Published in final edited form as:

Peptides. 2012 August ; 36(2): 272–279. doi:10.1016/j.peptides.2012.05.002.

Structure-activity and immunochemical data provide evidence of developmental- and tissue-specific myosuppressin signaling

M. Dickerson, J. McCormick, M. Mispelon, K. Paisley, and R. Nichols[#]

Biological Chemistry Department University of Michigan Medical School Ann Arbor, MI 48109-0600, USA

Abstract

Myosuppressin peptides dramatically diminish contractions of the gut and heart. Thus, delineating mechanisms involved in myosuppressin signaling may provide insight into peptidergic control of muscle contractility. *Drosophila* myosuppressin (DMS, TDVDHVFLRFamide) structure-activity relationship (SAR) was investigated to identify an antagonist and explore signaling. Alanyl-substituted, N-terminal truncated, and modified amino acid analogs identified residues and peptide length required for activity. Immunochemistry independently provided insight into myosuppressin mechanisms. DMS decreased gut motility and cardiac contractility dose dependently; the different effective concentrations at half maximal-response were indicative of tissue-specific mechanisms. Replacement of aspartic acid 2 (D2) generated an analog with different developmental- and tissue-specific effects; [A2] DMS mimicked DMS in adult gut (100% inhibition), yet decreased larval gut contractions by only 32% with increased potency in pupal heart (126% inhibition). The DMS active core differed across development and in tissues; adult (DHVFLRFamide) and larval gut (TDVDHVFLRFamide), and adult (VFLRFamide) and pupal heart (VFLRFamide). Substitution of D2 and D4 with a modified amino acid, p-benzoyl-phenylalanine, produced developmental- and tissue-specific antagonists. In the presence of protease inhibitors, DMS and VFLRFamide were more effective in adult gut, but lower or unchanged in pupal heart compared to peptide or analog alone, respectively. DMS-specific antisera stained neurons that innervated the gut or heart. This study describes novel antagonists and data to identify developmental- and tissue-specific mechanisms underlying the pleiotropic effects of myosuppressin in muscle physiology.

1. Introduction

Muscle contractions are important for survival; failure to properly regulate contractility may result in a serious disease or death. Peptides regulate muscle contractions, yet much remains to be discovered regarding the mechanisms underlying peptidergic regulation of contractility. Establishing structure-activity relationship (SAR) is a powerful approach to design molecular tools to delineate mechanisms and design antagonists to a myoactive peptide which serves as a high-affinity ligand. Antagonism of peptidergic ligands which interact with G protein-coupled receptors (GPCRs) can provide a promising avenue to identify progenitors and develop strategies to address muscle contractility-related disorders.

© 2012 Elsevier Inc. All rights reserved.

[#]Corresponding author at: Biological Chemistry Department, University of Michigan Medical School, Ann Arbor, MI 48109-0600, USA. Tel.: +1 734 764-4467; fax: +1 734 763-4581. nicholsr@umich.edu (R. Nichols).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Myosuppressins are invertebrate, myoactive peptides [5, 7, 8, 10, 18]. The consensus structure representing insect myosuppressins is X1DVX2HX3FLRFamide, where X1 = pE, P, T, A; X2 = D, G, V; X3 = V, S. Myosuppressins are members of a peptide family which contain an identical RFamide C terminus. The first RFamide peptide identified was the cardioactive tetrapeptide, FMRFamide [15]. The FMRFamide-related peptide (FaRP) family is subdivided based on XRFamide, where X defines the subgroup. Myosuppressins contain an LRFamide.

The first myosuppressin identified, leucomyosuppressin (LMS), was isolated based on its ability to decrease the frequency and amplitude of gut contractions [5]. The structurally-similar *Drosophila melanogaster* myosuppressin (dromyosuppressin, DMS, TDVDHVFLRFamide) was identified as a naturally-occurring peptide [10]. Following the discovery of LMS as a brain peptide which decreased gut motility, myosuppressins were found to regulate cardiovascular function [17]. DMS binds to two expressed GPCR proteins, DMS receptor-1 (DMSR-1) and -2 (DMSR-2) [4].

Numerous vertebrate FaRPs are known. The first vertebrate FaRP identified was an LRFamide-containing peptide, chicken brain LPLRFamide [2]. Human RFamide-related peptide-1 (hRFRP-1; MPHSFANLPLRFamide), also an LRFamide, dramatically decreases mammalian cardiac performance [13]. The cross-species conservation of structure and bioactivity provides the opportunity to utilize an interdisciplinary approach to delineate peptidergic regulation of muscle contractility. However, no published report describes a detailed SAR, functional antagonist, and developmental- and/or tissue-specific analysis of an LRFamide. Thus, DMS SAR was established in *D. melanogaster* adult and larval gut, and in adult, pupal, and larval heart. Additionally, analogs containing a modified amino acid were designed to identify antagonists. Finally, DMS-specific antisera were used to immunochemically map peptide expression.

This study tested the hypothesis that residues throughout the peptide may be involved in signaling. This prediction was based on the myosuppressin consensus structure. Furthermore, it was hypothesized that mechanisms underlying the influence of myosuppressin may be developmentally and/or tissue-specifically regulated. Additionally, it was hypothesized that unique brain cells innervated either the gut or heart to regulate peptidergic function. These predictions were based on the needs of an animal to respond to variant physiological and environmental cues and requirements for nutrient absorption and circulation across development and in tissues. Myosuppressin SAR, active cores, antagonists, and antisera provide powerful information and molecular tools to dissect mechanisms and establish function. Knowledge of mechanisms underlying the influence of myosuppressin aids in identifying molecular targets to develop strategies to regulate muscle contractility.

2. Materials and methods

2.1. Flies

D. melanogaster Oregon R strain flies were maintained on cornmeal molasses media at 24°C under a 12 hour light/dark cycle. Animals selected for analysis were wandering 3rd instar larvae for gut and heart bioassays, and white prepupae for heart bioassays. In addition, 4-7 day and newly eclosed to 2 hour adults were chosen for gut or heart bioassays, respectively. The effects of DMS, analogs, and physiological saline, the carrier, tested without peptide or analog as a control, were measured on both females and males; no sex-specific response was observed.

2.2. Chemicals

Alanyl-substituted, truncated, p-benzoyl-phenylalanine (Bpa)-containing, and free acid DMS analogs were synthesized using a standard Fmoc protocol and purified by reversed phase HPLC. Structures were confirmed by amino acid analysis and mass spectrometry. The nomenclature for the alanyl-substituted and N-terminal truncated analogs is found in Tables 1-5. Peptides were diluted in series with physiological saline to obtain the working solutions. The cocktail of water-soluble protease inhibitors had broad specificity for serine, cysteine, and metalloproteases (Sigma-Aldrich Co.; SKU P2714).

2.3 Bioassays

An adult gut assay was performed according to a previously reported protocol [3]. An *in vivo* larval gut assay was established. To measure the effect of substances on gut contractions wandering 3rd instar larvae were removed from media, washed, dried, and placed on double-sided sticky tape attached to a glass microscope slide. A micromanipulator was used to deliver 40 nl of experimental solution or saline, during and after which contractions were continuously recorded for a 10-minute time period. A minimum of 7 animals were analyzed for each developmental stage and each injectant. Gut contractions of each animal were recorded prior to application of the peptide, analog, or saline to yield basal gut motility.

Heart beat was observed in each developmental stage using a previously described microscope-based assay [12]. The rate of heart contractions for each animal was recorded prior to application of the peptide, analog, or saline to yield basal heart rate. Animals (n = 7) were injected with 40 nl of solution using drawn out glass micropipettes; each animal was used only once. Injections were made anterior of the CNS and dorsal vessel to promote diffusion of injectant in the hemolymph and avoid damage to neural and cardiac tissues. During and after delivery of the injectant, contractions were continuously recorded for a 10-minute time period.

To test whether an inactive analog acted as an antagonist it was applied and the 30-second and 1-minute data points were recorded, at which time DMS was applied and the response was recorded for 10 minutes. Controls included saline applied in place of the inactive analog or DMS or both.

A cocktail of protease enzyme inhibitors was included at a final concentration of 1% to investigate whether its presence led to a difference in the activity of DMS or VFLRFamide on adult gut and pupal heart. Saline in the presence of 1% inhibitor cocktail was a control. The application and recording were done as described above for DMS, analog, or saline alone.

2.4 Data analysis

Data analyzed were the maximum responses of peptide, analogs, or saline within the 10-minute recording period reported as mean values $\pm$ S.E.M. Data in the dose-response curves and graphs are reported as % basal gut motility or heart rate where the frequency of contractions before application of peptide, analog, or saline is considered a measure of the baseline or basal value. Data in the tables are reported as % inhibition relative to the effect of DMS.

Dose-response curves were generated and effective concentration at half-maximal response (EC50) values were calculated using a non-linear regression (GraphPad Prism version 3.0). Activities of analogs and saline were compared to DMS using Single Factor ANOVA

(Microsoft Excel) with significance at p value = 0.01. In the presence of protease inhibitors, DMS, analogs, and saline were compared to injectant alone to calculate a p value.

2.5 Immunochemical localization

Indirect immunochemical localization was performed according to a protocol previously described [8, 11, 14]. Second antibodies included cyanine 3-labeled goat anti-rabbit antibody (Jackson ImmunoResearch Laboratory) and fluorescein isothiocyanate-labeled phalloidin (Invitrogen). Phalloidin, a toxin that binds actin, was used to stain tissue and enhance visualization of the preparation. Fluorescent signals were imaged using a scanning confocal microscope. The z-series data were processed with Adobe Photoshop CS2.

3. Results

DMS dose response on gut motility and heart rate

Myosuppressins were first discovered based on their myoinhibitory effect on gut contractions and, subsequently, also found to decrease heart rate. The influence of DMS on the spontaneous contractions of adult *D. melanogaster* gut and pupal heart was measured to test our hypothesis that the peptide decreased muscle contractility.

Dromyosuppressin was a potent inhibitory agent on adult gut contractions with an EC_{50} = 0.8 μ M (Fig. 1A). DMS also decreased pupal heart contractions but with a decreased potency; EC_{50} = 30 nM (Fig. 1B). In addition to the magnitude of effectiveness, the response to the peptide over time was different in gut compared to heart (Fig. 2). Maximum effects typically occurred within 1 minute of peptide application. Saline, the carrier, did not significantly affect adult gut motility or pupal heart contractions (Fig. 2; Tables 1 and 2).

Structure-activity relationship elucidated for DMS on gut and heart

An aim of this study was to delineate SAR to identify amino acids critical for activity and an active core, the shortest length of residues not significantly different from the effect of DMS. A SAR provides data to identify antagonists for mechanistic and functional studies. Two sets of analogs, alanyl-substituted and N-terminal truncations, were chosen to provide independent but complementary SAR data. The importance of the C-terminal amide was examined by testing the free acid form of the peptide.

The influence of DMS was measured in two or more stages of the life cycle and gut and heart to gain insight into mechanisms underlying critical physiological processes on developmental- and tissue-specific levels. The effects of the analogs and saline were reported relative to DMS. Analogs were tested at 1 μ M because DMS is potent at this concentration (Fig. 1), thus, subtle responses, important in characterizing antagonists, would be detected.

Alanyl-substituted DMS analogs analyzed on gut and heart

Alanyl-substituted analogs were used to delineate SAR by replacing each residue, one by one, with alanine to investigate the contribution of the amino acid side chain to DMS activity in gut and heart (Fig. 2; Tables 1 and 2). Controls included the analysis of the unsubstituted parent peptide and physiological saline, the carrier, without DMS or analog (Fig. 2, Tables 1 and 2).

In adult gut, the replacement of only one residue, leucine 8, (L8), produced an inactive analog, [A8] DMS. Substitution of all other residues resulted in analogs with reduced but prominent levels of activity at 70-100% inhibition (Fig. 2A; Table 1). The free acid analog

retained activity to 71% inhibition compared to DMS. Application of saline was ineffective at 23% inhibition compared to DMS.

In larval gut, the alanine scan demonstrated numerous residues were important to activity. Substitution of D2, V3, D4, H5, F7, L8, and F10 produced analogs with significantly different responses compared to DMS at 32%, 26%, 10%, 14%, 10%, 42%, and 26% inhibition, respectively. The replacement of T1 and V6 generated analogs which mimicked DMS at 95% and 91% inhibition, respectively; [A9] DMS was effective at 74% inhibition (Fig. 2B; Table 1). The free acid analog and saline were significantly different from DMS at 19% and 8% inhibition, respectively.

To explore the tissue specificity of DMS SAR alanyl-substituted analogs were next tested on heart (Fig. 2C; Table 2). In adult heart, no individual residue was critical for the effect of DMS (Table 2). [A7] DMS and [A10] DMS did, however, show reduced effectiveness at 46% and 41% inhibition, respectively. Substitution of T1 and D4 resulted in enhanced activity at 108% and 114% inhibition. The free acid analog had a reduced effect compared to DMS at 30% inhibition. Saline was relatively ineffective on adult heart rate at 24% inhibition compared to DMS.

In pupal heart, replacement of F7 and F10 produced [A7] DMS and [A10] DMS with significantly different responses from DMS at 45% and 35% inhibition, respectively. Substitution of T1, D2, V3, and V6 generated analogs with enhanced activity at 120%, 126%, 108%, and 115% inhibition, respectively (Fig. 2C; Table 2). The free acid analog was significantly different from DMS at 22% inhibition. The effect of saline was significantly different from DMS at 0% inhibition.

In larval heart, replacement of only one residue, D4, resulted in a significantly different response from DMS at 27% inhibition (Table 2). The effect of no other analog was significantly different from DMS. Substitution of all other residues resulted in analogs with reduced but observable levels of activity at 27-77% inhibition. The free acid analog and saline were less active than DMS at 27% and 23% inhibition, respectively.

N-terminal DMS truncation analogs evaluated on gut and heart

The second approach to establish DMS SAR was to examine the peptide length required for DMS activity in gut and heart. N-terminal truncated analogs were generated by systematically removing a residue, one at a time. Analysis of N-terminal truncated analogs was the strategy taken because of the high degree of structure conservation in the C terminus of myosuppressins.

In adult gut, removing the first two amino acids produced [2-10] DMS and [3-10] DMS which did not significantly alter activity compared to DMS (Table 3). Truncation of the next residue, V3, decreased activity to 87% inhibition. Further trimming of the N terminus produced [5-10] DMS and [6-10] DMS which were significantly different from DMS at 49% and 54% inhibition, respectively. In larval gut, the influence of all N-terminal truncated analogs tested was significantly different from DMS (Table 3).

In adult heart, the truncation of the first five residues produced analogs which were not significantly different from DMS (Table 4). However, the removal of an additional residue, V6, produced an analog which was significantly different from DMS at 14% inhibition.

In pupal heart, [2-10] DMS, [3-10] DMS, [4-10] DMS, and [6-10] DMS were more effective than DMS at 126%, 108%, 137%, and 103% inhibition, respectively (Table 4). [5-10] DMS decreased activity at 89% inhibition compared to DMS. The sixth residue, V6, trimmed

from the N terminus generated [7-10] DMS which was significantly different from DMS at 8% inhibition.

In larval heart, truncation of the first three residues produced [2-10] DMS, [3-10] DMS, and [4-10] DMS which were more effective than DMS at 127%, 223%, and 150% inhibition, respectively (Table 4). [5-10] DMS decreased activity at 36% inhibition compared to DMS. [6-10] DMS, and [7-10] DMS were each significantly different from DMS at 14% inhibition.

p-Benzoyl-phenylalanine-containing DMS analogs evaluated

To further investigate DMS SAR a modified amino acid replaced D2 and D4 in a tyrosyl-extended analog. In adult gut and pupal heart, Y-[Bpa2] DMS mimicked the un-substituted peptide (Fig. 3). In adult gut, the analog decreased motility with an $EC_{50} = 0.01$ pM (Fig. 3A). In pupal heart, the analog decreased rate with an $EC_{50} = 0.01$ nM (Fig. 3B). In adult heart, the analog was more effective than DMS with an $EC_{50} = 10$ uM (Fig. 3C).

In adult gut Y-[Bpa4] DMS decreased contractility to 53% inhibition, yet in heart the analog was inactive at 0% inhibition compared to DMS. In adult heart, applying Y-[Bpa4] DMS and peptide at equal concentration (10 nM) reduced DMS activity from 100% to 16% inhibition, which suggest the analog acted as an antagonist. In larval gut, Y-[Bpa4] DMS was active to 97% inhibition; however, Y-[Bpa2] DMS was inactive at 3% inhibition.

DMS analog delivered with protease inhibitors

A 1% protease inhibitor cocktail was delivered with an analog to investigate mechanisms. [6-10] DMS was used in this analysis because it decreased adult gut motility and pupal heart rate at 54% and 103% inhibition, respectively. In adult gut, DMS plus 1% protease inhibitor cocktail (DMS + PI) was significantly different from DMS alone, at 146% versus 100% inhibition, respectively (Table 5). Also, [6-10] DMS + PI was significantly different in adult gut from analog alone, 124% inhibition versus 78% inhibition (Table 5). Saline + PI was not significantly different from saline alone in adult gut (Table 5).

In pupal heart, DMS + PI was not significantly different from DMS at 86% versus 100% inhibition, respectively (Table 5). Also, [6-10] DMS + PI was not significantly different from [6-10] DMS at 100% versus 103% inhibition, respectively (Table 5). Saline + PI was not significantly different from saline alone (Table 5). Taken together, the inclusion of protease inhibitor cocktail may be interpreted to suggest that mechanisms associated with DMS are under developmental- or tissue-specific regulation.

Antisera detect DMS-specific immunoreactivity in gut and heart

Peptide-specific antisera [8, 11, 14] were used to identify cells and neuronal processes containing DMS immunoreactive material. Immunostaining provides complementary yet independent data on mechanisms involved in signaling. DMS was expressed in neurons from which immunoreactive fibers arborized impinging throughout the adult brain including the cerebrum, optic lobes, and subesophageal ganglion (Fig. 4A). Fibers were observed to project from DMS-stained cells to innervate the gut. The delivery of the peptide was consistent with the effect of DMS on gut motility, and indicative of the gut receiving neuronal input.

Additionally, neurons in the superior protocerebrum were stained by DMS-specific antisera (Fig. 4B). Immunoreactive fibers projected from protocerebral neurons in the pupal brain to innervate the anterior dorsal vessel, the aorta. Thus, immunostaining data were consistent

with cardiac function receiving input from unique cells in the brain providing further evidence that DMS activity is regulated on a tissue-specific level.

4. Discussion

Our results strongly support the conclusion DMS plays a role in muscle physiology, and its pleiotropic effects on contractility involve mechanisms regulated on a developmental- and tissue-specific level. A detailed DMS SAR provided functional agonists and antagonists to gain insight into mechanisms regulating gut and heart contractions. DMS-specific antisera identified distinct brain cells from which immunoreactive fibers delivered the peptide to the gut and heart consistent with tissue-specific neuronal control of muscle contractility.

DMS influenced gut motility and heart rate dose dependently over a range of concentrations with physiological relevance, and analog activity was specific to adult, pupa, or larva and gut or heart. Individual single alanyl-substituted analogs were inactive, yet others were more potent than DMS (Tables 1 and 2). N-terminal truncated analogs showed increased activity (Tables 3 and 4) suggesting shorter peptide lengths may also be targets to increase potency. Previous studies of the relationship of structure to myosuppressin activity did not provide a complete profile of residues or peptide lengths required for activity in more than one stage of development or multiple tissues in one animal species [1, 9, 20]. Thus, there was no opportunity to investigate and compare mechanisms at different stages of the life cycle or in multiple tissues. The generation of analogs which act as developmental- or tissue-specific antagonists or analogs with increased potency may benefit understanding peptidergic control of muscle contractility.

The incorporation of a modified amino acid, Bpa, a phenylalanine analog, produced developmental- and tissue-specific antagonists and agonists with increased potency which was consistent with DMS SAR data obtained from alanyl-substituted and N-terminal truncation analogs. Analysis of the Bpa-containing analogs generated by replacing D2 and D4 supported other DMS SAR data; they indicated mechanisms were different in gut and heart, and adult and larva. Additionally, the position of the residue in the peptide sequence was critical for whether it played a role in activity or binding or both.

These results set the basis for an approach to probe myosuppressin receptor proteins because Bpa is a photoactivatable cross-linker [6] and the tyrosyl extension provides a site for labeling. Labeled Y-SchistoFLRFamide, a locust myosuppressin analog, suggested two receptors are associated with oviduct [19] which is in agreement with genome database mining that identified two DMSRs [4]. The existence of two GPCR proteins to which DMS binds is consistent with the presence of multiple signaling pathways which may contribute to the observed developmental- and tissue-specific mechanisms.

Analogues containing modified amino acids may also provide protection from peptidases. Peptide-based drugs delivered intravenously or orally are susceptible to proteolysis. An initial foray into reducing proteolysis employing inhibitors was consistent with the presence of mechanisms specific to development and unique to different tissues. However, the use of protease inhibitors to limit proteolysis is unattractive due to lack of specificity and cost, thus, resulting in harmful side effects. Development of a peptide-based drug typically incorporates modified residues or non-peptidyl moieties to address degradation in the blood stream or gastrointestinal tract.

An animal receives numerous physiological and environmental inputs which impact the requirements for nutrient absorption and circulation during its life cycle. Probing tissue with peptide-specific antisera provided an independent but complementary approach to explore mechanisms involved in myosuppressin control of muscle contractility relevant to gut and

heart physiology. DMS-specific antisera stained brain cells from which immunoreactive processes projected to uniquely innervate the gastrointestinal tract or the cardiovascular system showing gut motility and heart rate received independent neural input. Previously, we probed the blowfly nervous system with myosuppressin antisera and found the peptide was delivered from the CNS to the heart [1]. Also, we found blowfly gut was innervated by myosuppressin immunoreactive processes which originated in the CNS [16]. Thus, our results are in agreement with and expand past studies to provide independent and complementary data to support the conclusion myosuppressin acts through developmental- and tissue-specific mechanisms under neural regulation to play a role in gut and cardiovascular physiology. Knowledge of the mechanisms underlying the pleotropic effects of myosuppressin on muscle contractility may pave the way to identify targets and lead to the development of strategies to address related diseases.

Acknowledgments

This research was supported by National Institutes of Health funding R21HL093627, Concept to Commercialization Funding from the University of Michigan Medical Innovation Center, and a University of Michigan Cardiovascular Center Innovative Grant to RN.

References

1. Angioy AM, Muroi P, Barbarossa IT, McCormick J, Nichols R. Evidence dromyosuppressin acts at posterior and anterior pacemakers to decrease the fast and the slow cardiac activity in the blowfly *Protophormia terraenovae*. *Peptides*. 2007; 28:585–93. [PubMed: 17141921]
2. Dockray GJ, Reeve JR Jr, Shively J, Gayton RJ, Barnard CS. A novel active pentapeptide from chicken brain identified by antibodies to FMRFamide. *Nature*. 1983; 305:328–30. [PubMed: 6137771]
3. Duttlinger A, Berry K, Nichols R. The different effects of three *Drosophila melanogaster* dFMRFamide-containing peptides on crop contractions suggest these structurally related peptides do not play redundant functions in gut. *Peptides*. 2002; 23:1953–7. [PubMed: 12431733]
4. Egerod K, Reynisson E, Hauser F, Cazzamali G, Williamson M, Grimmelikhuijzen CJ. Molecular cloning and functional expression of the first two specific insect myosuppressin receptors. *Proc Natl Acad Sci USA*. 2003; 100:9808–13. [PubMed: 12907701]
5. Holman GM, Cook BJ, Nachman RJ. Primary structure and synthesis of a blocked myotropic neuropeptide isolated from the cockroach, *Leucophaea maderae*. *Comp Biochem Physiol C*. 1986; 85:219–24. [PubMed: 2877794]
6. Kauer JC, Erickson-Viitanen S, Wolfe HR Jr, DeGrado WF. p-Benzoyl-L-phenylalanine, a new photoreactive amino acid. Photolabeling of calmodulin with a synthetic calmodulin-binding peptide. *J Biol Chem*. 1986; 261:10695–700. [PubMed: 3733726]
7. Marciniak P, Audsley N, Kuczer M, Rosinski G. Identification of myotropic neuropeptides from the brain and corpus cardiacum-corpora allata complex of the beetle, *Zophobas atratus*. *J Insect Sci*. 2010; 10:1–19. [PubMed: 20569122]
8. McCormick J, Nichols R. Spatial and temporal expression identify dromyosuppressin as a brain-gut peptide in *Drosophila melanogaster*. *J Comp Neurol*. 1993; 338:278–88. [PubMed: 8308172]
9. Nachman RJ, Holman GM, Hayes TK, Beier RC. Structure-activity relationships for inhibitory insect myosuppressins: contrast with the stimulatory sulfakinins. *Peptides*. 1993; 14:665–70. [PubMed: 8234009]
10. Nichols R. Isolation and structural characterization of *Drosophila* TDVDHVFLRFamide and FMRFamide-containing neural peptides. *J Mol Neurosci*. 1992; 3:213–8. [PubMed: 1390001]
11. Nichols R, McCormick J, Lim I. Multiple antigenic peptides designed to structurally-related *Drosophila* peptides. *Peptides*. 1997; 18:41–5. [PubMed: 9114450]
12. Nichols R, McCormick J, Cohen M, Howe E, Jean C, Paisley K, et al. Differential processing of neuropeptides influences *Drosophila* heart rate. *J Neurogenet*. 1999; 13:89–104. [PubMed: 10858818]

13. Nichols R, Demers LA, Larsen BM, Robinson D, Converso K, Russell MW, et al. Human RFamide-related peptide-1 diminishes cellular and integrated cardiac contractile performance. *Peptides*. 2010; 31:2067–74. [PubMed: 20797420]
14. Posnett, DN.; Tam, JP. Multiple antigenic peptide method for producing antipeptide site-specific antibodies. In: Langone, JJ., editor. *Methods in Enzymology*. San Diego: Academic Press; 1989. p. 739-746.
15. Price DA, Greenberg MJ. Structure of a molluscan neuropeptide. *Science*. 1977; 197:670–1. [PubMed: 877582]
16. Richer S, Stoffolano JG Jr, Yin CM, Nichols R. Innervation of dromyosuppressin (DMS) immunoreactive processes and effect of DMS and benzethonium chloride on the *Phormia regina* (Meigen) crop. *J Comp Neurol*. 2000; 421:136–42. [PubMed: 10813776]
17. Robb S, Packman LC, Evans PD. Isolation, primary structure and bioactivity of SchistoFLRF-amide, a FMRF-amide-like neuropeptide from the locust, *Schistocerca gregaria*. *Biochem Biophys Res Comm*. 1989; 160:850–6. [PubMed: 2719702]
18. Stevens JS, Cashman CR, Smith CM, Beale KM, Towle DW, Christie AE, et al. The peptide hormone pQDLDHVFLRFamide (crustacean myosuppressin) modulates the *Homarus americanus* cardiac neuromuscular system at multiple sites. *J Exp Biol*. 2009; 212:3961–76. [PubMed: 19946074]
19. Wang Z, Orchard I, Lange AB. Identification and characterization of two receptors for SchistoFLRFamide on locust oviduct. *Peptides*. 1994; 15:875–82. [PubMed: 7984508]
20. Wang Z, Orchard I, Lange AB. Binding affinity and physiological activity of some HVFLRFamide analogues on the oviducts of the locust, *Locusta migratoria*. *Regul Pept*. 1995; 57:339–46. [PubMed: 7480883]

Highlights

DMS decreased gut motility and cardiac contractility dose dependently.

Truncated and Y-[Bpa₂] DMS analogs were increased agonists in pupal and adult heart.

Adult, pupal, and larval heart and gut active cores were developmental- and tissue specific.

Y-[Bpa_x] DMS activity and binding differed on a developmental- and tissue-specific level.

DMS-specific antisera stained unique cells and fibers that innervated gut or heart.

Fig. 1. Dose-response curves of DMS on adult gut and pupal heart. The y-axis is DMS activity as percent basal (A) gut motility or (B) heart rate. The x-axis is log DMS concentration (molarity, M). Mean values $\pm$ S.E.M. of the maximal response are reported.

Fig. 2.

The effects of alanyl-substituted analogs are reported as mean values $\pm$ S.E.M. (A) Adult gut, — DMS, —□— [A1]DMS, —△— [A2]DMS, —○— [A3]DMS, —▲— [A6]DMS, —×— [A9]DMS, —◇— [A4]DMS, —■— [A10]DMS, —■— [A5]DMS, —●— [A7]DMS, —▲— DMS-OH, —◆— [A8]DMS, and —●— saline, (B) larval gut, DMS, —□— [A1]DMS, —▲— [A6]DMS, —×— [A9]DMS, —◆— [A8]DMS, —○— [A3]DMS, —■— [A10]DMS, —▲— DMS-OH, —■— [A5]DMS, —△— [A2]DMS, —◇— [A4]DMS, —●— [A7]DMS, and —●— saline and (C) pupal heart, —△— [A2]DMS, —□— [A1]DMS, —▲— [A6]DMS, — DMS, —×— [A9]DMS, —◆— [A8]DMS, —■— [A5]DMS, —●— [A7]DMS, —○— [A3]DMS, —■— [A10]DMS, —▲— DMS-OH, —◇— [A4]DMS, and —●— saline.

Fig. 3. Dose-response curves of Y-[Bpa2]-DMS on (A) adult gut, (B) pupal heart, and (C) adult heart are reported as mean values $\pm$ S.E.M. of the maximal response. The y-axis is Y-[Bpa2]-DMS activity as percent basal (A) gut motility or (B and C) heart rate. The x-axis is log [Y-Bpa2]-DMS concentration (M). The effects of DMS on (C) adult heart are included (gray line).

Fig. 4. Staining of (A) adult CNS and gut and (B) pupal CNS and anterior dorsal vessel (aorta) identify immunoreactive material as fluorescent signal (white). DMS-specific antisera stained neurons in the (A) subesophageal ganglion and (B) protocerebrum which sent immunoreactive fibers to innervate the gut and aorta, respectively. The bars in the lower right-hand corner represent 50 μ M.

The effects of alanine-substituted analogs on adult and larval gut are reported as mean values calculated as percent inhibition relative to the effect of DMS. The symbol † indicates p 0.00001.

Table 1

			% Inhibition (gut)			
			Adult	p value	Larval	p value
DMS		TDVDHVFLRFamide	100		100	
[A1]DMS		ADVVDHVFLRFamide	100	1	95	0.6
[A2]DMS		TAVVDHVFLRFamide	100	1	32	0.002
[A3]DMS		TDADHVFLRFamide	100	1	26	0.00008
[A4]DMS		TDVAHVFLRFamide	91	1	10	0.00002
[A5]DMS		TDVDADVFLRFamide	78	0.01	14	†
[A6]DMS		TDVDHAFALRFamide	100	1	91	0.5
[A7]DMS		TDVDHVAFALRFamide	70	0.008	10	†
[A8]DMS		TDVDHVFAFARFamide	14	†	42	0.0005
[A9]DMS		TDVDHVFLAFamide	100	1	74	0.1
[A10]DMS		TDVDHVFLRAamide	90	0.08	26	0.0002
DMS-OH		TDVDHVFLRF-OH	71	0.002	19	†
Saline			23	†	8	†

Table 2

The effects of alanine-substituted analogs on adult, pupal, and larval heart are reported as mean values calculated as percent inhibition relative to the effect of DMS. The symbol † indicates p < 0.00001.

		% Inhibition (heart)					
		Adult	p value	Pupal	p value	Larval	p value
DMS		100		100		100	
[A1]DMS		108	0.8	120	0.2	64	0.3
[A2]DMS		95	0.9	126	0.1	50	0.1
[A3]DMS		73	0.3	108	0.6	73	0.4
[A4]DMS		114	0.7	77	0.2	27	0.01
[A5]DMS		76	0.5	78	0.3	45	0.07
[A6]DMS		100	1	115	0.3	55	0.08
[A7]DMS		46	0.2	45	0.005	27	0.02
[A8]DMS		86	0.7	83	0.3	55	0.2
[A9]DMS		103	0.9	98	0.9	55	0.3
[A10]DMS		41	0.06	35	0.0002	77	0.6
DMS-OH		30	0.05	22	†	27	0.02
Saline		24	0.04	0	†	23	0.04

Table 3

The effects of N-terminal truncated analogs on adult and larval gut are reported as mean values calculated as percent inhibition relative to the effect of DMS. The symbol † indicates p < 0.0001.

			% Inhibition (gut)			
			Adult	p value	Larval	p value
DMS		TDVDHVFLRFamide	100		100	
[2-10]DMS		DVDHVFLRFamide	100	1	17	†
[3-10]DMS		VDHVFLRFamide	100	1	29	0.0004
[4-10]DMS		DHVFLRFamide	87	0.09	8	†
[5-10]DMS		HVFLRFamide	49	0.0001	0	†
[6-10]DMS		VFLRFamide	54	†	14	†
Saline			23	†	8	†

Table 4

The effects of N-terminal truncated analogs on adult, pupal, and larval heart are reported as mean values calculated as percent inhibition relative to the effect of DMS. The symbol † indicates p < 0.00001.

		% Inhibition (heart)			
		Adult	p value	Pupal	p value
DMS		100		100	
[2-10]DMS		86	0.7	126	0.1
[3-10]DMS		68	0.4	108	0.5
[4-10]DMS		81	0.5	137	0.08
[5-10]DMS		86	0.7	89	0.5
[6-10]DMS		65	0.4	103	0.8
[7-10]DMS		14	0.04	8	†
Saline		24	0.04	0	†

Table 5

The effects of DMS and an analog in the presence of protease inhibitor (PI) on adult gut (0.1 nM) and pupal heart (1 uM) are reported as mean values calculated as percent inhibition relative to the effect of DMS. The p values compare DMS, [6-10] DMS, or saline with versus without PI. The symbol † indicates $p < 0.00001$.

	% Inhibition			
	Adult gut	p value	Pupal heart	p value
DMS	100		100	
DMS + PI	146	0.003	86	0.4
[6-10]DMS	78		103	
[6-10]DMS + PI	124	0.004	100	0.8
Saline	20		0	
Saline + PI	39	0.8	22	0.05