervous system regulates movement and digestion in the lobster's foregut. The foregut of the digestive system is separated into two areas: the cardiac stomach, where food initially enters from the esophagus and is broken down by the three calcified teeth known as the gastric mill, and the pyloric stomach, where the pyloric filter further filters food particles to aid in digestion. Each portion of the lobster foregut is controlled by a distinct, tightly coupled central pattern generator – the gastric mill circuit, or the pyloric filter circuit. Cell bodies of the neurons in both circuits are housed in the stomatogastric ganglion (STG), with axons extending posteriorly to innervate striated muscles (Marder & Bucher, 2007, Figure 1). Activity in these circuits can be measured by sequential "bursts" of neuronal activity, where several action potentials will fire in one neuron before another neuron in the circuit becomes active. Chemical synapses as well as electrical gap junctions work together to govern activity both within and between these circuits (Figure 2 A & B).

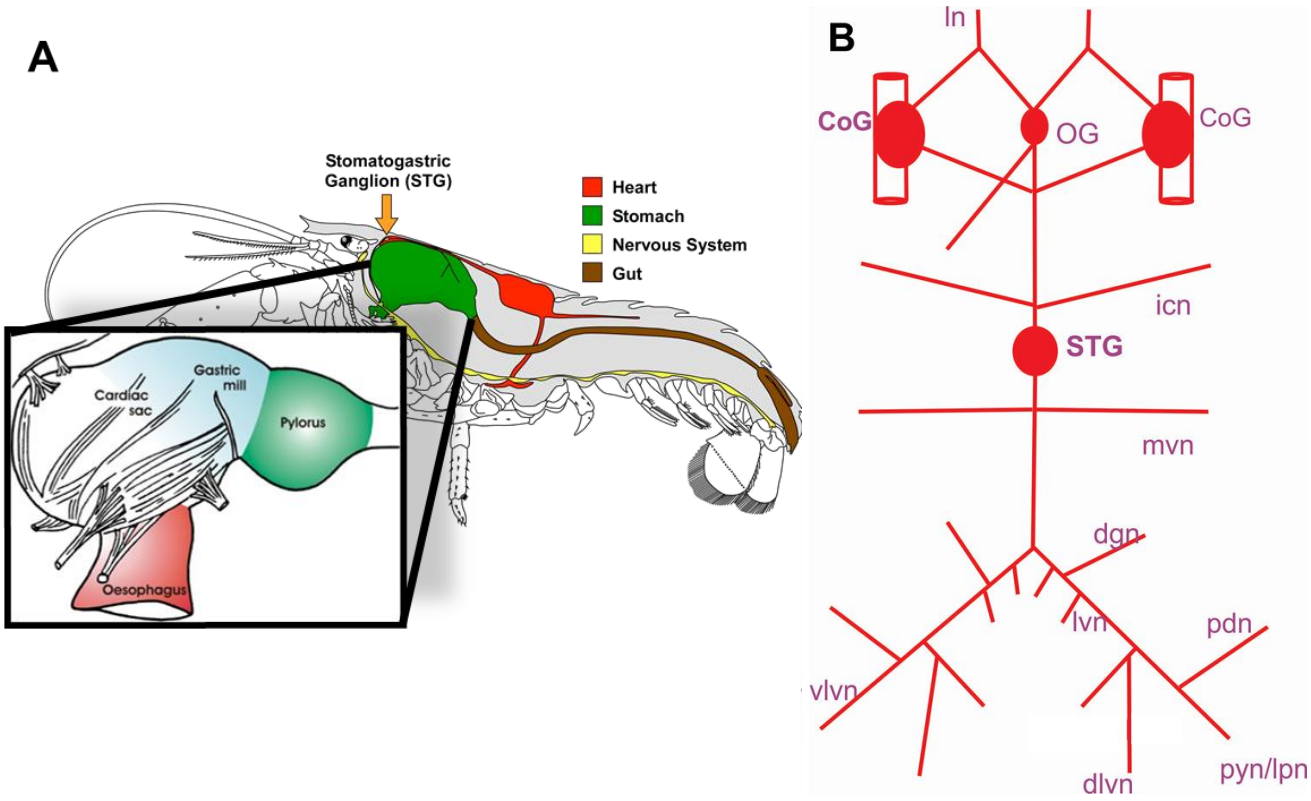

Figure 1. The stomatogastric nervous system (STNS). (A) Side view of whole lobster showing relative stomach, heart, and STNS positions. *Inset:* Isolated lobster foregut and midgut. Note the pyloric stomach located posterior to the gastric mill. (B) Simplified STNS schematic, organized as typically studied *in vitro* once dissected from the foregut. Red lines represent nerves containing individual neurons that comprise the gastric mill and pyloric CPGs. STG, stomatogastric ganglion; CoG, commissural ganglia; OG, oesophageal ganglion; *icn*, inferior cardiac nerve; *mvn*, medial ventricular nerve; *dgn*, dorsal gastric nerve; *lvn*, lateral ventricular nerve; *pdn*, pyloric dilator nerve; *dlvn*, dorsal lateral ventricular nerve; *pyn*, pyloric nerve; *lpn*, lateral pyloric nerve; *vlvn*, ventral lateral ventricular nerve. (A) is adapted from Marder & Bucher, 2007, *Inset* and (B) are adapted from the Dickinson lab.

1.3.1 The gastric mill circuit

The gastric mill circuit is a network consisting of 11 neurons that operate on a flexible multi-phase, several second-long bursting pattern, and are only intermittently active based on feeding, with a long ~8-20 second period (Selverston et al., 2009, Table 1, Figure 2). Likely due to the multifaceted omnivorous diet of the lobster, the intermittent gastric mill fires in phased patterns that vary by time and individual. This CPG controls one medial and two lateral teeth within the cardiac stomach, where food is initially broken down before being passed to the pyloric stomach. The gastric mill pattern can be studied as two subcircuits, connected by a single interneuron (Int 1); one subcircuit controls the medial tooth movement, and one controls the lateral teeth. Int 1 plays a key role in generating an initial rhythm and coordinating these subcircuits; however, the exact drivers of gastric mill activity are still unknown. Nusbaum & Beenhakker (2002) have found that while neurons outside this circuit are not required to operate the gastric mill, descending modulatory inputs may nonetheless drive or coordinate much of the pattern's activity.

Within each of the two subcircuits described above, certain neurons bring the teeth together to scoop and crush food (known as a power stroke), while reciprocal neurons withdraw the teeth (return stroke). The medial tooth is activated by four gastric mill (GM) neurons, and scoops food towards the center of the gastric mill. The dorsal gastric (DG) and anterior median (AM) neurons then fire together to draw back the medial tooth, acting reciprocally with the GM neurons. Food is then crushed into small pieces when the lateral gastric (LG) and medial gastric (MG) neurons fire synchronously via the lateral teeth subcircuit. When the gastric mill is not active, the lateral posterior gastric neurons (LPG) are believed to fire tonically to hold the lateral

teeth open; the synchronous LG and MG firing therefore occurs between tonic LPG bursts (Selverston, 1992).

Name	Abbreviation	# Neurons	Muscle innervated	Action
Interneuron 1	Int 1	1	–	
Gastric mill	GM	4	gm 1b,2a,b	Medial tooth power
Dorsal gastric	DG	1	gm 4a,b,c	Medial tooth return
Anterior median	AM	1	c6, c7	Medial tooth return
Lateral gastric	LG	1	gm 5b, 6a	Lateral teeth closer
Medial gastric	MG	1	gm 9a,9c	Lateral teeth closer
Lateral posterior gastric	LPG	2	gm 3	Lateral teeth opener

Table 1. Neurons in the crustacean gastric mill circuit. Adapted from Selverston et al., 2009.

1.3.2 The pyloric filter circuit

The triphasic pyloric pattern is generated by 14 neurons that control the continuously active pyloric filter, which passes food through the pyloric stomach into the gut. This pattern occurs on a fast, ~1-2 second period (Nagy & Dickinson, 1983, Table 2, Figure 2). The specific mechanism of action of the pyloric filter is not well understood, but the bursting activity of individual neurons in this circuit has been well studied. The core triphasic pattern consists of a burst from the pyloric dilator (PD) neuron, followed by bursts in the lateral pyloric (LP) and the pyloric (PY) neurons. The ventricular dilator (VD) neuron often fires synchronously with PY neurons, while the inferior cardiac (IC) neuron fires with LP (Selverston, 2008). An additional interneuron, the anterior burster (AB), is electrically coupled to each PD and projects anterior to the rest of the circuit, travelling to the commissural ganglia (CoGs) via the stomatogastric nerve

(*stn*). This interneuron is considered a pacemaker neuron due to its robust intrinsic activity, even when isolated from the rest of the pyloric system (Selverston, 2008).

Name	**Abbreviation**	**# Neurons**	**Muscle innervated**
Anterior burster	AB	1	interneuron
Pyloric dilator	PD	2	cpv 1&2
Lateral pyloric	LP	1	p1
Ventricular dilator	VD	1	cv 2
Inferior cardiac	IC	1	cv 3
Pyloric	PY	8	p 2-4, 7-8, 10-11

Table 2. Neurons in the crustacean pyloric circuit. Adapted from (Selverston, 2008).

1.3.3 Circuitry and neuromodulation

Despite often being studied as isolated circuits, neurons that control the gastric mill and pylorus nonetheless interact with each other (Figure 2C). Past studies have found that the numerous electrical and chemical connections between these two systems can yield complex and confounding effects on patterned activity (Marder & Bucher, 2007). For example, some research in crustaceans has shown that when the gastric mill is not active, neurons that are typically part of the gastric mill circuit will instead fire in synchrony with the pyloric network (Heinzel et al., 1993; Weimann et al., 1991).

Although portions of the STNS will fire intrinsically, they are nonetheless subject to modulation. Despite the early notion that central pattern generators were fixed circuits that exhibited no flexibility, neuromodulation from external inputs via neuroendocrine pathways

(e.g., sinus gland release), or direct inputs (e.g., commissural ganglia) can provide functional flexibility to physically fixed circuits in the STNS (Selverston, 1995; Selverston et al., 1992).

Removing descending projection inputs to these circuits by transecting or blocking the stomatogastric nerve anterior to the STG can be used to study the isolated effects of a specific neuromodulator. Cutting these inputs in the American lobster typically slows or fully stops the intrinsic pyloric pattern, which can then be recovered to different degrees with certain neuromodulators (Marder, 2012). Cutting the *stn* therefore shows the degree to which an isolated circuit can flexibly respond to different neuromodulators, and helps characterize specific activity caused by any one modulator. While several previous studies have considered the effects of neuromodulators on both intact and cut *stn* preparations, the large family of peptides considered in this study, combined with concerns about long-term STNS viability once removed from the lobster led us to focus solely on intact STNS systems.

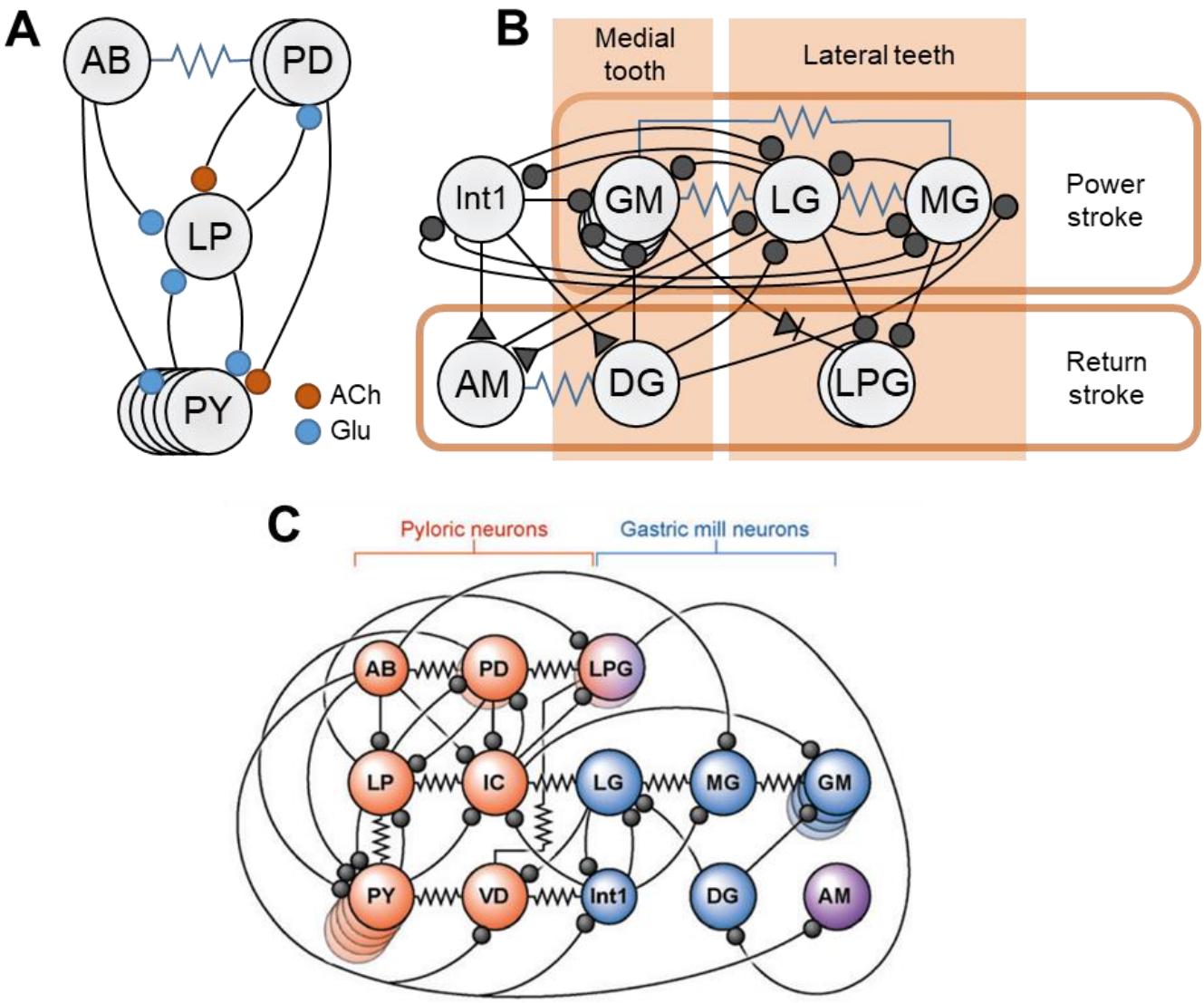

Figure 2. Circuit diagrams of putative crustacean pyloric and gastric mill circuits. (A) Simplified pyloric circuit without ventricular dilator (VD) and inferior cardiac (IC) neurons. AB, anterior burster; PD, pyloric dilator; LP, lateral pyloric; PY, pyloric. Chemical synapses are shown in red (cholinergic) and blue (glutamatergic). (B) Simplified gastric mill circuit, with neurons grouped by which tooth they control in power and return chewing motion phases. Int1; interneuron 1; GM, gastric mill; DG, dorsal gastric; LG, lateral gastric; MG, medial gastric; LPG, lateral posterior gastric. The anterior median (AM) neuron controls a muscle in the cardiac sac. (C) Connections between the pyloric and gastric mill circuits. In (A), (B), and (C), circles indicate inhibitory synapses, triangles indicate excitatory synapses, resistor symbols indicate electrical coupling by gap junction, and diode symbols indicate a rectifying electrical synapse that likely travels only in one direction. Adapted from Marder & Bucher (2007).

1.4 GSEFLamides

This project focused on a family of neuropeptides, the GSEFLamides, which was first identified in *H. americanus* in 2017 in collaboration with the Christie lab (Christie et al., 2017). Neuropeptides are short chains of amino acids that play a key role in modulating neural activity within circuits to generate behavioral flexibility, and are therefore often studied in the lobster. New peptides continue to be revealed through techniques like genetic sequencing and mass spectrometry, which can determine the identities of several isoforms in one peptide family. While isoforms in a peptide family only vary slightly in amino acid sequence and structure, they are capable of producing drastically varied functional effects (or no effect at all), depending on how they are received by a system. The *H. americanus* GSEFLamide family contains six isoforms: I-, M-, AL-, AM-, AV-, and VM-GSEFLamides (Table 3). Each isoform in this family varies by only one or two amino acids, and all are characterized by the conserved C-terminal – GSEFLamide motif.

Peptide isoform sequence	# isoform copies in transcript
IGSEFLa	1
MGSEFLa	2
AMGSEFLa	6/7
ALGSEFLa	1
VMGSEFLa	1
AVGSEFLa	1

Table 3. Putative *H. americanus* GSEFLamide isoforms. AMGSEFLamide was copied 6 to 7 times more than most other familial isoforms, depending on the transcriptome assembly splice variant (Christie et al., 2017).

1.4.1 in-silico identification

Due to the key role neuropeptides play in modulating central pattern generating systems, many studies have attempted to identify endogenous peptides in the American lobster. Early work used mass spectral techniques to identify novel neuropeptides (Cape et al., 2008; Fu et al., 2005). While these techniques prove quite useful for identifying specific peptides, they are limited to relatively small, high abundance, easily-ionized sequences (Christie et al., 2010, 2017). The development of *in-silico* transcriptome mining techniques in the past two decades has thus propelled research in crustaceans and other models to new heights, allowing for in-depth neuropeptidome predictions. An *in-silico* technique initially used for transcriptome mining involved creating short mRNA sequences, called expressed sequence tags (ESTs), from specific tissues or from a whole organism based on known DNA templates. Those sequences would be uploaded to large online databases, and could be mined for mRNA precursor transcripts that encode for known orthologous proteins in other species (Christie et al., 2008). While this technique was successful in identifying several neuropeptides from any one species, it was also limited by some factors. The established EST databases often would not encompass the full transcriptome of any one crustacean, and additionally were typically only formed from "single pass" sequences, leaving the possibility for imperfect sequencing to yield erroneous predicted peptide structures (Christie et al., 2010).

In recent years, high-throughput sequencing via RNA-seq techniques has allowed for much more rapid and accurate *de novo* transcriptome assembly (Pandey & Williams, 2014). RNA-seq can provide a precise count of putative endogenous peptide mRNA copies by assembling a transcript from RNA-seq reads. Peptide expression becomes particularly pertinent when studying the degree to which different familial isoforms might affect a system

independently, as is considered in the present study. Specifically regarding crustacean neuropeptides, recent methodology has focused on using known endogenous crustacean proteins as a query input into transcriptome mining software, which is used to identify predicted matching peptide structures from a *de novo* transcriptome. Post-translational modifications can then be predicted for those matching structures. However, even when a novel mRNA sequence is correctly translated and matches a known crustacean peptide, predictive post-translational techniques are not perfect, and might not accurately represent *in-vivo* post-translational modifications (Christie et al., 2015, 2017). Acknowledging these limitations, this study nonetheless focused on the GSEFLamide peptide family because it has been found with varied isoform expression across several crustacean species.

1.4.2 H. americanus neuropeptidome

The GSEFLamides were first discussed in an *in-silico* transcriptome mining study by Christie (2014), in which he found novel GSEFLamide sequences present in the copepod *Tigriopus californicus*. No prior literature had noted this particular sequence in any species, but a further database search using the predicted *T. californicus* preprohormone identified transcriptome shotgun assembly sequences that encode GSEFLamide preprohormones in two other crustaceans; *Eriocheir sinensis*, the brachyuran crab, and *Litopenaeus vannamei*, the penaeid shrimp. In a later effort to further expand the American lobster neuropeptidome via transcriptome mining, Christie et al. (2015) identified specific GSEFLamide isoforms AVG- and AMGSEFLamide as predicted neuropeptides. This 2015 study utilized several neural tissues, including the brain, ventral nerve cord, cardiac ganglion, and STNS to build a *de novo* American lobster transcriptome. Recently, Christie et al. (2017) assembled another transcriptome from *H. americanus* eyestalk ganglia, which play a key role in the neuroendocrine system via X-organ-

sinus-gland hormone production and release. This work identified the six GSEFLamide isoforms used in the present study via translations from the eyestalk transcriptome that encoded for putative arthropod GSEFLamides. Two nearly identical full-length prepro-GSEFLamide transcripts were found, likely representing two splice variants of a single gene (Figure 3). As identified in Table 3, AMGSEFLamide was the most common putative GSEFLamide copied in the mRNA transcripts, occurring six or seven times depending on the splice variant considered. Additionally, mRNA encoding MGSEFLamide was present twice in each splice variant, while the remaining four isoforms all appeared once in each splice variant.

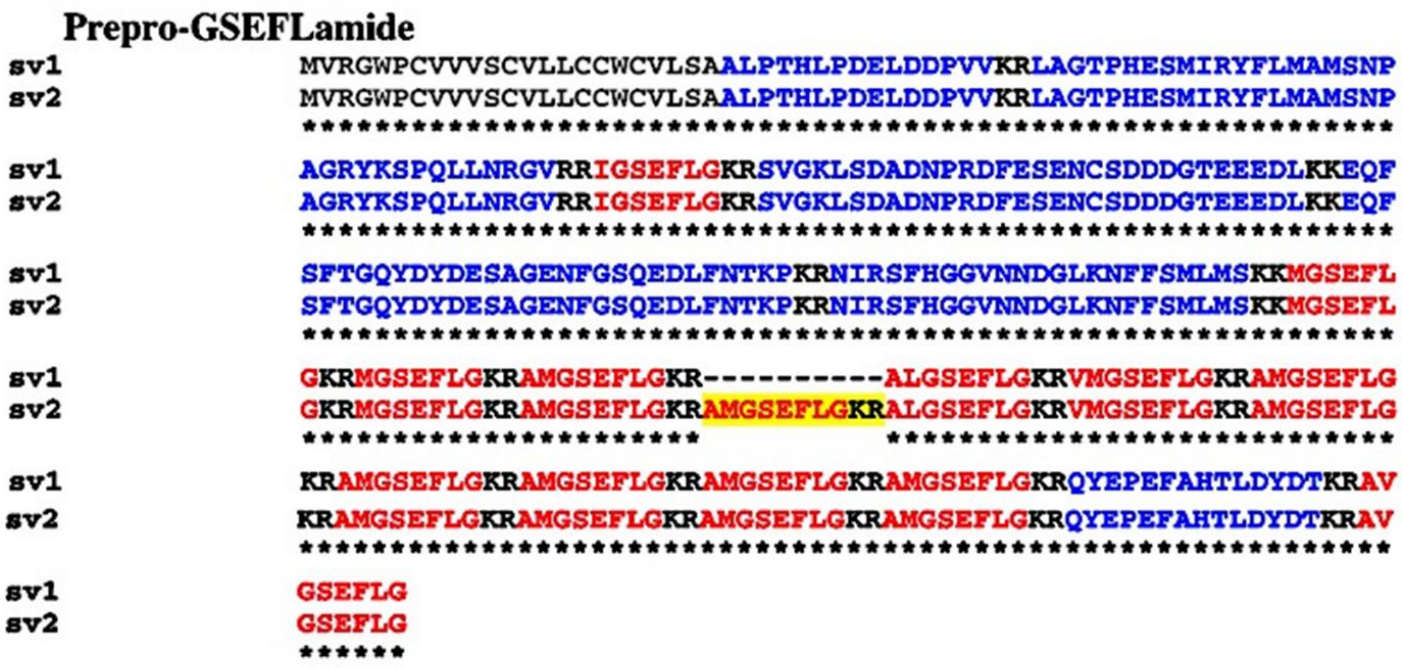

Figure 3. Two predicted splice variants of the GSEFLamide neuropeptide family. Identified from the *H. americanus* eyestalk ganglia transcriptome, familial isoforms are shown in red, linker peptides in blue, and cleavage sites in black. Asterisks indicate conserved sequences between splice variants. Extra AMGSEFLG sequence from second splice variant highlighted in yellow. From: Christie et al. (2017).

1.4.3 Recent GSEFLamide research

Although the proposed GSEFLamide family was identified through *in-silico* transcriptome mining in 2017, only very recent studies considered this family in an *in vitro* setting, and confirmed its endogenous presence in *H. americanus*. Dickinson et al. (2019) first identified all six GSEFLamide isoforms endogenously, and then characterized the effects of the most abundant isoform in this family, AMGSEFLamide, on CPGs in the American lobster. That study used reverse transcription PCR (RT-PCR) of eyestalk ganglia and brain RNA, in addition to mass spectrometry to concretely identify all six GSEFLamide isoforms endogenously. The RT-PCR prepro-GSEFLamide translations revealed one splice variant with as many as 17 copies of AMGSEFLamide, with the second and third splice variants containing 15 and nine copies of that isoform, respectively. Across all three splice variants, the RT-PCR found two copies of MGSEFLamide and one copy of each of the remaining isoforms, similar to the number of copies of these isoforms predicted in Christie et al. (2017). The mass spectrometry data from this Dickinson lab study supported the RT-PCR data, identifying AMGSEFLamide in nine out of nine *H. americanus* brain extractions, in addition to finding ALG-, AVG-, MG-, VMG-, and IGSEFLamide in 7, 6, 5, 4, and 1 out of 9 extractions, respectively. The combination of RT-PCR and mass spectrometry data therefore confirm that the GSEFLamides exist endogenously in *H. americanus*, and highlight some potential relative abundances of each isoform. These experiments were thus the first to identify endogenous *H. americanus* GSEFLamide isoforms. Through further transcriptome mining and comparison with past published transcriptomes, they also determined that the GSEFLamides are widely conserved among arthropods, with the exception of insect suborders Endopterygota and Exopterygota.

In addition to confirming the presence of GSEFLamides in several crustacean species, Dickinson et al. (2019) began to assess the physiological role this family plays in the American lobster. Their initial work identified the GSEFLamides endogenously, with mass spectrometry and RT-PCR data explicitly finding isoforms or putative isoform mRNA expression in the brain and eyestalk ganglion. However, given the possibility of neurohormonal release of this family, this study also examined activity in two motor systems; the heart via the cardiac ganglion, and the foregut via the stomatogastric nervous system. Concentrations of AMGSEFLamide 10^{-8}M and higher applied to the isolated cardiac ganglion elicited a decrease in burst frequency in the heart, but increased burst duration and duty cycle outputs.

Interestingly, when this isoform was applied to the isolated STNS (*stn* intact), it activated the gastric mill pattern when not already active, and enhanced this pattern when it was already present, increasing burst duration and spike frequency. When the *stn* was intact, AMGSEFLamide also activated the pyloric pattern, as measured by pyloric frequency, at concentrations greater than 10^{-7}M, but did not affect burst duration. When the *stn* was cut, typical patterned gastric firing was unrecoverable; activity from LPG was still observed on a pyloric timescale, but this was likely due to its weak electrical coupling to PD (Figure 2C). However, a cut *stn* did not impede AMGSEFLamide's effects on the pyloric circuit, where it increased PD neuron cycle frequency, and occasionally recovered the full pyloric pattern, even in absence of other modulatory inputs. This series of differential effects has been observed in other studies, where peptides have varied modulatory effects on pattern generating systems (Saideman et al., 2007). Because Dickinson et al. (2019) considered only one of six GSEFLamide isoforms, the present project examined the degree to which the other GSEFLamide isoforms act in the STNS –

would they all activate intact STNS CPGs in the same way AMGSEFLamide did, or would different isoforms act individually, if at all?

1.5 Pyrokinin studies

Previous research from the Dickinson lab has examined scenarios in which structurally similar isoforms of one peptide family have differential effects on different pattern generating systems. For example, a set of recent studies (Dickinson et al., 2015a; Dickinson et al., 2015b) determined that one family of neuropeptides can differentially affect CPGs in the lobster heart and stomatogastric nervous system. This work focused on the pyrokinins, a subgroup of neuropeptides involved in moth sexual behavior as part of the Pheromone-Biosynthesis-Activating Neuropeptide (PBAN) family (Rafaeli, 2009). The pyrokinins are characterized by a C-terminal –FXPRLamide group, in which X represents various amino acid residues.

The first of the pyrokinin studies (Dickinson et al., 2015b) focused on the whole *H. americanus* heart, where only an isoform endogenous to the shrimp, ADFAFNPRLamide (named PevPK2), increased heart contraction amplitude. Initial immunostaining in *H. americanus* using an antibody to the conserved FSPRLamide isoform revealed the potential for pyrokinin release through both direct projections from neuropil onto pacemaker and motor neurons, and neuroendocrine release from the pericardial organ and sinus gland. Despite this pyrokinin immunoreactivity, neither FSPRLamide nor other pyrokinin isoforms elicited effects on the lobster heart. Even a structurally similar known crustacean peptide, DFAFSPRLamide (named PevPK1), which differs from PevPK2 only by an added alanine at the N-terminus and a serine at the variable X amino acid, produced no change in heart activity. Seeing how similar PevPK1 and PevPK2 were in structure but not in function, researchers decided to test several

synthetic pyrokinin length and X residue variants from PevPK2. They found that changing either of these criteria resulted in a loss of bioactive effects on the heart, indicating that pyrokinin receptors in the *H. americanus* heart are likely highly sensitive to both peptide length and X residue identity.

Although pyrokinins exhibited an apparent specificity in the lobster heart, Saideman et al. (2007) had considered the effects of several pyrokinin variants on the crab *Cancer borealis* STNS, and found that both endogenous and non-native pyrokinin sequences similarly modulated the gastric mill pattern, but not the pyloric pattern. In an attempt to determine whether this different pyrokinin action was due to either differing species attributes or differing systems within a species, Dickinson et al. (2015a) examined the effects of pyrokinins in the *H. americanus* STNS. Again, immunostaining using an anti-FSPRLamide antibody found pyrokinin immunoreactivity in a neuropil region, as well as the aforementioned endocrine sources, indicating the presence of at least potential STNS pyrokinins. Unlike what was observed with pyrokinins in the lobster heart, however, this study indicated effects similar to the Saideman et al. (2007) study. Five tested crustacean pyrokinins, including FSPRLamide, enhanced activity in the *H. americanus* gastric mill in a similar fashion, increasing burst duration and cycle frequency in weakly or moderately active preparations. However, none of the tested pyrokinins elicited any effects in the pyloric pattern. This study therefore raises the possibility that within the *H. americanus* nervous system, separate CPGs may express receptors with unique specificities for the same family of neuromodulators. As Jiang et al. (2014) noted, it is possible for peptides to have low-specificity relationships to multiple receptors, possibly explaining the varied pyrokinin responses. It is also of note, though, that none of the endogenous *H. americanus* pyrokinins had been identified at the time of this study, and were therefore not tested in this species.

1.6 Present work

From the pyrokinin studies, we see that there is precedent for a peptide family to differentially affect central pattern generating systems. Whereas all but one crustacean pyrokinin modulated the lobster heart, and no tested isoforms modulated the pyloric pattern, all of the isoforms modulated the gastric mill pattern. This selective action on multiple systems from one family of peptides thus led us to ask whether the GSEFLamides would all act similarly or have individual effects in the American lobster.

This project both expanded on research from Dickinson et al. (2019), and mimicked the pyrokinin studies by determining the degree to which the GSEFLamide peptide family modulates the STNS, as a family or individually. The present work only focused on characterizing these effects in the intact STNS due to the length of the STNS dissection and the large number of isoforms in this family; however, recent unpublished research from another lab member has considered these peptides in the lobster cardiac system, and that project is expected to continue in the near future. Their work indicated that five of the six GSEFLamide isoforms elicited similar increases in contraction amplitude when perfused through the whole *H. americanus* heart, while one (AVGSEFLamide) elicited no effect. The present study therefore aimed not only to elucidate the GSEFLamide effects in the STNS, but also to place those effects in the context of the broader *H. americanus* nervous system activity. We hoped to determine broader systemic impacts of the GSEFLamides in order to locate where they are specifically active, and hopefully provide some insight into their functional endogenous effects.

METHODS

2.1 Animals

Adult American lobsters, *Homarus americanus* (Milne-Edwards, 1837), were purchased from local (Brunswick, ME, USA) seafood providers. Lobsters were kept in recirculating natural seawater tanks at 10-12°C, and fed chopped squid or shrimp on a weekly basis. Hardness of shell and sex were random; most lobsters were small, in the ~500g range, on average.

2.2 Dissection

Lobsters were put on ice for 30 minutes before performing a gross dissection to remove the stomach. The appendages and tail were removed, and the carapace surrounding the stomach was cut and removed. Connective tissues and structures were separated from the stomach, which was removed, and cut open ventrally. The full stomach was separated from the body and pinned in a Sylgard-170 (Dow Corning, Midland, MI USA) lined dish in saline. Cold physiological saline was used throughout the experiment, with a composition of (in mM/l): 479.12 NaCl, 12.74 KCl, 13.67 CaCl2, 20.00 MgSO4, 3.91 Na2SO4, 11.45 Trizma base, and 4.82 maleic acid [pH = 7.45]

Using fine dissecting scissors and forceps, the STNS was removed from the stomach, beginning at the ganglia and dissecting towards the motor nerves. Glass probes and scissors were used to pull nerves away from muscle without damaging them. The STNS was transferred to a shallow clear Sylgard-184 (Dow Corning, Midland, MI USA) lined plate to be pinned down for recording, where the stomatogastric ganglion was first de-sheathed to allow peptide access. Throughout an entire dissection, saline was changed at a regular interval of 10-20 minutes.

2.3 Saline and solutions

All six GSEFLamide isoforms (Table 3) were synthesized by GenScript (Piscataway, NJ, USA). Dry peptides were first dissolved in dH_2O at 10^{-3}M and separated into aliquots. They were then prepared from frozen (-20°C) aliquots into 10^{-6} M solutions in lobster saline. The 10^{-6}M concentration was determined from previous experiments in the Dickinson lab. Control saline and peptide solutions were applied through perfusion at 5ml/min^{-1} across the length of the stomatogastric ganglion with a Gilson Minipuls peristaltic pump (Gilson, Middleton, WI, USA). Temperature was held constant (around 10-12˚C) with an in-line Peltier temperature regulator (CL-100 bipolar temperature controller and SC-20 solution heater/cooler; Warner Instruments, Hamden, CT, USA).

2.4 Recordings

To avoid neuronal damage by microelectrodes, electrical activity from the system was recorded extracellularly. Petroleum jelly wells were constructed around relevant motor nerves on either side of the STNS. At each desired nerve, two stainless steel pin electrodes were inserted into the Sylgard-184 plate, one in the petroleum well and the other nearby (but outside of the well) to record extracellular potentials. Neuronal activity was amplified from the electrodes using a 1700 A-M Systems Differential AC amplifier (A-M Systems, Sequim, WA, USA), and a Brownlee Precision amplifier (Brownlee Instruments, San Jose, CA, USA). Data were monitored and recorded onto a computer using a Cambridge Electronic Design Micro 1401 digitizer, and Spike2 version 9 multi-channel continuous data acquisition software (Cambridge Electronic Design, Cambridge, UK), at a sample rate of 10kHz.

A 10-20-minute baseline saline recording was taken in each preparation before 10 minutes (~60ml) of one peptide was applied through the perfusion system. After peptide application, the preparation was washed in control saline for ~60 minutes, to allow a return to baseline activity. Another peptide solution was then prepared and applied via the same process, which was repeated for all six GSEFLamide isoforms. The order of application of the peptides was random.

2.5 Burst characterization and analysis

All recordings were analyzed in Spike2, where spikes (action potentials) from each neuron were identified and then sorted into bursts of activity using scripts from the Bucher lab at NJIT (http://www.stg.rutgers.edu/Resources.html). Sets of at least five spikes were typically considered bursts. A burst was generally defined as started when two consecutive spikes occurred within 0.1 seconds of each other, and likewise bursts typically ended when two spikes occurred greater than 0.1 seconds apart. However, the specific ranges under which bursts were characterized varied between preparations. To assess modulation by the GSEFLamides, sets of ten sequential bursts from each neuron recording channel were chosen from the control saline condition, after which ten more bursts were drawn from the end of peptide application. Ten additional bursts were taken from the end of the saline wash condition, to confirm that preparations had returned to baseline. Sets of bursts ideally contained low noise, with individual neurons easily distinguishable from each other. Occasionally, a low-pass filter was applied to a neuron recording channel to attempt to attenuate noise.

After identifying ten control and ten peptide bursts, a Bucher lab script was used to analyze each burst for a number of parameters, including burst frequency (Hz), burst duration

(sec), and duty cycle (sec). Burst frequency was commonly used to represent activity of a whole pattern (i.e., gastric mill or pyloric filter circuits), whereas burst duration and duty cycle were considered characteristics of an individual neuron.

Burst parameters from control saline and peptide conditions were sorted by preparation and peptide in Microsoft Excel (2016), where parameters of the ten bursts in each set were averaged. The average parameters from each preparation (sorted by peptide and neuron) were compiled in GraphPad Prism 8. The modulatory effect of each peptide on a neuron was assessed using a paired *t*-test (two-tailed) of control saline and peptide parameters across all preparations for one GSEFLamide isoform, with significance defined as a *P*-value less than 0.05. Traces from selected preparations were additionally copied to CorelDRAW (2018), to represent qualitative assessments of the GSEFLamides' STNS effects.

RESULTS

3.1 Lateral gastric and pyloric dilator neurons

The intermittently active gastric mill pattern can be measured via activity in the lateral gastric (LG) neuron, which is located in the *mvn*. Under control conditions, the LG neuron can be completely silent, or it can fire with a period of 8-20 seconds, with anywhere from 10 to upwards of 50 spikes per burst.

The consistently bursting triphasic pyloric pattern can be measured via activity in the pyloric dilator (PD) neuron, which can be recorded from several nerves in the STNS. The PD neuron is typically active under control conditions, and bursts with a period of 1-2 seconds, usually with fewer than 10 spikes per burst.

The data below assess modulatory ability of the six GSEFLamide isoforms on the gastric mill and pyloric systems as measured by LG and PD neuron activity, respectively. Burst frequency was calculated as the average number of bursts per second over the course of ten bursts, and burst duration was the average time per burst over ten bursts. Cycle period was defined as the duration of one burst cycle (i.e., start of one burst to the start of the next burst). Duty cycle was defined as the duration of a neuron's burst divided by the cycle period.

Additionally, changes in patterned activity in these neurons was often distinguished as "enhanced" or "activated." Patterns that were "enhanced" were defined as bursting activity that increased in burst duration and/or density upon peptide application. Patterns that were "activated" would exhibit no neuronal activity during baseline recordings, but begin bursting in a patterned manner upon peptide application.

3.2 Gastric mill circuit

Application of 10^{-6}M AMGSEFLamide – putatively the most prevalent isoform in this family – to the isolated STNS appeared to activate or enhance gastric mill activity irrespective of the state of this intermittent circuit prior to application. Figure 4 illustrates how gastric mill patterned activity, as measured by burst duration and density in the lateral gastric (LG) neuron, was enhanced by 10^{-6}M AMGSEFLamide when the pattern was already active. Moreover, AMGSEFLamide often would activate the lateral gastric neuron, as recorded on the medial ventricular neuron (*mvn),* when this neuron was not already active (Figure 5). Quantitatively, 10^{-6}M AMGSEFLamide did not significantly change the cycle frequency of the gastric mill pattern. However, this isoform did increase the burst duration, as well as the duty cycle – the fraction of a burst period during which the neuron is firing – of the LG neuron (Figure 6).

In contrast, in over eight preparations in which the gastric mill was inactive, 10^{-6}M AVGSEFLamide did not qualitatively activate this pattern, nor did it appear to enhance previously active gastric circuits (Figure 7). Furthermore, this isoform did not elicit any quantitative changes in the gastric mill; whether applied to already active or to inactive gastric patterns, there were no instances in which 10^{-6}M AVGSEFLamide altered LG neuron bursting characteristics (Figure 8).

Application of 10^{-6}M ALGSEFLamide to the isolated STNS qualitatively activated gastric mill activity from an inactive state in eight out of ten observed preparations. However, only three preparations were recorded in which the gastric mill was initially active during the control period, so there is limited quantitative data on this isoform's gastric effects (Figure 9). Across those three preparations, no significant changes were observed in the pattern as measured

by LG neuron burst frequency, duty cycle, or burst duration. Nonetheless, this isoform elicited activity from inactive gastric mill preparations in similar fashion to activity produced by AMG-, VMG-, MG-, and IGSEFLamides, indicating ALGSEFLamide is capable of modulating the gastric mill circuit.

When 10^{-6}M VMGSEFLamide was applied to the isolated STNS, it activated gastric mill activity irrespective of the circuit's state prior to application. Figure 10 illustrates that the duty cycle of lateral gastric (LG) neuron bursts significantly increased with VMGSEFLamide application across several preparations. Although the pattern overall as measured by LG neuron burst frequency did not change, nor did LG neuron burst duration, 10^{-6}M VMGSEFLamide elicited activity from five out of eight inactive gastric mill preparations, indicating that this isoform does modulate the gastric mill circuit.

Application of 10^{-6}M MGSEFLamide – the isoform with the second highest number of copies in the *H. americanus* prepro-GSEFLamide transcripts – to the isolated STNS likewise activated the gastric mill irrespective its intermittent state prior to application. Figure 11 illustrates that the duty cycle of lateral gastric (LG) neuron firing significantly increased with MGSEFLamide application on average, as did burst duration. The gastric mill pattern as measured by LG neuron burst frequency, however, did not significantly change. Like several other familial isoforms, MGSEFLamide not only enhanced already active gastric mill activity, but also activated an initially inactive gastric mill in six out of nine observed preparations.

Application of 10^{-6}M IGSEFLamide to the isolated STNS also activated gastric mill activity irrespective of the state of the intermittent gastric pattern prior to application. Figure 12 illustrates that the mean duty cycle of lateral gastric (LG) neuron firing significantly increased

with IGSEFLamide application. Burst frequency in this pattern did not significantly change, although burst duration of the LG neuron trended toward increasing (*P*=0.0924, *n=5*). Similar to the rest of the GSEFLamide family (excluding AVGSEFLamide), IGSEFLamide both increased already active LG activity, and elicited activity in six out of eight non-active preparations. The quantitative effects of the GSEFLamides on lateral gastric neuron activity are summarized below in Table 4.

Isoform (-SEFLamide)	**Frequency**	**Duty Cycle**	**Burst Duration**
AMG-	X	sig. increase	sig. increase
AVG-	X	X	X
ALG-	X	X	increasing trend
VMG-	X	sig. increase	X
MG-	X	sig. increase	sig. increase
IG-	X	sig. increase	increasing trend

Table 4. Summary of 10^{-6}M GSEFLamide effects on already active lateral gastric neurons. No changes in LG neuron burst frequency were elicited by any GSEFLamide isoforms. LG neuron duty cycle significantly increased with 10^{-6}M AMGSEFLamide application (Fig. 6, *n*=7), VMGSEFLamide application (Fig. 10, *n*=5), MGSEFLamide application (Fig. 11, *n*=4), and IGSEFLamide application (Fig. 12, *n*=5). LG neuron burst duration significantly increased with 10^{-6}M AMGSEFLamide (*n*=7) and MGSEFLamide (*n*=4) application, and this parameter trended toward increasing ($p<0.1$) with 10^{-6}M ALGSEFLamide application (Fig. 9, *n*=3) and IGSEFLamide application (*n*=5). 10^{-6}M AVGSEFLamide did not enhance already active lateral gastric neuron patterns (Fig. 8, *n*=6).

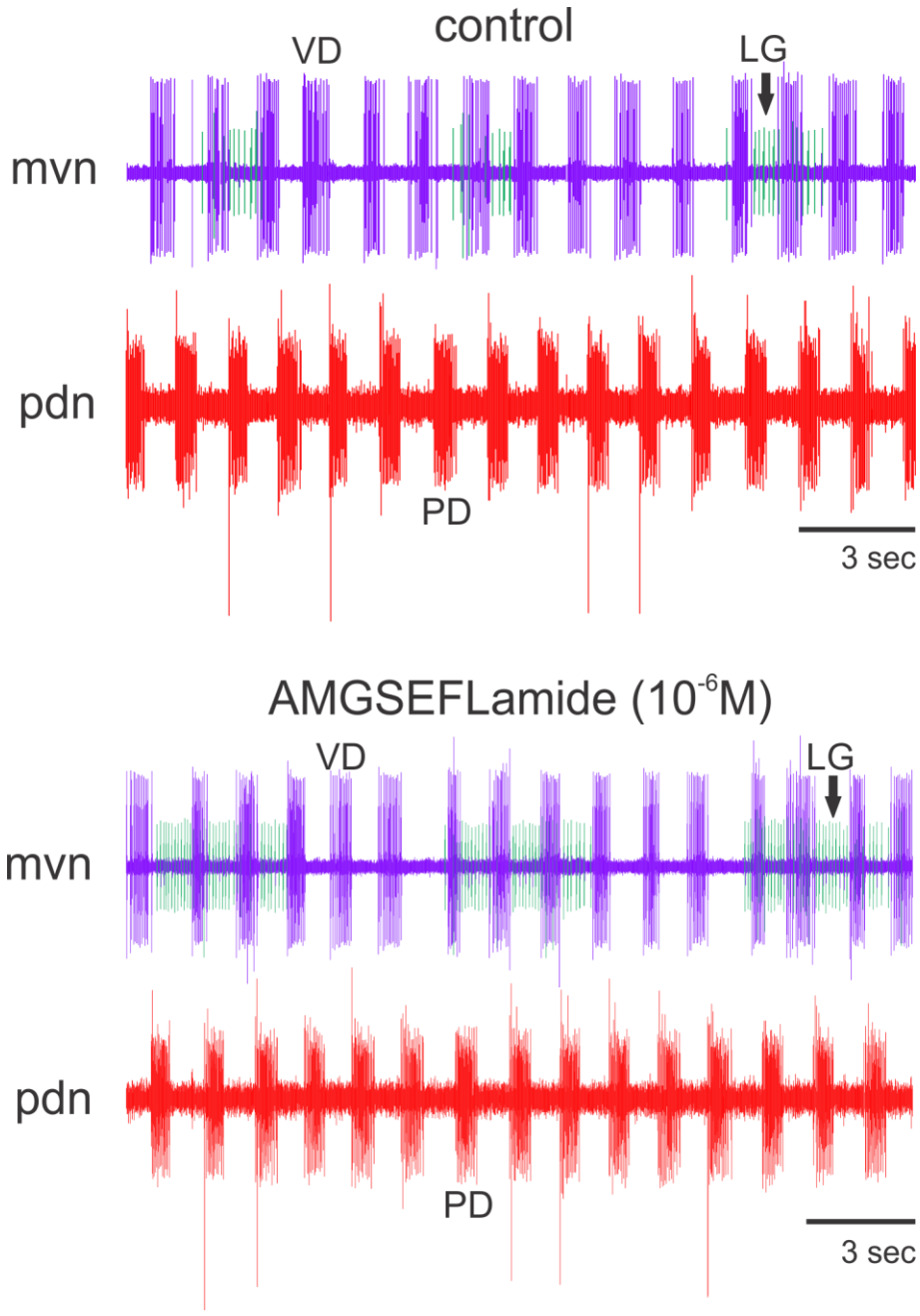

Figure 4. AMGSEFLamide enhanced an already active gastric pattern, but not pyloric pattern. Upon application of 10^{-6}M AMGSEFLamide, lateral gastric neuron bursts (green) appeared to increase in burst duration and density. The pyloric pattern, represented here by ventricular dilator (VD, purple) and pyloric dilator (PD, red) neurons, did not appear to qualitatively change with AMGSEFLamide application. *mvn*, medial ventricular nerve; *pdn*, pyloric dilator nerve.

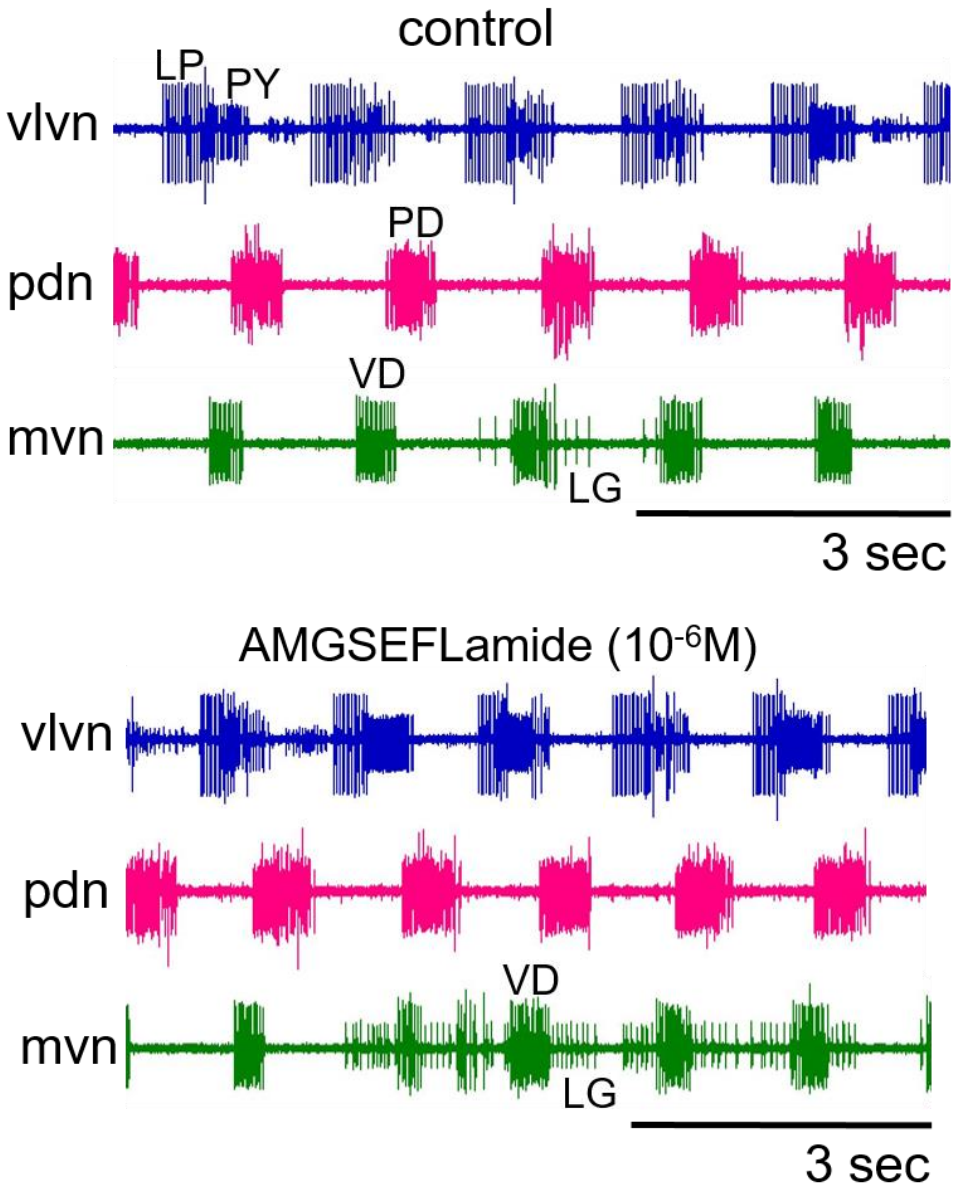

Figure 5. AMGSEFLamide visibly activated an inactive gastric pattern. Lateral gastric (LG) activity increased from an inactive state independent of pyloric pattern bursting, indicating gastric pattern activation with 10^{-6}M AMGSEFLamide. Out of seven preparations in which the LG neuron was not active during control saline application, 10^{-6}M AMGSEFLamide induced activity in this neuron five times. The triphasic pyloric pattern consisting of LP, PY, and PD neurons did not qualitatively substantially change upon application of 10^{-6}M AMGSEFLamide. *mvn*, medial ventricular nerve; *pdn*, pyloric dilator nerve; *vlvn*, ventral lateral ventricular nerve.

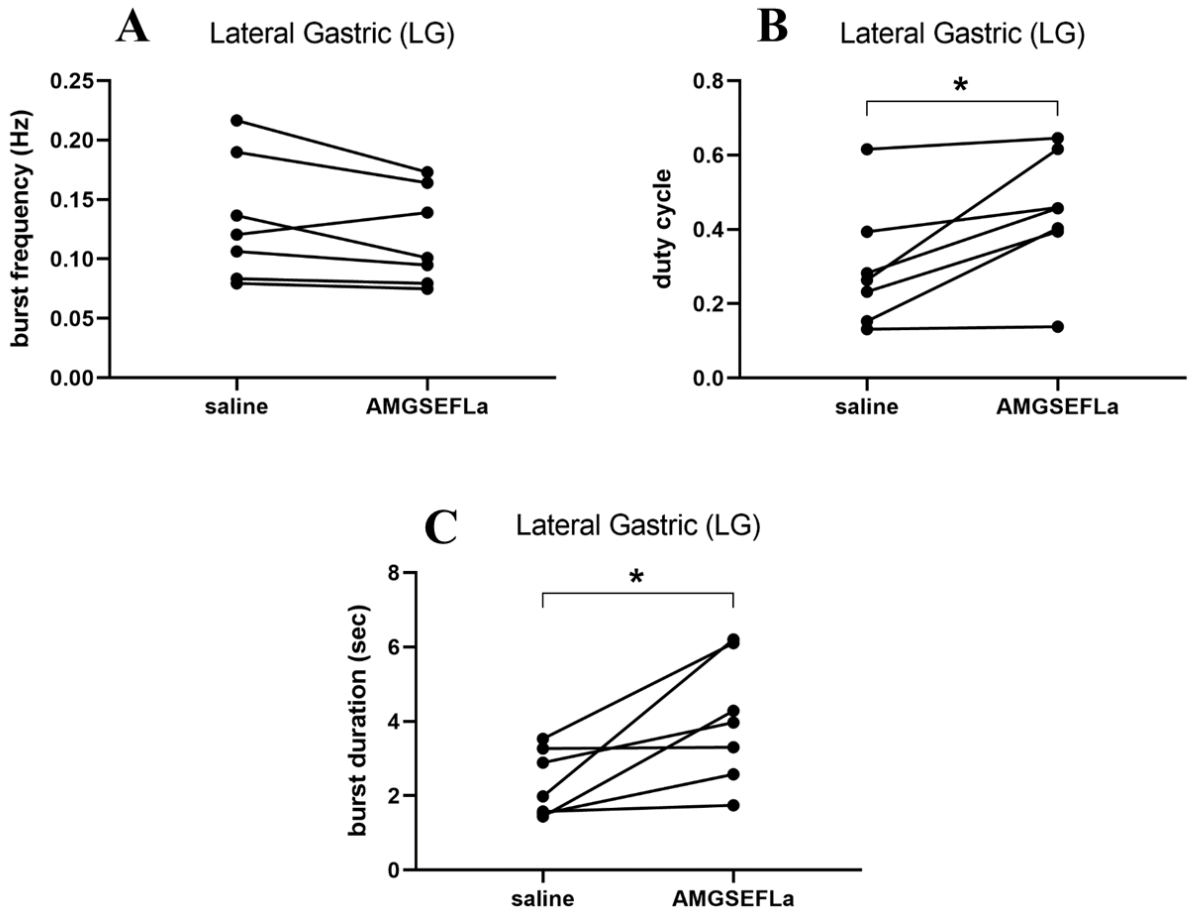

Figure 6. AMGSEFLamide significantly activated the lateral gastric neuron. (A) AMGSEFLamide applied at 10^{-6}M did not alter burst frequency of the gastric pattern as measured by the lateral gastric (LG) neuron when it was already active; paired *t*-test, *n*=7, *P*=0.1076. (B) AMGSEFLamide applied at 10^{-6}M increased activity in the LG neuron as measured by duty cycle; asterisk (*) indicates mean values significantly different from each other; paired *t*-test, *n*=7, *P*=0.0200. (C) AMGSEFLamide applied at 10^{-6}M increased burst duration of the LG neuron; paired *t*-test, *n*=7, *P*=0.0264. In 5/7 preparations in which the LG neuron was initially inactive, 10^{-6}M AMGSEFLamide application prompted LG neuron activity.

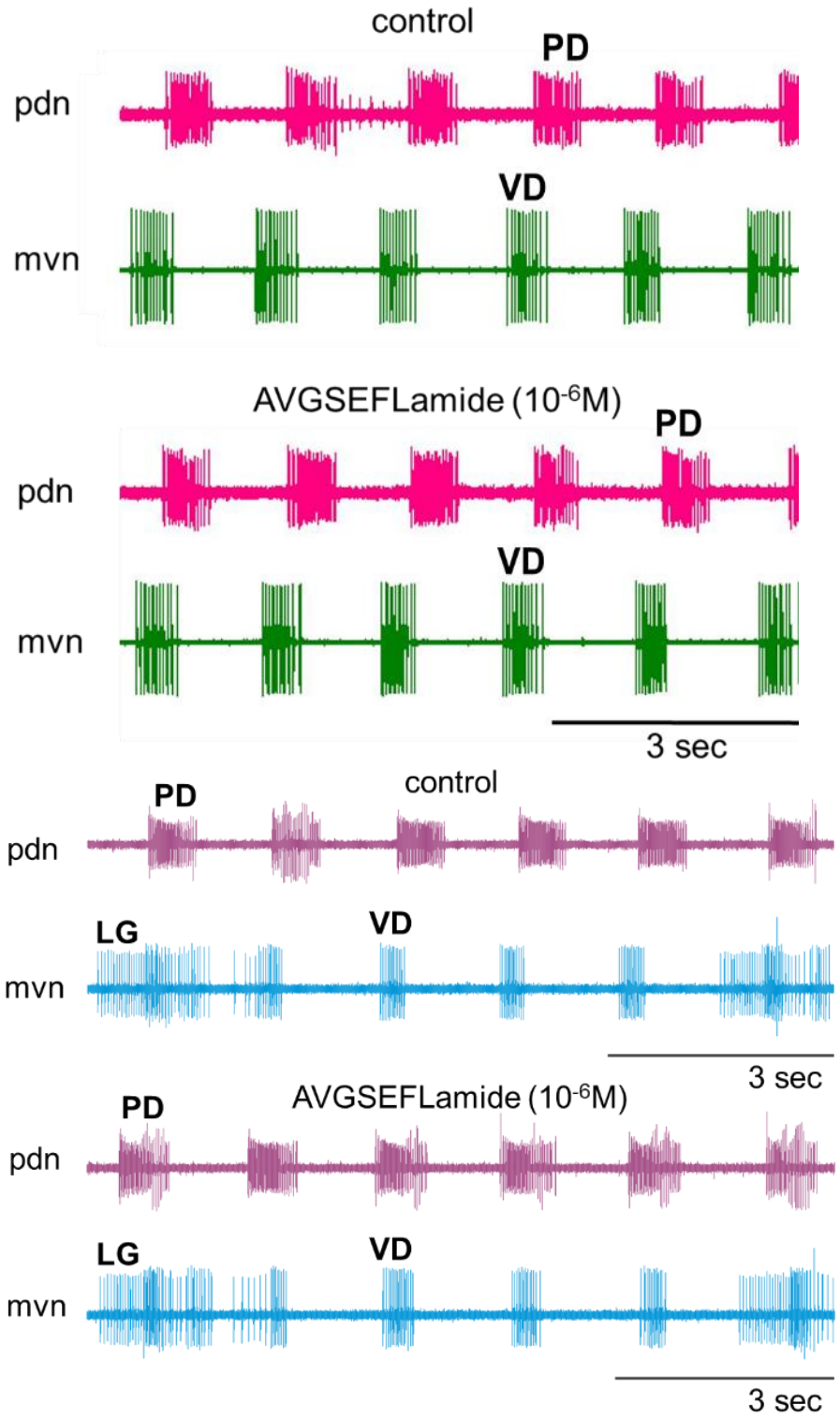

Figure 7. AVGSEFLamide did not appear to activate the gastric mill or pyloric pattern. Gastric activity was not visibly activated from an inactive state (top), nor enhanced if already active (bottom, LG neuron), when 10^{-6}M AVGSEFLamide was applied to the system. Pyloric activity did not appear to change with 10^{-6}M AVGSEFLamide application (PD, VD neurons). Bursting activity was determined by visible changes in burst density or duration as compared to control. *mvn*, medial ventricular nerve; *pdn*, pyloric dilator nerve.

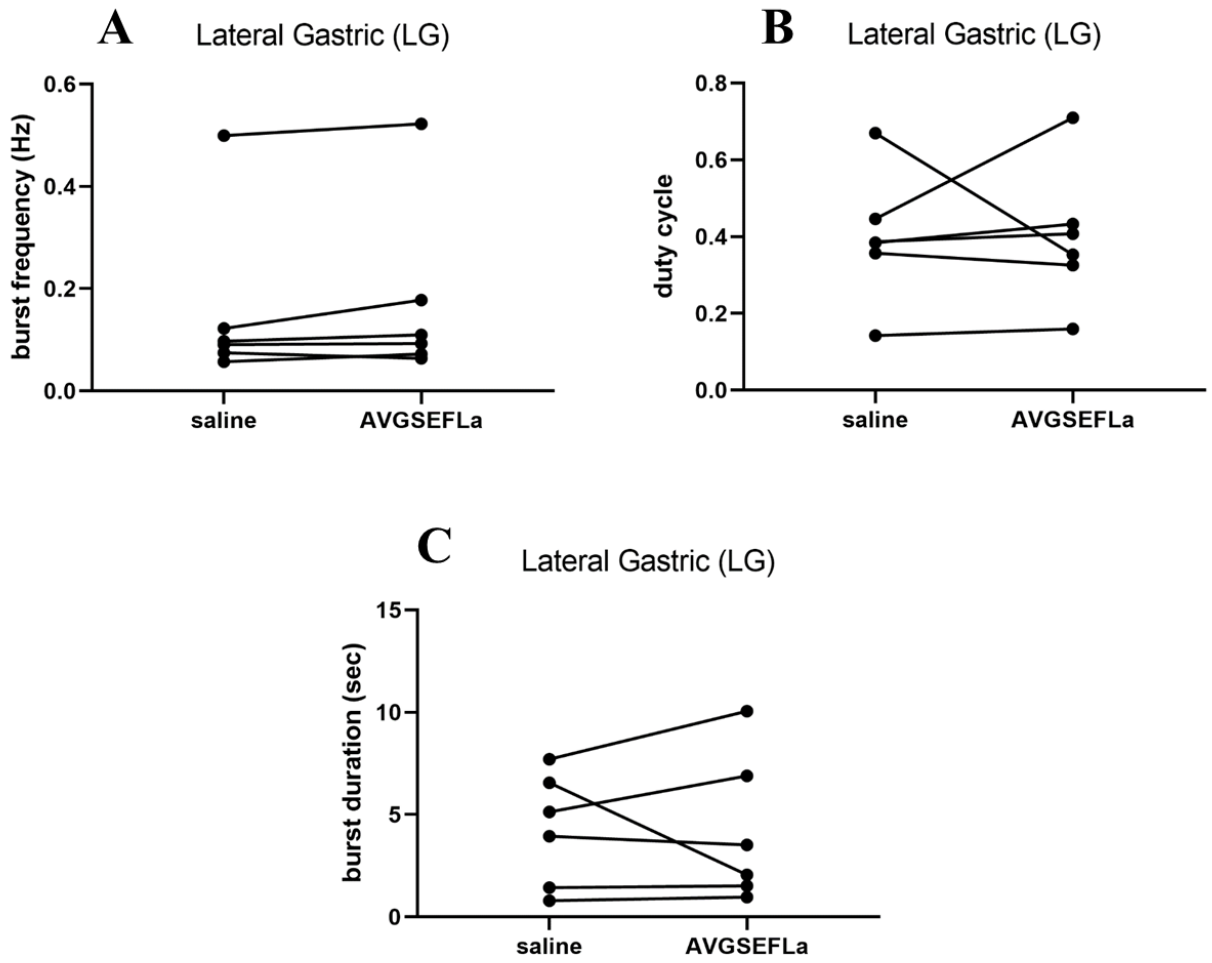

Figure 8. AVGSEFLamide did not activate the gastric mill circuit. (A) AVGSEFLamide applied at 10^{-6}M did not alter burst frequency of the gastric pattern as measured by the lateral gastric (LG) neuron when it was already active; paired *t*-test, *n*=6, *P*=0.1408. (B) AVGSEFLamide applied at 10^{-6}M did not change activity in the LG neuron as measured by duty cycle; paired *t*-test, *n*=6, *P*=0.9971. (C) AVGSEFLamide applied at 10^{-6}M did not alter burst duration of the LG neuron; paired *t*-test, *n*=6, *P*=0.9283. In 8/8 preparations in which the LG neuron was initially inactive, 10^{-6}M AVGSEFLamide application did not prompt LG neuron activity.

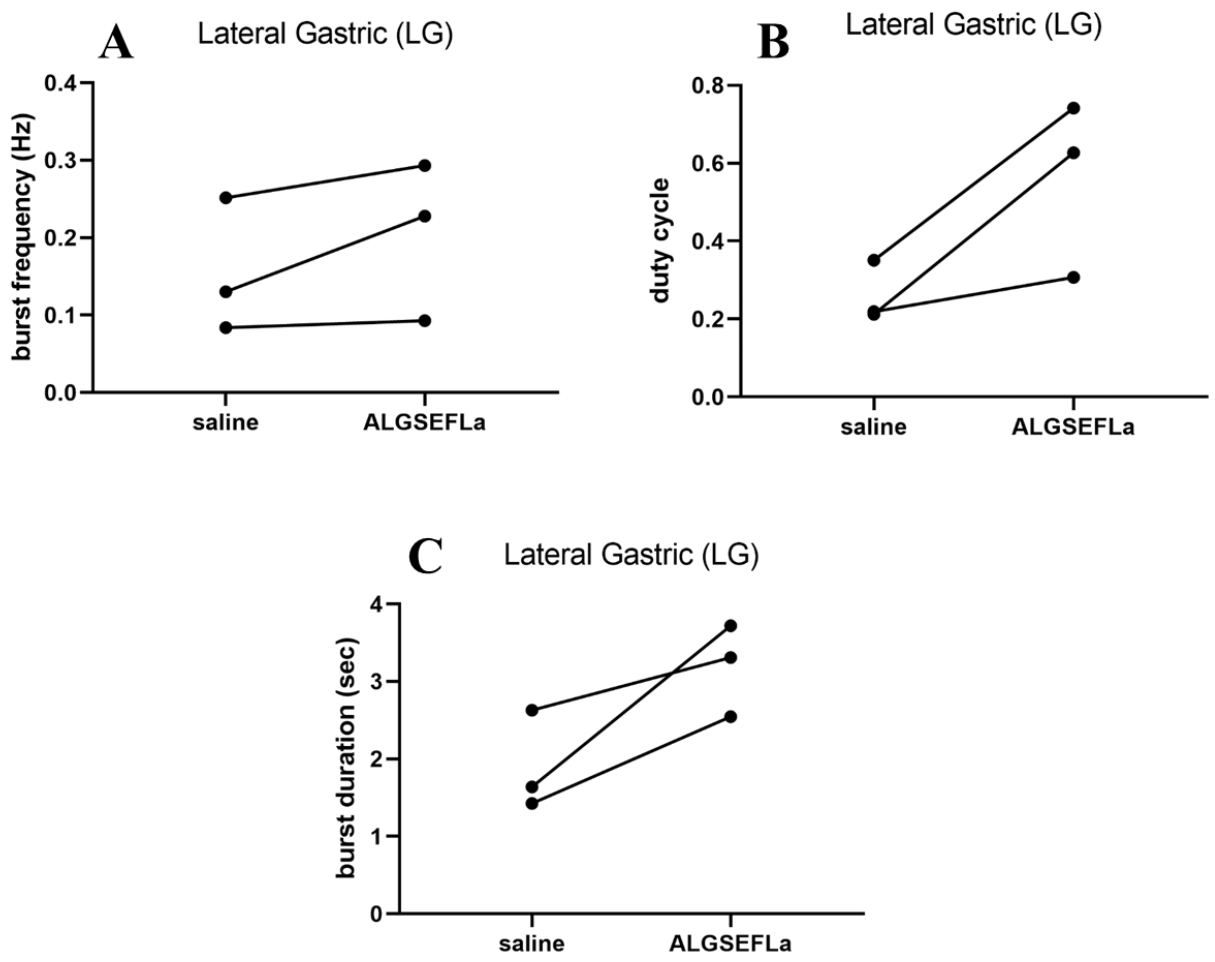

Figure 9. ALGSEFLamide modulated the lateral gastric neuron. (A) ALGSEFLamide applied at 10^{-6}M did not alter burst frequency of the gastric pattern as measured by the lateral gastric (LG) neuron when it was already active; paired *t*-test, *n*=3, *P*=0.1973. (B) ALGSEFLamide applied at 10^{-6}M did not change activity in the LG neuron as measured by duty cycle; paired *t*-test, *n*=3, *P*=0.1054. (C) ALGSEFLamide applied at 10^{-6}M did not change burst duration of the LG neuron; paired *t*-test, *n*=3, *P*=0.0888. Although the LG neuron was only initially active in three observed controls prior to ALGSEFLamide application, in 8/10 initially inactive preparations, application of 10^{-6}M ALGSEFLamide elicited patterned activity.

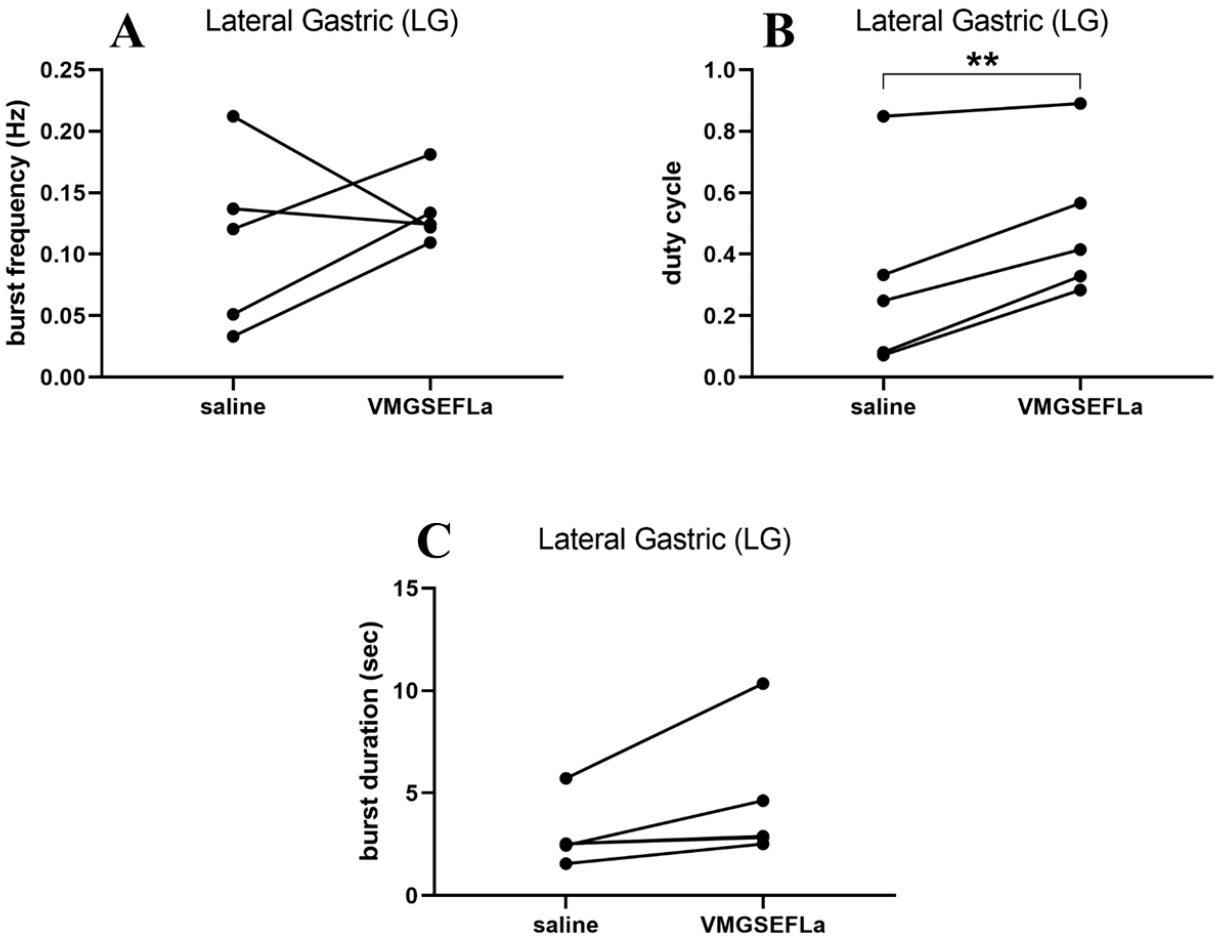

Figure 10. VMGSEFLamide significantly modulated the lateral gastric neuron. (A) VMGSEFLamide applied at 10^{-6}M did not alter burst frequency of the gastric pattern as measured by the lateral gastric (LG) neuron when it was already active; paired *t*-test, *n*=5, *P*=0.5193. (B) VMGSEFLamide applied at 10^{-6}M increased activity in the LG neuron as measured by duty cycle; asterisk (*) indicates mean values significantly different from each other; paired *t*-test, *n*=5, *P*=0.0084. (C) VMGSEFLamide applied at 10^{-6}M did not alter burst duration of the LG neuron; paired *t*-test, *n*=5, *P*=0.1061. In 5/8 preparations in which the LG neuron was initially inactive, 10^{-6}M VMGSEFLamide application prompted some LG neuron activity.

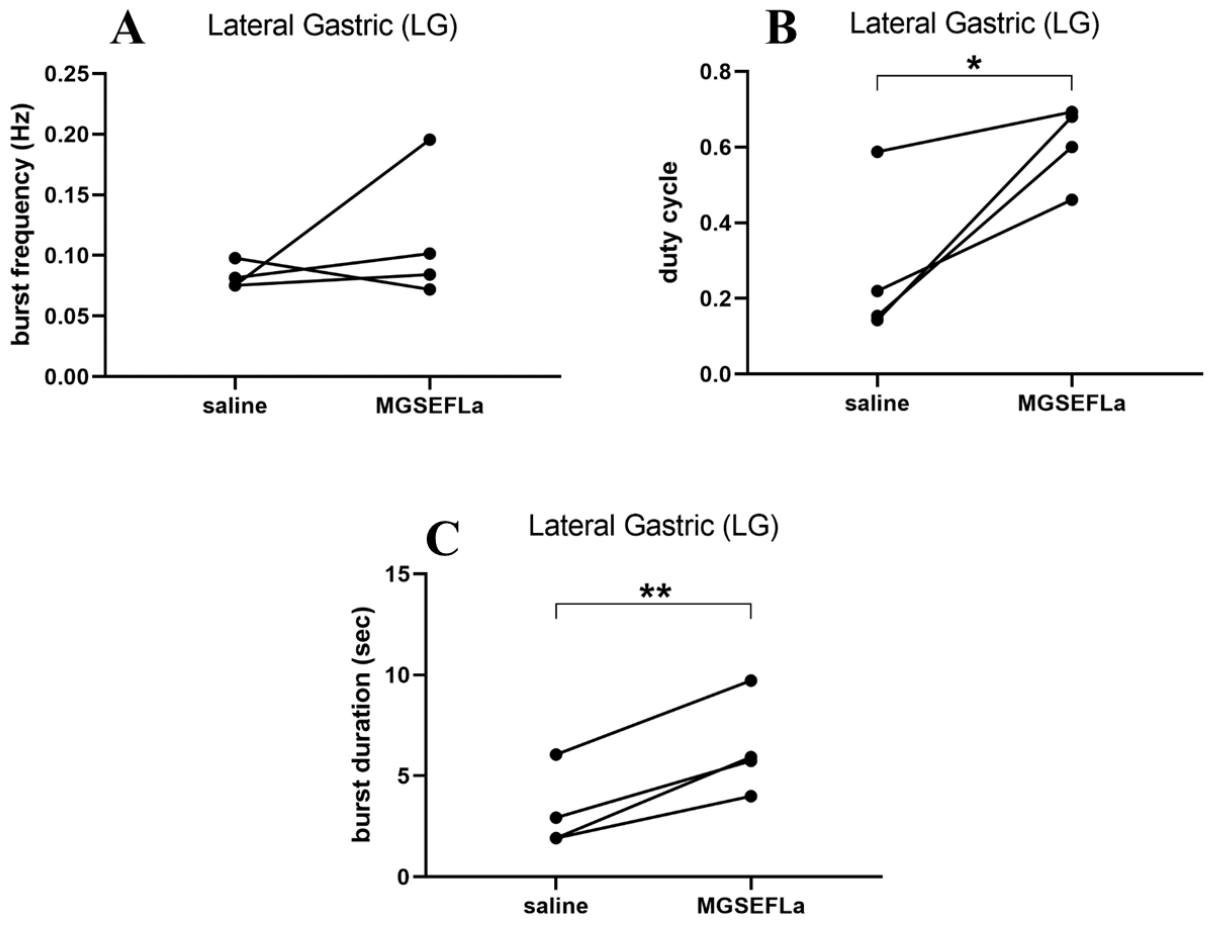

Figure 11. MGSEFLamide significantly modulated the lateral gastric neuron. (A) MGSEFLamide applied at 10^{-6}M did not alter burst frequency of the gastric pattern as measured by the lateral gastric (LG) neuron when it was already active; paired *t*-test, *n*=4, *P*=0.3979. (B) MGSEFLamide applied at 10^{-6}M increased activity in the LG neuron as measured by duty cycle; asterisk (*) indicates mean values significantly different from each other; paired *t*-test, *n*=4, *P*=0.0424. (C) MGSEFLamide applied at 10^{-6}M increased mean burst duration of the LG neuron; paired *t*-test, *n*=4, *P*=0.0055. In 6/9 preparations in which the LG neuron was initially inactive, 10^{-6}M MGSEFLamide application prompted LG neuron activity.

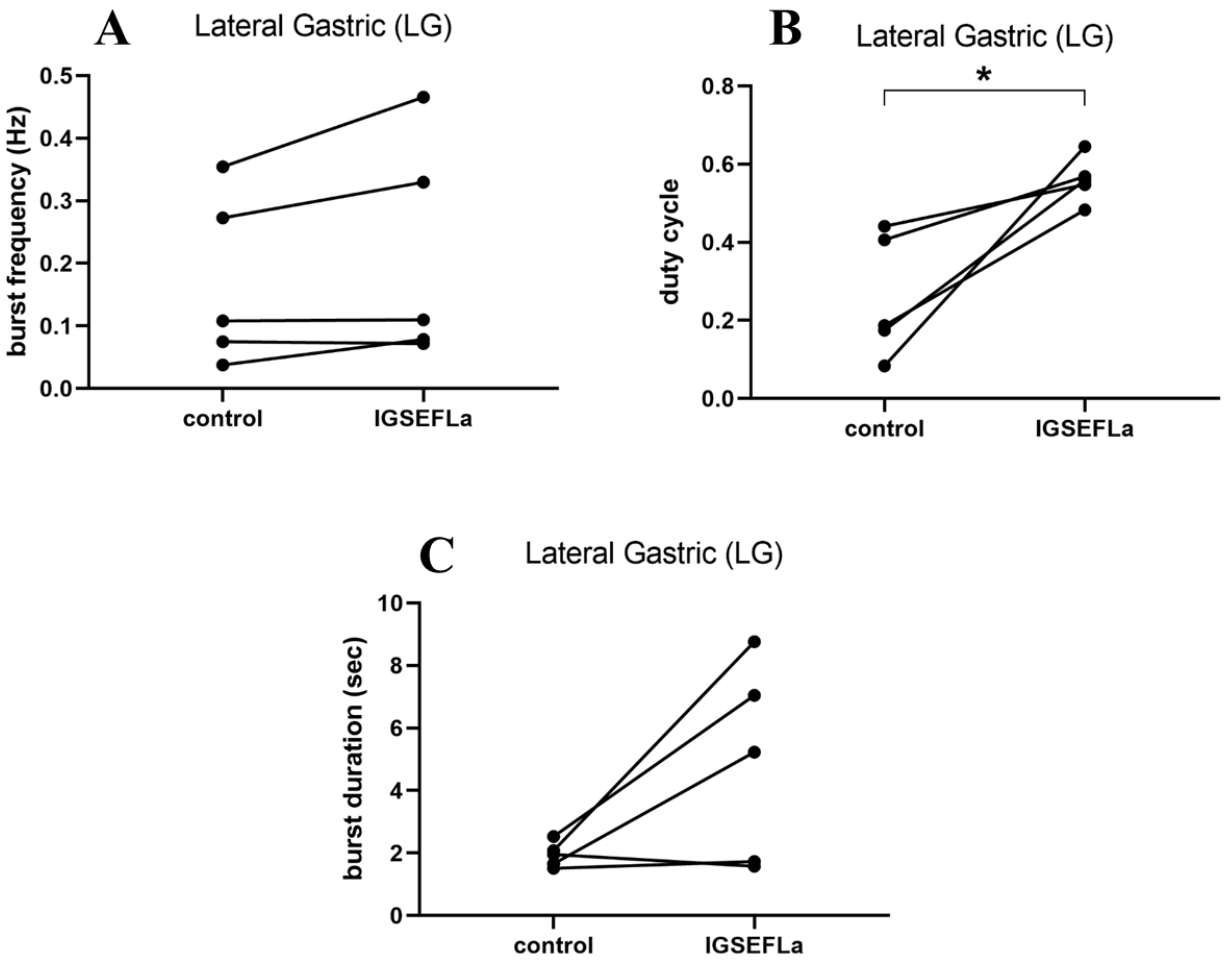

Figure 12. IGSEFLamide significantly modulated the lateral gastric neuron. (A) IGSEFLamide applied at 10^{-6}M did not alter burst frequency of the gastric pattern as measured by the lateral gastric (LG) neuron when it was already active; paired *t*-test, *n*=5, *P*=0.1153. (B) IGSEFLamide applied at 10^{-6}M increased activity in the LG neuron as measured by duty cycle; asterisk (*) indicates mean values significantly different from each other; paired *t*-test, *n*=5, *P*=0.0203. (C) IGSEFLamide applied at 10^{-6}M did not significantly increase mean burst duration of the LG neuron; paired *t*-test, *n*=5, *P*=0.0924. In 6/8 preparations in which the LG neuron was initially inactive, 10^{-6}M MGSEFLamide application prompted some LG neuron activity.

3.3 Pyloric circuit

In Figures 4 and 5 above, bursting patterns in the lateral pyloric (LP), pyloric (PY), pyloric dilator (PD), and ventricular dilator (VD) neurons were not visibly altered when 10^{-6}M AMGSEFLamide was applied. Similarly, no other GSEFLamide isoforms elicited strong, qualitatively obvious effects in the pyloric pattern. Quantitatively, this family never significantly changed pyloric cycle frequency as measured by PD neuron activity. However, most GSEFLamide isoforms did elicit more minor effects on the pattern, as represented by changes in burst duration or duty cycle of the PD neuron. The quantitative effects of this family on the pyloric dilator neuron are summarized below in Table 5.

AMGSEFLamide applied at 10^{-6}M significantly increased the duty cycle of pyloric dilator (PD) neurons, but did not alter burst frequency or burst duration (Figure 13). Much like what was observed in the gastric mill pattern, AVGSEFLamide applied at 10^{-6}M did not activate the pyloric circuit visibly (Figure 7) or quantitatively (Figure 14). ALGSEFLamide applied at 10^{-6}M significantly increased the duty cycle of pyloric dilator (PD) neurons, but did not change burst frequency or burst duration (Figure 15). VMGSEFLamide applied at 10^{-6}M also increased the mean duty cycle of pyloric dilator (PD) neurons (Figure 16); this represented the most significant change in PD neuron activity (as measured by duty cycle) of any member of the GSEFLamide family. Again, no changes in burst frequency and burst duration of the PD neuron were observed with this peptide.

MGSEFLamide applied at 10^{-6}M did not significantly change any bursting parameters of the pyloric filter circuit as measured by PD neuron activity (Figure 17). However, application of this peptide showed a trend toward higher average PD neuron duty cycle ($p<0.1$). IGSEFLamide

applied at 10^{-6}M significantly increased burst duration of the PD neuron, and elicited a trend towards higher mean PD neuron duty cycle ($p<0.1$), but did not alter burst frequency (Figure 18).

Isoform (-SEFLamide)	**Frequency**	**Duty Cycle**	**Burst Duration**
AMG-	X	sig. increase	X
AVG-	X	X	X
ALG-	X	sig. increase	X
VMG-	X	sig. increase	X
MG-	X	increasing trend	X
IG-	X	increasing trend	sig. increase

Table 5. Summary of 10^{-6}M GSEFLamide effects on activity in pyloric dilator neurons. No changes in PD neuron burst frequency were elicited by any GSEFLamide isoforms (an X represents no change). PD neuron duty cycle significantly increased with 10^{-6}M AMGSEFLamide application (Fig. 13, *n*=10), ALGSEFLamide application (Fig. 15, *n*=6) and VMGSEFLamide application (Fig. 16, *n*=7), and trended toward increasing with MGSEFLamide application (Fig. 17, *n*=6) and IGSEFLamide application (Fig. 18, *n*=7). PD neuron burst duration significantly increased with 10^{-6}M IGSEFLamide (*n*=7). AVGSEFLamide did not enhance pyloric dilator neuron patterns (Fig. 14, *n*=10).

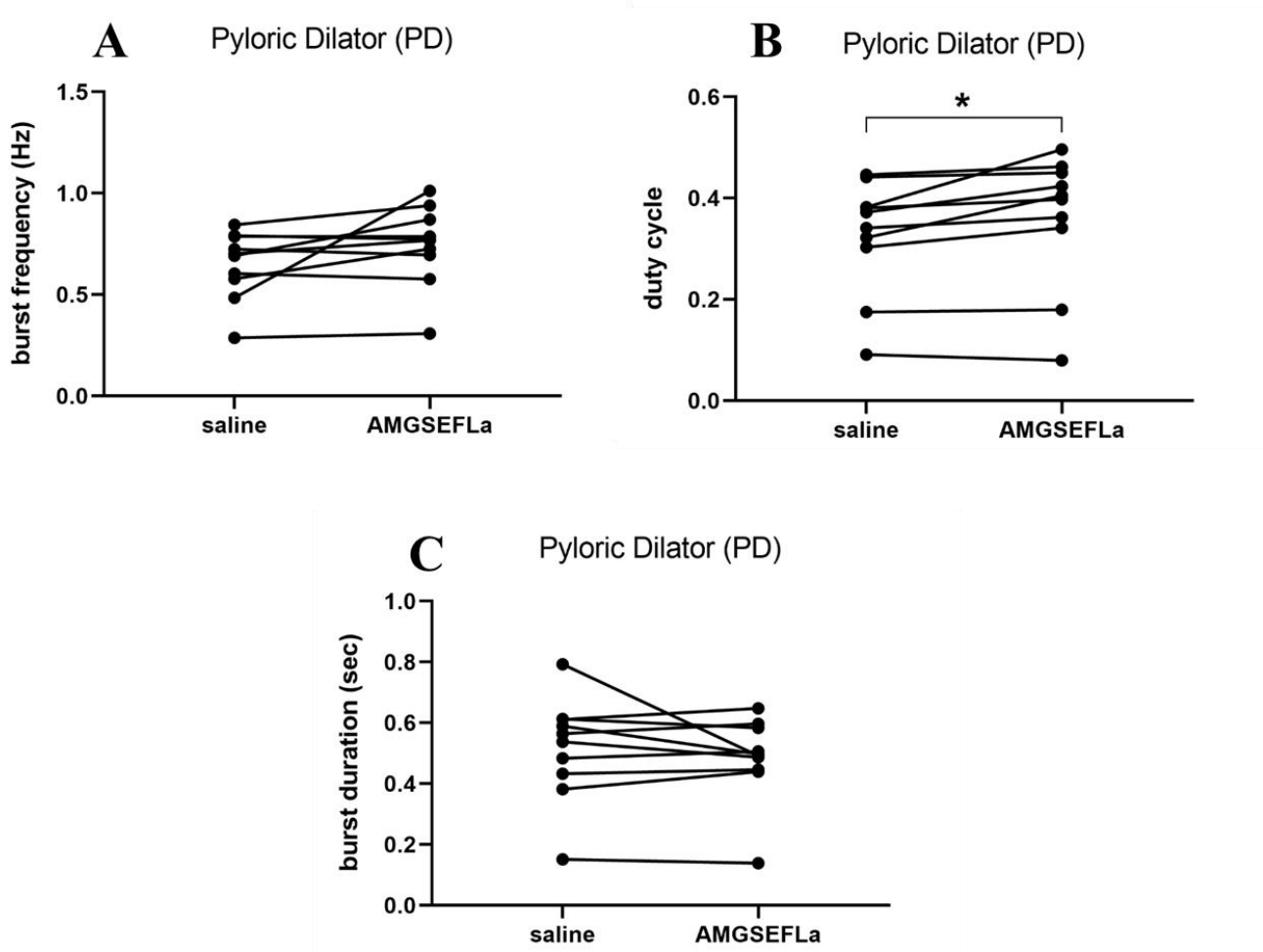

Figure 13. AMGSEFLamide enhanced activity in the pyloric dilator neuron. (A) AMGSEFLamide applied at 10^{-6}M did not alter burst frequency of the pyloric pattern as measured by activity in the pyloric dilator (PD) neuron; paired *t*-test, *n*=10, *P*=0.1040. (B) AMGSEFLamide applied at 10^{-6}M increased activity in the PD neuron as measured by duty cycle; asterisk (*) indicates mean values significantly different from each other; paired *t*-test, *n*=10, *P*=0.0207. (C) AMGSEFLamide applied at 10^{-6}M did not change burst duration of the PD neuron; paired *t*-test, *n*=10, *P*=0.3539.

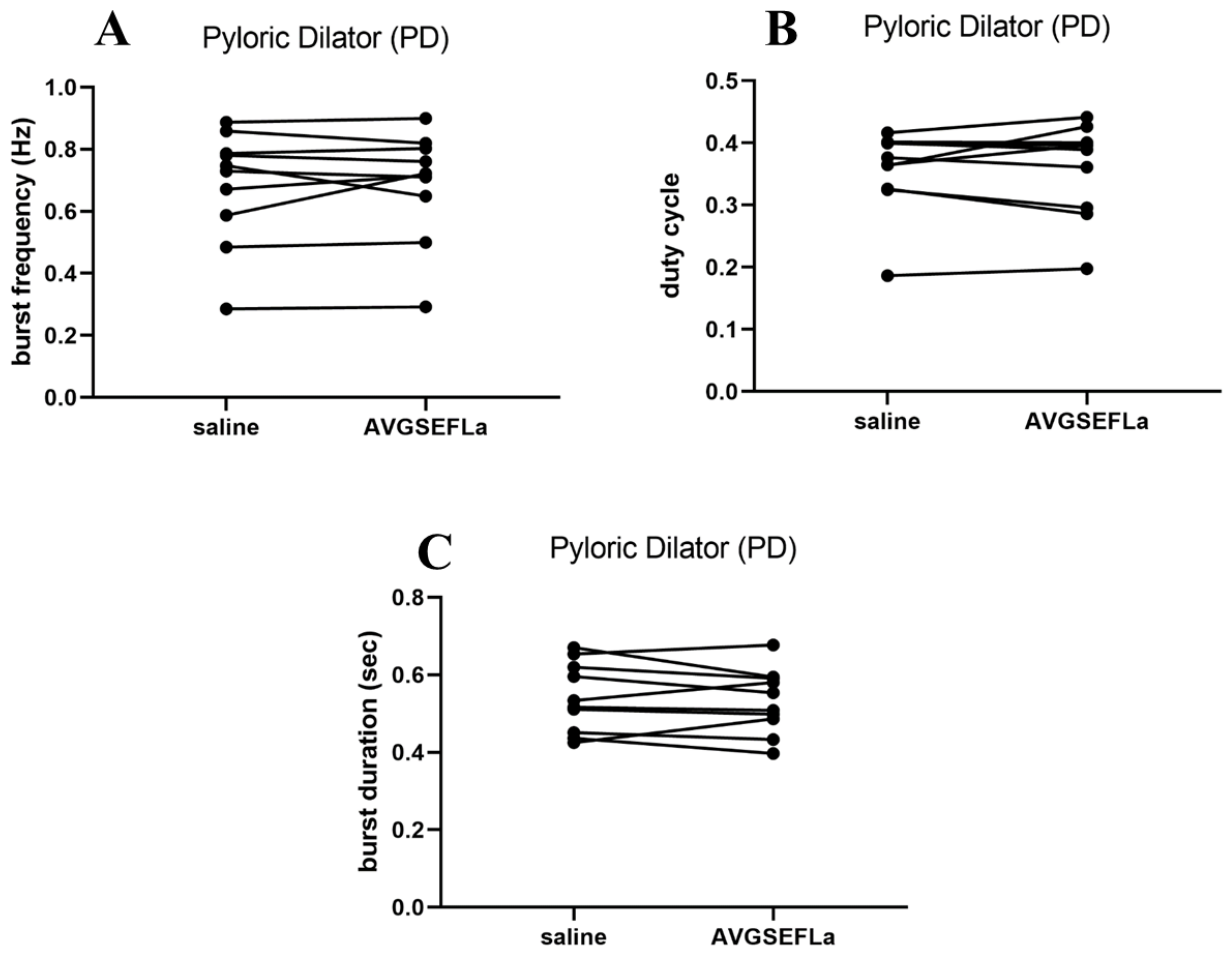

Figure 14. AVGSEFLamide did not alter activity in the pyloric circuit. (A) AVGSEFLamide applied at 10^{-6}M did not alter burst frequency of the pyloric pattern as measured by activity in the pyloric dilator (PD) neuron; paired *t*-test, *n*=10, *P*=0.7833. (B) AVGSEFLamide applied at 10^{-6}M did not change activity in the PD neuron as measured by duty cycle; paired *t*-test, *n*=10, *P*=0.7585. (C) AVGSEFLamide applied at 10^{-6}M did not change average burst duration of the PD neuron; paired *t*-test, *n*=10, *P*=0.5032.

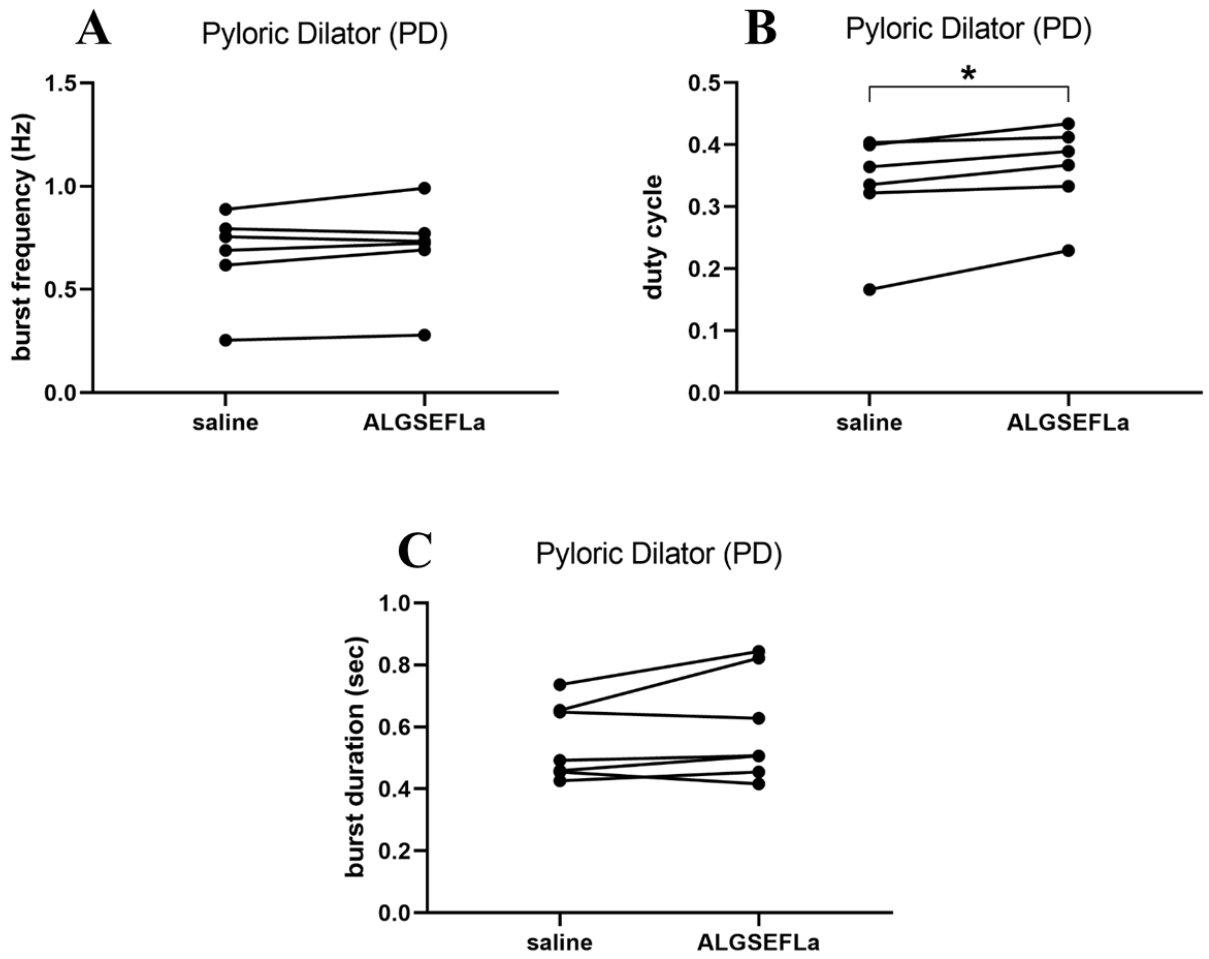

Figure 15. ALGSEFLamide enhanced activity in the pyloric dilator neuron. (A) ALGSEFLamide applied at 10^{-6}M did not alter burst frequency of the pyloric pattern as measured by activity in the pyloric dilator (PD) neuron; paired *t*-test, *n*=6, *P*=0.1862. (B) ALGSEFLamide applied at 10^{-6}M increased activity in the PD neuron as measured by duty cycle; asterisk (*) indicates mean values significantly different from each other; paired *t*-test, *n*=6, *P*=0.0158. (C) ALGSEFLamide applied at 10^{-6}M did not change burst duration of the PD neuron; paired *t*-test, *n*=7, *P*=0.1576.

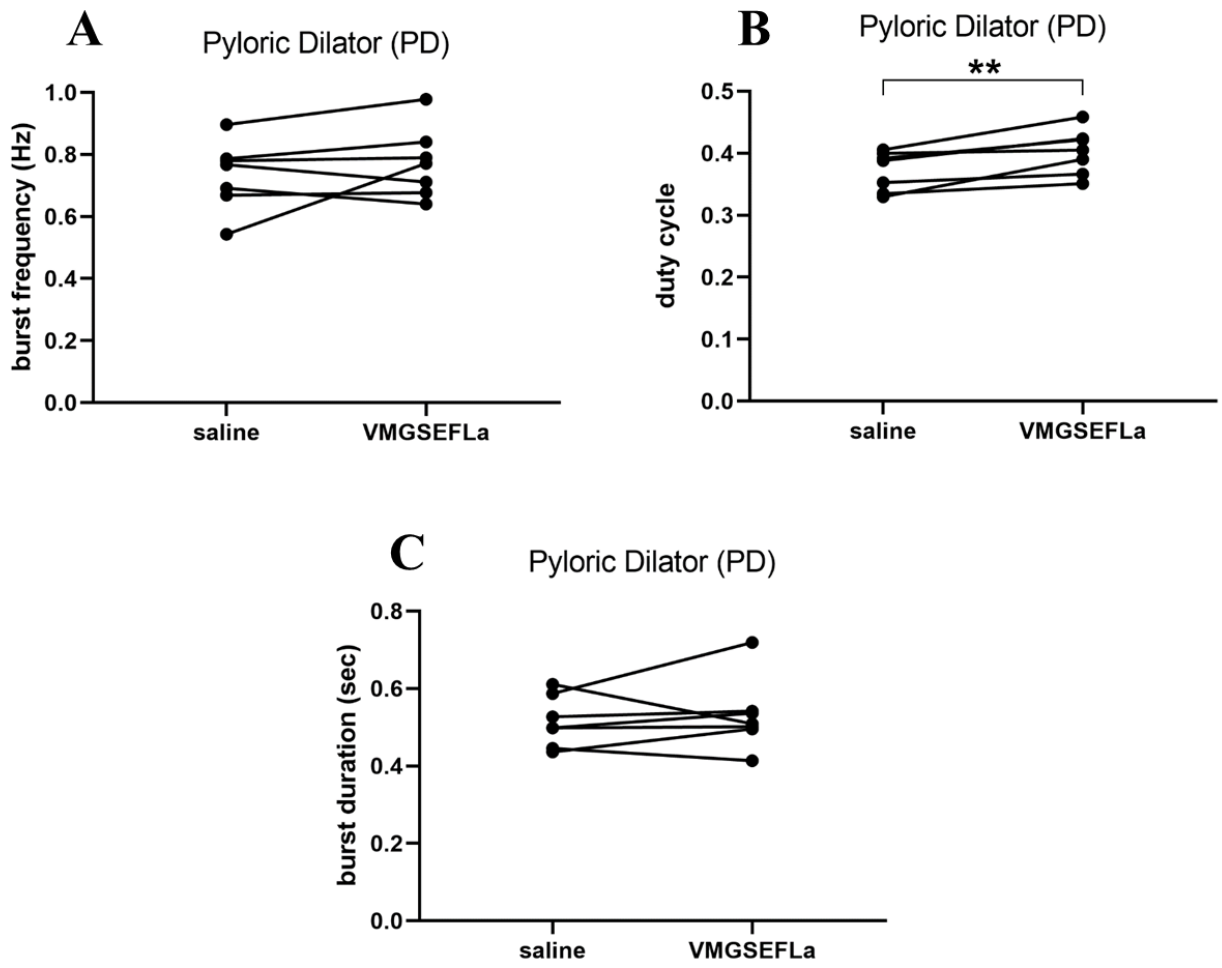

Figure 16. VMGSEFLamide enhanced activity in the pyloric dilator neuron. (A) VMGSEFLamide applied at 10^{-6}M did not alter burst frequency of the pyloric pattern as measured by activity in the pyloric dilator (PD) neuron; paired *t*-test, *n*=7, *P*=0.3256. (B) VMGSEFLamide applied at 10^{-6}M increased activity in the PD neuron as measured by duty cycle; asterisk (*) indicates mean values significantly different from each other; paired *t*-test, *n*=7, *P*=0.0077. (C) VMGSEFLamide applied at 10^{-6}M did not change burst duration of the PD neuron; paired *t*-test, *n*=7, *P*=0.5812.

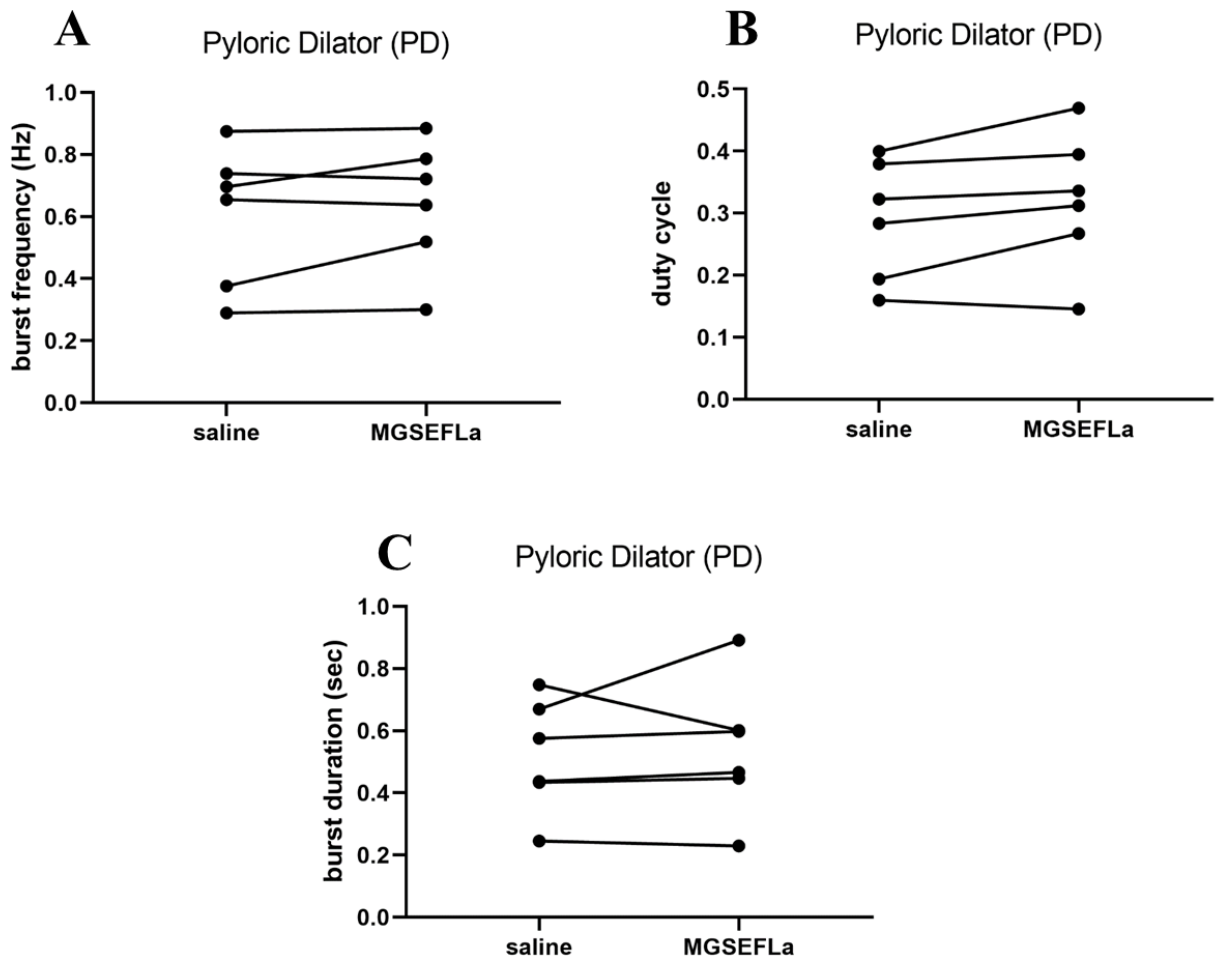

Figure 17. MGSEFLamide did not significantly alter activity in the pyloric circuit. (A) MGSEFLamide applied at 10^{-6}M did not alter burst frequency of the pyloric pattern as measured by activity in the pyloric dilator (PD) neuron; paired *t*-test, *n*=6, *P*=0.2327. (B) MGSEFLamide applied at 10^{-6}M did not significantly change activity in the PD neuron as measured by duty cycle; paired *t*-test, *n*=6, *P*=0.0776. (C) MGSEFLamide applied at 10^{-6}M did not change average burst duration of the PD neuron; paired *t*-test, *n*=6, *P*=0.6861.

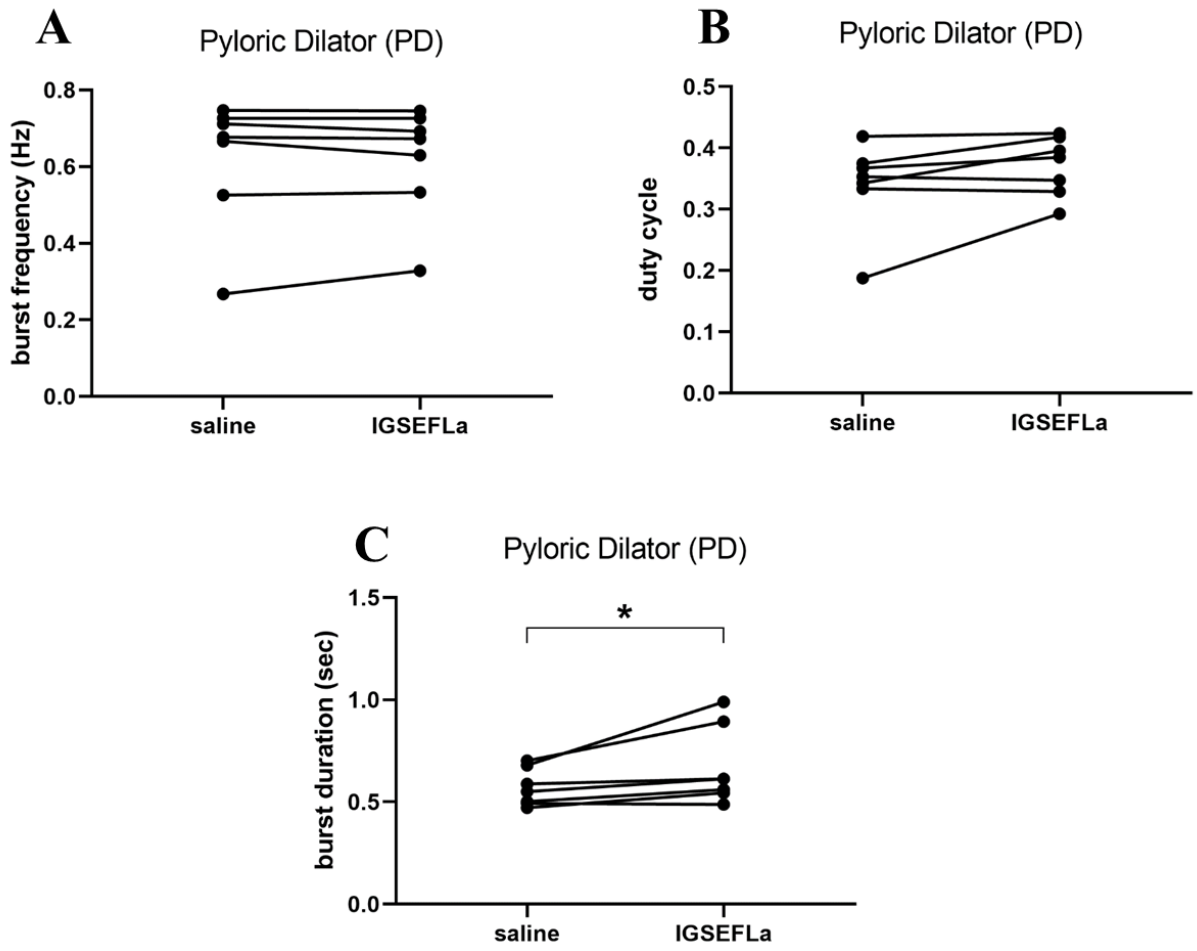

Figure 18. IGSEFLamide enhanced activity in the pyloric dilator neuron. (A) IGSEFLamide applied at 10^{-6}M did not alter burst frequency of the pyloric pattern as measured by activity in the pyloric dilator (PD) neuron; paired *t*-test, *n*=7, *P*=0.9366. (B) IGSEFLamide applied at 10^{-6}M did not significantly change activity in the PD neuron as measured by duty cycle; paired *t*-test, *n*=7, *P*=0.0902. (C) IGSEFLamide applied at 10^{-6}M increased mean burst duration of the PD neuron; asterisk (*) indicates mean values significantly different from each other; paired *t*-test, *n*=7, *P*=0.0498.

DISCUSSION

The goal of this project was to characterize the effects of a novel family of neuropeptides, the GSEFLamides, on the stomatogastric nervous system of the American lobster. Initially identified endogenously in 2017, the GSEFLamides are produced in at least one center of neurohormone synthesis, the lobster's eyestalk, in which the X-organ-sinus-gland is located. While this family of peptides may be released into hemolymphatic circulation in order to modulate the nervous system, it is also feasible that the GSEFLamides are released locally onto the STNS. Using electrophysiological recordings, we were able to assess the potential functional modulatory effects of this family on the stomatogastric nervous system. Two central pattern generators drive and regulate movement in the lobster's foregut; the continuously active gastric mill, and the intermittently active pyloric filter. These two CPGs have cell bodies housed in the stomatogastric ganglion, one of four ganglia in the STNS. Activity in each CPG was measured via bursting patterns from individual neurons, and six GSEFLamide isoforms were applied in random order to each preparation to determine their effects.

The STNS preparations each responded uniquely (if at all) to GSEFLamide isoforms, and these responses were likely impacted by several variables, such as the precision and speed of gross and microdissections, saline temperature, and electronics setups. Nonetheless, some notable patterns in activity were observed in the gastric mill and pyloric CPGs when the GSEFLamides were applied. Overall, data from these experiments indicated that the GSEFLamides variably activated CPGs in the STNS, but that AVGSEFLamide consistently had no effect. These findings are underscored by recent unpublished work from the Dickinson lab, which highlighted that every isoform in this family except AVGSEFLamide increased contraction amplitude in the whole lobster heart. All GSEFLamide isoforms, with the exception

of AVGSEFLamide, activated or enhanced activity in the gastric mill CPG, particularly when activity was measured by lateral gastric neuron duty cycle. Although less quantifiable, in many preparations, gastric neurons were observed to initiate patterned activity when a peptide isoform was applied; this occurred several times with each isoform except AVGSEFLamide.

This family produced more subtle effects on the pyloric filter CPG, but nonetheless increases in pyloric dilator neuron activity (also measured by duty cycle) were observed in three out of six GSEFLamide isoforms, with two more of those isoforms (MG- and IGSEFLamide) causing mean trends toward increased duty cycle. Across both circuits, however, AVGSEFLamide failed to elicit increased or new activity, which parallels its lack of effect observed in the whole heart.

While the lack of effect produced by AVGSEFLamide is consistent, the physiological purpose of this peptide isoform in either the heart or the foregut remains unclear. Additionally, it is notable that any significant effects elicited by isoforms in this peptide family were activating – none of the GSEFLamides caused a decrease in activity as measured by the examined parameters. This could suggest that a broader physiological role of this family accelerates or assists in coordinating the lobster's digestive activity.

4.1 Five of six GSEFLamide isoforms modulate the gastric mill circuit

Our data on AMGSEFLamide were consistent with findings from Dickinson et al. (2019); when applied at 10^{-6}M, this peptide isoform increased bursting activity in the gastric mill pattern as measured by increased burst duration and duty cycle in the LG neuron. Additionally, analysis of VMG-, MG- and IGSEFLamide isoforms revealed increases in LG neuron activity as measured by duty cycle. MGSEFLamide was also observed to increase LG neuron burst

duration. Although by quantitative parameters ALGSEFLamide did not appear to enhance the gastric circuit, this isoform did activate LG neurons in several preparations in which it was not active, and it has therefore been counted as a peptide that elicits activity from the gastric mill. Together, these data indicate that most GSEFLamides modulate the gastric mill CPG, but that AVGSEFLamide has no notable effect on this circuit.

4.2 Three of six GSEFLamide isoforms modulate the pyloric filter circuit

Again consistent with findings from Dickinson et al. (2019), AMGSEFLamide increased bursting activity in the pyloric filter circuit, in this case measured by duty cycle of PD neuron bursts. Although this increased pyloric activity is consistent, the parameters by which this activity was measured differ between the Dickinson et al. study and the present work. The 2019 study found increases in pyloric frequency when AMGSEFLamide was applied at 10^{-6}M; however, the same conditions here did not show a consistent trend in pyloric frequency as measured by PD activity (Figure 13). Nonetheless, the present data are consistent with Dickinson et al. (2019) in finding that AMGSEFLamide increases pyloric activity.

ALGSEFLamide and VMGSEFLamide both followed AMGSEFLamide in their consistent pyloric dilator activation as measured by duty cycle. While MG- and IGSEFLamide isoforms did not significantly activate any aspect of the pyloric circuit, p-values of 0.0902 and 0.0776, respectively, indicate trends toward increases in PD neuron duty cycle elicited by these two family members. Additionally, IGSEFLamide increased PD neuron burst duration. Across the board, however, no significant increases in pyloric burst frequency were observed. Typically, burst frequency can be extrapolated to represent a whole pattern's activity, whereas burst duration and duty cycle are characteristics of individual neurons. The lack of change in burst

frequency in the pyloric pattern might therefore suggest that the whole pyloric circuit is not activated by this family to the same degree that its individual constituent neurons are. Again, as seen in the whole heart and in the gastric mill circuit, AVGSEFLamide elicited no significant effects on the pyloric pattern.

These pyloric data, in conjunction with qualitative assessments of the pyloric pattern before and after peptide application (e.g., Figures 4, 5) suggest that while the GSEFLamides may modulate the pyloric circuit, they prompt more drastic changes in the gastric mill circuit. It is possible, though, that these changes are more drastic due to the long cycle period and only intermittently-active nature of the gastric mill, making cumulative modulatory effects more obvious than in the constantly-bursting pyloric circuit.

4.3 Receptors

The lack of activity produced by AVGSEFLamide across multiple central pattern generators and systems is unique given the fairly robust effects of its structurally similar family members. Considering this isoform also did not modulate activity in the lobster heart, it is plausible that AVGSEFLamide does not play any role in either of these two systems. This raises the question of the broader functional role of this isoform (if any), given that its five family members all appear to modulate both the cardiac system and the STNS.

Due to the novelty of the GSEFLamide peptide hormones, there are no known receptors for this family, and therefore determining more specific mechanisms or locations of modulatory activity is difficult. However, there are some steps that could be taken to further elucidate the functionality of AVGSEFLamide. One such step would be to apply AVGSEFLamide to a system like the STNS in conjunction with a familial isoform that is known to affect the system, such as

AMGSEFLamide. Doing this could provide insight as to whether AVGSEFLamide is binding to the same receptors as its family members, and simply not having an effect, or if it is not binding to receptors in this system at all.

We had hoped to complete experiments along these lines during the final months of this project; however, due to the COVID-19 pandemic-shortened 2020 Spring semester, only one such trial was performed. In that experiment, a solution containing both AVGSEFLamide and AMGSEFLamide (each at $5x10^{-7}$M) was perfused through the isolated STNS. In this single trial, the combined AVG- and AMGSEFLamide solution was observed to activate the gastric mill pattern in the same way AMGSEFLamide had done alone previously. This gastric activation occurred despite each isoform being applied at a slightly lower concentration ($5x10^{-7}$M) than was typically used in this project. These data suggested the possibility that AVGSEFLamide either does not bind to the same receptors as its family members, or binds in an uncompetitive or less selective way, or perhaps that our AVGSEFLamide isoform was synthesized in a faulty way and thus is inert. In any case, further experiments that apply AVGSEFLamide in conjunction with known activating GSEFLamide family members could be utilized to elucidate this isoform's functional purpose.

4.4 Potential AVGSEFLamide roles

If further research indicates that GSEFLamide isoforms can modulate *H. americanus* central pattern generators even in the presence of simultaneously applied AVGSEFLamide, an interesting analysis might compare effects of those combinatory solutions to the effects elicited by the five activating isoforms in this study. If combination AVGSEFLamide solutions significantly differed in effect from single GSEFLamide isoforms, it might be possible that

AVGSEFLamide acts exclusively in an allosteric manner on GSEFLamide receptors, and has no role of its own. Receptors in the STNS are often G-protein coupled receptors (GPCRs), which have been seen to be subject to allosteric modulation in other organisms (Gregory et al., 2011); however, there is no established evidence of a neuropeptide having an exclusively allosteric effect on GPCRs in American lobster central pattern generators. Interestingly, though, there is an abundance of research on the allosteric modulation of crustacean haemocyanin, the respiratory protein that carries oxygen in hemolymph. Several studies have found that otherwise non-physiologically functional molecules and ions like L-lactate (lactic acid) and caffeine are able to elicit increased oxygen binding affinity in crustacean haemocyanin (Menze et al., 2005; Zeis et al., 1992). Thus, if AVGSEFLamide appeared to alter GSEFLamide activity when combined with other familial isoforms, this could suggest an isolated allosteric role for an otherwise non-functional peptide. It is also plausible, however, that AVGSEFLamide is simply an inactive peptide in the lobster, and only is present in the neuropeptidome due to a mutation like a missense point mutation, creating an unwanted peptide in what should be another GSEFLamide isoform.

4.5 Future directions

Past research on STNS central pattern generators in the American lobster have consistently utilized preparations in which the stomatogastric nerve has been blocked or cut, in order to isolate the gastric mill and pyloric networks from anterior modulatory inputs. Therefore, steps to further elucidate the functional effects of the GSEFLamides on the STNS should include examining isolated systems in which the *stn* has been blocked or cut. Doing so might provide more detailed insight as to the activating capacity of this peptide family in the STNS.

This study also only considered the effects of the GSEFLamides at a concentration of 10^{-6}M; this concentration was used based on information from the Dickinson et al. 2019 study, which found this concentration of AMGSEFLamide to be effective in modulating the cardiac ganglion and STNS without overwhelming either system. Future experiments could consider increasing the concentration at which GSEFLamide isoforms are applied, particularly in order to determine whether AVGSEFLamide might exhibit modulatory effects when applied at high concentrations. Finally, mass spectrometry studies like those done on the *H. americanus* brain (Dickinson et al., 2019) might be considered in the STNS, in order to look for GSEFLamide presence in local ganglia. This could support a hypothesis that the GSEFLamides act locally, in addition to hormonally.

Overall, this project has met its initial goals of characterizing the effects of the newly discovered GSEFLamide peptide family in the American lobster's stomatogastric nervous system. Given the novelty of this family, however, more can be done to elucidate its comprehensive functional effects in *Homarus*. The above work may therefore serve as a starting point from which the GSEFLamides can be further studied.

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