Distinct or shared actions of peptide family isoforms: II. Multiple pyrokinins exert similar effects in the lobster stomatogastric nervous system

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ABSTRACT

Many neuropeptides are members of peptide families, with multiple structurally similar peptide isoforms frequently found even within a single species. This raises the question of whether the individual peptides serve common or distinct functions. In the companion paper to this article (Dickinson, P.S., Sreekrishnan, A., Kwiatkowski, M.A. and Christie, A.E. 2015. Distinct or shared actions of peptide family isoforms: I. Peptide-specific actions of pyrokinins in the lobster cardiac neuromuscular system. J. Exp. Biol, submitted), we found high isoform specificity in the responses of the lobster (Homarus americanus) cardiac neuromuscular system to members of the pyrokinin peptide family: only one of five crustacean isoforms showed any bioactivity in the cardiac system. Because previous studies in other species had found little isoform specificity in pyrokinin actions, we examined the effects of the same five crustacean pyrokinins on the lobster stomatogastric nervous system (STNS). In contrast to our findings in the cardiac system, the effects of the five pyrokinin isoforms on the STNS were indistinguishable: they all activated or enhanced the gastric mill motor pattern, but did not alter the pyloric pattern. These results, in combination with those from the cardiac ganglion, suggest that members of a peptide family in the same species can be both isoform-specific and highly promiscuous in their modulatory capacity. The mechanisms that underlie these differences in specificity have not yet been elucidated; one possible explanation, which has yet to be tested, is the presence and differential distribution of multiple receptors for members of this peptide family.

SUMMARY STATEMENT

Crustaceans typically possess multiple pyrokinins; in the lobster, all isoforms tested similarly activated the gastric mill rhythm, suggesting that the pyrokinin receptor(s) in the stomatogastric nervous system is relatively promiscuous.
INTRODUCTION

It is now well established that central pattern generators, although they are anatomically “hard-wired”, are functionally flexible; they are capable of generating multiple motor outputs under the influence of a wide variety of neuromodulators (e.g., Blitz and Nusbaum, 2011; Blitz and Nusbaum, 2012; Dembrow and Johnston, 2014; Dickinson, 2006; Fenelon et al., 2003; Hooper and DiCaprio, 2004; LeBeau et al., 2005; LeBeau and Whittington, 2005; Marder and Bucher, 2001; Marder et al., 2014; Miles and Sillar, 2011; Mitchell and Johnson, 2003; Nusbaum and Beenakker, 2002; Nusbaum et al., 2001; Rauscent et al., 2006; Selverston and Ayers, 2006; Sillar et al., 2008; Stein, 2009). The compounds that serve as neuromodulators are highly diverse, and include small molecule transmitters (e.g., acetylcholine, glutamate and GABA), amines (e.g., dopamine and serotonin) and diffusible gases (e.g., nitric oxide), as well as a variety of different peptides, which represent the largest single class of neuroactive molecules. The number of different peptides that has been identified within the nervous system of a single species is remarkably high. Recent studies of the neuropeptidomes of several decapod crustaceans, for example, have shown that these species often contain upwards of 100 or more distinct peptides (e.g., Fu et al., 2005; Hui et al., 2013; Hui et al., 2012; Ma et al., 2009; Ma et al., 2010; Ma et al., 2008; Saideman et al., 2007; Torfs et al., 2001; Christie and Chi, 2015). The pyrokinins are members of the PBAN/diapause hormone/pyrokinin/periviscerokinin superfamily of peptides, which typically possess the carboxy (C)-terminal sequence –FXPRLamide, or a close approximation thereof (e.g., Predel and Wegener, 2006; Rafaeli, 2009). Members of this peptide superfamily are well studied in insects and have been implicated in the control of a large number of biological processes, including, but not limited to, pheromone biosynthesis, ecdysone biosynthesis, visceral muscle contraction, cuticle melanization, acceleration of pupation and induction of diapause (e.g., Predel and Wegener, 2006; Rafaeli, 2009). In insects, members of the PBAN/diapause hormone/pyrokinin/periviscerokinin superfamily are encoded by two genes: capability and
hugin (e.g., Baggerman et al., 2002; Hewes and Taghert, 2001; Kean et al., 2002; Meng et al., 2002; Vanden Broeck, 2001). Twenty different pyrokinins have been identified in six different decapod crustaceans (Table 1 (Christie, 2014a; Hui et al., 2012; Ma et al., 2009; Ma et al., 2010; Ma et al., 2008; Saideman et al., 2007; Torfs et al., 2001; Christie and Chi, 2015)), with each species having from one (the lobster Homarus americanus (Ma et al., 2008) and the crab Callinectes sapidus (Hui et al., 2012)) to eleven (the penaeid shrimp Litopenaeus vannamei (Christie, 2014a; Ma et al., 2010; Torfs et al., 2001)) different isoforms. However, only two studies to date (Saideman et al., 2007; Dickinson et al., 2015 (a companion paper to this report)) have examined the biological activity of these peptides in the crustaceans; both studies found that the pyrokinins are active neuromodulators.

The stomatogastric nervous system (STNS) contains the four ganglia that generate the rhythmic motor patterns that control the movements of the foregut (i.e., the oesophageal, cardiac sac, gastric mill and pyloric rhythms). A previous study conducted on the STNS of the crab (Cancer borealis) examined the modulatory effects of three different pyrokinins (Saideman et al., 2007), two native to C. borealis (Saideman et al., 2007) and one derived from the cockroach Rhyparobia (formerly Leucophaea) maderae (Holman et al., 1986). All three pyrokinins exerted virtually identical effects on the crab stomatogastric system, suggesting that the structural differences between these peptides are functionally unimportant, and that the pyrokinin receptor does not distinguish between these different isoforms (Saideman et al., 2007). In contrast, of four crustacean pyrokinins tested in the lobster (H. americanus) cardiac ganglion (CG), which controls the rhythmic beating of the neurogenic heart in crustaceans, only the shrimp L. vannamei isoform (ADFAFNPRLamide; PevPK2), exerted any significant modulatory effects (Dickinson et al., 2015 (the companion article to the study presented here)). This suggests that the pyrokinin receptor in the lobster CG is highly selective, and that small changes in the pyrokinin sequence have considerable functional consequences.

Despite the contrasting selectivity of the pyrokinin response in these two species and central pattern generators, immunohistochemical studies suggest that pyrokinin is in fact present within both neural networks. Pyrokinin-like immunoreactivity was found in the neuropil of the stomatogastric ganglion (STG) in the crab (Saideman et al., 2007); it was also found in neuroendocrine organs and throughout the H. americanus CG, including in terminals surrounding both the small pacemaker and large motor neurons of this ganglion (Dickinson et al., 2015). These contrasting results led us to ask (1) whether pyrokinin is distributed similarly in the crab and the lobster STNS, and (2) whether the neural circuits in the STG of
the lobster would respond broadly to pyrokinins, as they do in the *C. borealis* STG, or selectively, as they do in the *H. americanus* CG.

**RESULTS**

**Distribution of pyrokinin-like immunoreactivity in the Homarus STNS**

To determine the distribution of pyrokinin-like peptides in the STNS of *H. americanus* (N=15 animals), wholemount immunohistochemistry was conducted using an antibody generated against the sequence –FSPRLamide, the only pyrokinin thus far isolated from *H. americanus* (Ma et al., 2008). As in all decapod species, the STNS of *H. americanus* consists of the unpaired STG, the unpaired oesophageal ganglion (OG) and the paired commissural ganglia (CoGs), as well as a number of interconnecting and motor nerves (Fig. 1). Within all STGs examined (n=15 ganglia), pyrokinin-like labeling was restricted to the neuropile, with none of the approximately 30 intrinsic somata exhibiting pyrokinin immunoreactivity (Fig. 2A). The immunopositive STG neuropile originates from axons projecting into the ganglion from the stomatogastric nerve (*stn*) via the paired superior oesophageal nerves (*sons*). These axons are likely derived from somata present in the CoGs (Fig. 2B); in each CoG (n=29 ganglia) approximately 14 somata exhibit pyrokinin-like labeling (range 6-30 somata per ganglion), as does an extensive neuropile, which appears to be composed of processes originating from both the intrinsic immunopositive cell bodies, as well as from immunoreactive axons projecting into the ganglion from the circumoesophageal connective (*coc*), a nerve that links the STNS to the brain and fused thoracic and abdominal ganglia via each CoG. Due to the weak intensity and punctate nature of labeling of the axons in the *stn* and *sons*, as well as their tendency toward fasciculation, it was not possible to unambiguously count the number of axons that give rise to the pyrokinin-like immunoreactivity in the STG neuropile, though we estimate that there are approximately eight axons contributing to it.

At least a subset (and perhaps all) of the pyrokinin-immunopositive axons that produce the labeling in the STG exit the ganglion via the dorsal ventricular nerve (*dvn*), where they project approximately halfway to the branch point of the lateral ventricular nerves (*lvns*) and terminate in putative neuropile-like profiles. The axons that give rise to the pyrokinin-immunopositive STG neuropile are also likely to be the source of an extensive endocrine-like plexus that covers much of the anterior portion of the STNS (Fig. 3). This plexus is superficially located, with immunoreactivity within or just below the sheath. The immunoreactivity in the structure is distinctly bark-like in appearance, and extends from the
branch point of the dorsal posterior oesophageal nerve (dpon) on each son to the junction of the sons and stn, down the stn approximately half way to the STG, and up the oesophageal nerve (on) and inferior oesophageal nerves (ions) to the branch point of the labral nerve (ln) on each ion.

All of the pyrokinin-immunopositive structures reported here were seen in all preparations, though the intensity of labeling in them was quite variable between animals.

### Specificity of pyrokinin-like labeling

To strengthen our confidence that the pyrokinin-like immunoreactivity reported here is due to the presence of native pyrokinins, two sets of specificity controls were conducted. To ensure that the lobster ganglia did not react with non-specific components of the rabbit serum, CoGs incubated in pre-immune serum were compared to those incubated in serum R3-30 (N=3 CoG pairs). For all CoG pairs, only the ganglion incubated in immune serum showed immunopositive structures (data not shown).

To ensure that the antibodies we used interacted specifically with pyrokinins, and not with other lobster neuropeptides, we conducted a series of antibody adsorptions, in which serum R3-30 was incubated with a number of peptides, including both pyrokinins and other crustacean peptides, particularly those that have significant sequence similarity to the pyrokinins. For these experiments, serum R3-30 was incubated with a $10^{-3}$ M concentration of FSPRLamide (the *H. americanus* peptide against which the antibody was directed; Ma et al., 2008), SGGFAFSPRLamide (*Cancer borealis* pyrokinin-2 (CabPK2); Saideman et al., 2007), DFAFSPRLamide (*Litopenaeus (Penaeus) vannamei* pyrokinin-1 (PevPK1); Torfs et al., 2001), DPSPEPFNPYNRFKIPRIamide (an ecdysis-triggering hormone (ETH) predicted from the cladoceran crustacean *Daphnia pulex* (Dappu-ETH); Gard et al., 2009), DTSTPALRLRFamide (a short neuropeptide F isoform from *H. americanus* (Homam-sNPF); Ma et al., 2008) or SGRNFLRFamide (a native *H. americanus* FLRFamide; Ma et al., 2008) for 3 hr at room temperature. Antiserum adsorption by FSPRLamide, CabPK2 or PevPK1 completely abolished all staining in the CoG (N=3 CoG pairs per peptide) whereas adsorption of R3-30 by Dappu-ETH, Homam-sNPF or SGRNFLRFamide (N=3 CoG pairs per peptide) had no effect on immunolabeling (data not shown).

### Qualitatively, pyrokinins resulted in enhanced gastric mill activity

The only pyrokinin peptide that has been identified in *H. americanus* is FSPRLamide, the conserved sequence found in all pyrokinins (Ma et al., 2008), whereas all other
crustaceans in which they have been identified generally include longer pyrokinins, as well as more pyrokinins. Moreover, only the shrimp pyrokinin ADFAFNPRLa (Litopenaeus (Penaeus) vannamei) pyrokinin-2 (PevPK2; Torfs et al., 2001) had any effect on the H. americanus heart (Dickinson et al., 2015), whereas the crab stomatogastric system responds equally to multiple pyrokinins (Saideman et al., 2007). Thus, we examined the effects of not only FSPRLamide, but also of 4 other identified crustacean pyrokinins (Table 1), two identified from the shrimp L. vannamei (PevPK1 and PevPK2) and two from the crab C. borealis (CabPK2 and TNFAFSPRLamide (Cancer borealis pyrokinin-1 or CabPK1); Saideman et al., 2007) on the activity of the stomatogastric networks in the lobster.

In preparations in which the gastric mill pattern was weakly or moderately active, superfusion of FSPRLamide and the other crustacean pyrokinins at 10^{-6} M (PevPK1 shown) enhanced gastric activity in gastric mill neurons. In such preparations (e.g., Fig. 4), most of the gastric motor neurons fired in patterns that included both gastric and pyloric components in control saline. Upon superfusion with the pyrokinins, activity became predominantly gastric in these neurons. For example, in the recording shown in Figure 4A, the lateral posterior gastric (LPG) and dorsal gastric (DG) neurons fired primarily in shorter, pyloric-timed bursts, with gastric modulation, in saline; in response to PevPK1 superfusion, lateral gastric (LG) neuron bursts increased in duration, and spike frequency within bursts increased as well. Similarly, bursts in the DG neuron, which were not well-defined and included weak pyloric characteristics in saline, were stronger, with a higher spike frequency and an increase in the gastric mill timing, in the presence of pyrokinin. The LPG neuron similarly began to fire strongly and primarily in gastric mill time. Additionally, the inferior cardiac (IC) neuron, which fired with weak pyloric rhythmicity in saline (Fig. 4B), often increased its firing frequency and began to fire more focused bursts in gastric time in the presence of pyrokinin (Fig. 4B).

All crustacean pyrokinins excite gastric mill neurons to a similar extent

Superfusion with any of the pyrokinins tested exerted excitatory effects on a number of gastric mill and gastropyloric neurons (Fig. 5). At least superficially, the effects exerted by the five pyrokinins were very similar: bursting in all of the gastric mill neurons we recorded from became more intense and the pattern appeared to be more robust. Moreover, all five pyrokinins appeared to have similar state-dependent effects on gastric mill cycle frequency, with increases in frequency when the pattern was slow in control saline, but decreases when the initial frequency was higher and the pattern was robust (Fig. 6).
residuals from the non-linear fit of percent change in frequency as a function of baseline cycle frequency did not differ significantly among the five pyrokinins, nor did they vary systematically as a function of baseline conditions, indicating that all five pyrokinins had similar effects on the overall burst frequency of the gastric pattern.

Although the details of the patterns evoked by each peptide often differed slightly (Fig. 5), this was likely due to the fact that the control pattern changed slightly over time, and at least some of the effects of the peptides are state-dependent, so that they differ depending on the initial pattern. Thus, to determine whether there were differences in the response of the STNS to the different pyrokinins that were not evident visually, we quantified the effects of the five pyrokinins on two gastric mill motor neurons, one lateral tooth motor neuron, the LG neuron, and one medial tooth motor neuron, the DG neuron.

Regardless of the initial conditions, both burst duration and spike frequency in the LG neuron most often increased during pyrokinin application (Fig. 7A,B; pooled data, single sample t-test, p<0.0001, n=77). However, like cycle frequency, the percent change in these values tended to be inversely correlated with the starting value (Fig. 7C,D), although these correlations were not strong, with $R^2$ values of only .26 (spike frequency) to .46 (burst duration). Moreover, in some preparations, particularly those with very long initial durations, burst duration decreased in the presence of the pyrokinins. Nonetheless, mean residuals from the fit curve did not differ significantly between the 5 peptides (ANOVA, p= 0.31 for duration, p=0.84 for spike frequency), suggesting that they enhanced LG activity similarly.

Interestingly, in the DG neuron, while the pyrokinins clearly enhanced gastric activity in individual preparations, their effects on burst duration were not consistent, nor were they strongly dependent on the starting values (Fig. 8A,B). In fact, when all data were averaged together, there was not a significant change in DG burst duration in any of the peptides (pooled data from all peptides, single sample t-test, p=0.98, n=56). Nonetheless, in individual preparations, changes in DG burst duration were often evident, with increases of over 90% in some preparations and decreases of as much as 65% in others. This variability was seen in all of the peptides, with no differences among peptides in their effects on this parameter (i.e., residuals did not differ significantly from one another). In contrast, all of the pyrokinins consistently increased the spike frequency within bursts (Fig. 8C,D; pooled % change from all peptides, single sample t-test, p<0.0001, n=53), regardless of the starting value. These differences were seen in all of the peptides, and there were again no differences among peptides in their effects on this parameter. This was borne out by comparisons of the
residuals to a nonlinear fit of the change in duration as a function of starting duration; residuals did not differ significantly among peptides.

**Pyrokinins do not alter pyloric activity**

In contrast to the activation of the gastric pattern by all of the crustacean pyrokinins, the pyloric motor pattern did not appear to be modulated by any of the pyrokinins (Fig. 9); we recorded no apparent changes in cycle frequency or in the activity of the pyloric dilator (PD) neurons, the ventricular dilator (VD) neuron, or the lateral pyloric (LP) neuron (Fig. 9). To ensure that the apparent lack of effect of the pyrokinins was not due to variability in the response to the peptides, as was the case for DG burst duration and has been seen in other systems (e.g., Wiwatpanit et al., 2012), we plotted the effects of the peptides on pyloric cycle frequency (Fig. 10A) as well as on burst duration and spike frequency in the PD neurons (Fig. 10B,C) and in the LP neuron (Fig. 10D,E) for individual preparations. Averages did not change significantly for any parameter, and changes in individual preparations were generally quite small, suggesting that the pyrokinins have little or no modulatory effect on the pyloric motor pattern.

**DISCUSSION**

The distribution of pyrokinin-like labeling in the crab and lobster STNS are remarkably similar

The distribution of pyrokinin-like immunoreactivity has now been mapped in the STNS of two decapods: the crab *C. borealis* (Saideman et al., 2007) and the lobster *H. americanus* (this study). Despite *C. borealis* and *H. americanus* being from different infraorders, Brachyura and Astacidea, respectively, the distribution of pyrokinins in their STNSs is remarkably similar. Specifically, in both species, labeling in the STG is restricted to the neuropile and is derived from small number of axons (or fascicles of axons) projecting to the ganglion via the *stn* and *sons*. Within the CoGs, similar numbers of pyrokinin-immunopositive somata and an extensive region of neuropile were seen in both the crab and lobster; a subset of these labeled somata are proposed as the source of the STG neuropile in both species. Similarly, in neither species were any somata labeled in the OG, nor were any immunopositive axons seen exiting the CoGs via the *ions*. In fact, the only major differences seen between crab and lobster staining patterns were the presence of an endocrine-like plexus...
covering the nerves of the anterior portion of the STNS in *H. americanus*, a structure not labeled in *C. borealis*, and a small region of neuropil in the *dlvn*, again seen in the lobster but not in the crab. Thus, the high degree of conservation seen in the STNS distribution of pyrokinins in these two decapods is suggestive of conserved physiological roles being played by this peptide family in rather distantly related species, particularly in their local modulation of the neural circuits present in the STG.

**Pyrokinins are likely to function as both local and hormonal modulators of the stomatogastric neural circuits**

In the lobster, pyrokinin-like immunoreactivity was found in the STNS itself, as well as in several neuroendocrine organs, notably the pericardial organs and the sinus gland (Dickinson et al., 2015), suggesting the possibility that members of this peptide family might act as modulators that are delivered to the STNS both locally and hormonally. Here, we examined and compared the effects of the pyrokinins at concentrations of $10^{-6}$ M, which is generally considered to be a concentration that would mimic local rather than hormonal release (e.g., Christie et al., 1995). However, an earlier study in the crab (Saideman et al., 2007) found that the gastric mill pattern generator responded to concentrations as low as $10^{-8}$ M, which is in the range of peptide concentrations that can be considered as hormonal (e.g., Christie et al., 1995). For example, while measurements of some peptides (e.g., those in the FLRFamide family) in hemolymph are on the order of $10^{-9}$ M in both insects and snails (Price et al., 1985; Robb and Evans, 1990), others, such as ETH and vitellogenin inhibiting hormone, have been measured at concentrations as high as 3-4 x$10^{-8}$ M, suggesting that the pyrokinins, consistent with their distribution, might well serve as both local and hormonal modulators.

**Pyrokinins activate the gastric mill but not the pyloric motor pattern**

While the effects of pyrokinins in the insects have been examined extensively, and they have been shown to have pleiotropic effects, most of these studies have focused on their ability to activate pheromone synthesis (e.g., Predel and Wegener, 2006; Rafaeli, 2009). Nonetheless, pyrokinins have been found within the central nervous systems of many species (e.g., Predel and Wegener, 2006; Rafaeli, 2009), suggesting the possibility that they have direct effects on behavior as well as indirect effects via the activation of pheromone synthesis. In the only study of the functional effects of pyrokinins on the crustacean STNS, Saideman et al. (2007) found that pyrokinins excite gastric mill motor neurons and the gastric
mill pattern as a whole, including gastro-pyloric neurons, but they do not alter the pyloric motor pattern in the crab *C. borealis*. Our results suggest that these peptides have effects in the lobster *H. americanus* that are strikingly parallel to those seen in the crab. As was the case in the studies of crab (Saideman et al., 2007), pyrokinins strengthened the gastric mill pattern, and enhanced the gastric mill-timed firing of gastro-pyloric neurons (e.g., the LPG neuron), but had no effect on purely pyloric motor neurons such as the PD and LP neurons. Nonetheless, there were minor differences in the responses of the crab and lobster STNS to pyrokinins. Specifically, in the rare cases in which the gastric mill pattern was not active in control saline in the lobster, pyrokinin application was not able to activate the pattern, while it did so in the crab (data not shown). Additionally, while the DG neuron in the crab most often fired in strong bursts that were independent of the remaining gastric mill neurons, the enhanced firing induced by pyrokinins in the DG neuron in the lobster was always coordinated with the other gastric mill neurons. Despite these differences, the overall pattern of pyrokinin effects was remarkably similar between the crab (Saideman et al., 2007) and the lobster.

**Multiple crustacean pyrokinins have similar effects on the gastric mill pattern**

As was the case with the three pyrokinins, *i.e.*, two native isoforms and the cockroach isoform, *Leucophaea maderae* pyrokinin, that were tested in the crab stomatogastric system (Saideman et al., 2007), the effects of the five pyrokinins examined in this study did not differ significantly from one another. The only pyrokinin that has been identified from *Homarus* is an amidated pentapeptide, FSPRLamide (Ma et al., 2008), corresponding to the conserved sequence that characterizes the pyrokinins family. This contrasts sharply with the case in most other crustaceans, as well as most insects. To date, pyrokinins have been identified in six decapod crustacean species, including a shrimp (*L. vannamei*), three species of crab (*C. borealis* and *Carcinus maenas, C. sapidus*), the crayfish *Procambarus clarkii*, and the lobster *H. americanus*, (Christie, 2014a; Hui et al., 2012; Ma et al., 2009; Ma et al., 2010; Ma et al., 2008; Saideman et al., 2007; Torfs et al., 2001; Christie and Chi, 2015); in all of these except *Homarus*, pyrokinins with extended C-termini have been identified. In contrast to the case in insects, where many functions have been ascribed to pyrokinins (e.g., Predel and Wegener, 2006; Rafaeli, 2009), the function of these peptides in the crustaceans has been examined minimally. In one such study, the two shrimp pyrokinins (PevPK1 and PevPK2) were shown to increase the amplitude and frequency of hindgut contractions (Torfs et al., 2001); interestingly, these studies were done not on the shrimp from which the peptides were
isolated, but instead on a crayfish (*Astacus leptodactylus*) and on the cockroach *R. maderae*, from which the first pyrokinins had been identified and shown to be myotropic (Holman et al., 1986). The effects of the two pyrokinins in this study were very similar to one another, although the threshold for effects on the crayfish was lower than that on the cockroach (Torfs et al., 2001). The other previous study of pyrokinin function in the crustaceans showed that the two native crab pyrokinins and the cockroach pyrokinin had virtually indistinguishable effects on the crab (*C. borealis*) stomatogastric nervous system (Saideman et al., 2007). In the present study, five different crustacean pyrokinins had virtually identical effects, suggesting that the pyrokinin receptor is relatively promiscuous, and does not distinguish between the different pyrokinins. An earlier study in insects reported similarly that changes to the N-terminus of the pyrokinin peptide sequence, where the largest differences between the crustacean pyrokinins are found, had only minor effects on activity. However, while the pyrokinin receptor required only the conserved sequence, –FXPRLamide, for binding and activation, the response to the conserved sequence was only 30% of that recorded with the longer peptide sequences (Nachman et al., 1986), whereas in the lobster STNS, there was no apparent difference between the response to FSRPRLamide and the other crustacean pyrokinins. It should be noted, however, that the present study used suprathreshold concentrations of all of the pyrokinins, whereas Nachman et al. focused on identifying the threshold concentration. It is possible that quantitative differences in response might be seen if lower concentrations, mimicking hormonal rather than local release, were used in the lobster STNS.

**The specificity of pyrokinin effects in the lobster STNS differs from that in the lobster cardiac ganglion**

In contrast to the similarity of the responses of the STNS to the different crustacean pyrokinins, the lobster CG responds to only one of these peptides (Dickinson et al., 2015). Moreover, the peptide that is active on the cardiac system is not the short sequence that has been identified in the lobster nervous system using mass spectrometry (Ma et al., 2008). This suggests the possibility that there are multiple pyrokinins in *H. americanus*, including in the STNS. In most crustaceans thus far examined, this is the case (Christie, 2014a; Christie, 2014b; Christie et al., 2013; Hui et al., 2012; Ma et al., 2009; Ma et al., 2010; Ma et al., 2008; Saideman et al., 2007; Torfs et al., 2001; Christie and Chi, 2015), with at least one species (the shrimp *L. vannamei*) possessing 11 distinct PBAN/diapause hormone/pyrokinin/periviscerokinin superfamily members (Christie, 2014a; Ma et al., 2010;
Torfs et al., 2001). The differences in responses of the cardiac and stomatogastric ganglia to different pyrokinins would also be explained if there were at least two pyrokinin receptors, one of which was highly specific, while the other(s) was much more promiscuous in its binding properties, a situation that exists in at least some insects (e.g., Hariton-Shalev et al., 2013; Jiang et al., 2014). In this case, differential distribution of the receptors, with the specific receptor located in the CG and the less specific one(s) (or both) present in the STNS would account for the different specificities of the responses to pyrokinins. Similarly, there could be multiple pyrokinin receptors, all of which are relatively specific, with just one in the CG, but multiple receptors present in the STNS. In our opinion, the former seems more likely, since it seems less likely that multiple receptors would all have the same distribution and effects on the various neurons that constitute the gastric mill pattern generator.

Alternatively, it is possible that all of the pyrokinins bind to the same receptor, but that each pyrokinin alters that receptor differentially, thus activating different second messenger pathways. In this case, the pathways that are activated by most of the pyrokinins might be present in the STNS but not in the CG, or those pathways may not alter the parameters (contraction amplitude and frequency, burst duration) that were modified by PevPK2 in this study (Dickinson et al., 2015). Although it is not common for the same receptor to mediate different effects in response to activation by different ligands, such a situation exists in the responses to tachykinins in insects (Poels et al., 2005). Poels et al. found that four different tachykinins were able to activate the same receptor (\textit{Stomoxys calcitrans} tachykinin-related peptide receptor (STKR), a neurokinin/tachykinin receptor identified from the stable fly). STKR is a G-protein coupled receptor that activates both a cyclic AMP pathway and a phospholipase C/Ca$^{++}$ pathway (Torfs et al., 2000), and can do so when expressed in an insect cell line. Strikingly, when challenged with 4 different tachykinins, this receptor responded with different patterns of increases in the two second messengers; some tachykinins had much greater effects on the cAMP pathway while others had larger effects on the Ca pathway, leading Poels et al., (2005) to suggest that the receptor may be able to adopt one of two distinct functional states, which might then interact with different G proteins (reviewed in Nusbaum and Blitz, 2012). Similar reports of multiple states for neurokinin (Holst et al., 2001; Palanche et al., 2001; Sachon et al., 2002) and muscarinic (Gurwitz et al., 1994) receptors from mammals, as well as for aminergic receptors from \textit{Drosophila} (Reale et al., 1997; Robb et al., 1994), have likewise been reported.
MATERIALS AND METHODS

Animals and tissue collection

Animals

American lobsters, *H. americanus* Milne-Edwards, were purchased from local (Bar Harbor and Brunswick, ME, USA) seafood retailers. All animals were housed in flow-through or recirculating natural seawater aquaria at 10–12°C and were fed approximately weekly on a diet of chopped squid.

Dissections

For the isolation of the STNS (Fig. 1) for both immunohistochemistry and physiological experiments, animals were cold-anesthetized by packing in ice for approximately 20-30 min. After anesthetization, the foregut was removed and the STNS was manually dissected from the surrounding musculature in chilled (approximately 4°C) physiological saline (composition in mM/L: 479.12 NaCl, 12.74 KCl, 13.67 CaCl$_2$, 20.00 MgSO$_4$, 3.91 Na$_2$SO$_4$, 11.45 trizma base, and 4.82 maleic acid (pH = 7.45)).

Wholemount immunohistochemistry

Development of pyrokinin antisera

In order to map the distribution of pyrokinin-like peptides in the lobster nervous system, custom antibodies were designed and generated against the only known *H. americanus* member of this peptide family (*i.e.*, FSPRLamide (Ma et al., 2008)); antibody production was contracted through LAMPIRE Biological Laboratories, Inc. (Pipersville, PA, USA). In brief, a synthetic peptide with the sequence CFSPRLamide was synthesized and conjugated to bovine serum albumin (BSA) via the amino (N)-terminal cysteine using $m$-maleimidobenzoyl-$N$-hydroxysuccinimide ester (GenScript Corporation, Piscataway, NJ, USA). New Zealand white rabbits (three in total; animal codes 15417 (rabbit 1, R1), 15418 (rabbit 2, R2) and 15419 (rabbit 3, R3)) were injected subcutaneously and intradermally at multiple locations with 500 mg of peptide-BSA conjugate emulsified in 500 ml of complete Freund’s adjuvant. Rabbits were boosted with 500 mg of peptide-BSA conjugate emulsified in 500 ml of Freund’s incomplete adjuvant at 3-week intervals.

Preimmune serum was drawn from each rabbit prior to the initial injection of immunogen, with post-injection bleeds taken 30, 50 and 57 days after the initial immunogen injection. Preimmune sera and sera produced from all post-injection bleeds were stored at -
80°C. The immune sera from all three rabbits and all three bleeds produced apparently identical labeling in the STNS and neuroendocrine organs; however, all descriptions of immunoreactivity reported in this study were derived from tissues labeled using serum from the Day 30 bleed of rabbit 3 (serum code R3-30).

**Immunoprocessing**

Immunolabeling was done using wholemount preparations. In brief, dissected tissues were pinned out in Sylgard-lined Petri dishes and fixed for 12-24 hr in a solution of 4% paraformaldehyde (EM grade; Electron Microscopy Sciences; Hatfield, PA, USA; catalog #15710) in 0.1 M sodium phosphate buffer, pH 7.4 (P). Following fixation, tissues were rinsed five times at 1-hr intervals in P containing 0.3% Triton-X 100 (P-Triton), after which they were incubated for approximately 72 hr in pyrokinin antibody (see above) diluted to a final concentration of 1:10000-1:15000 in P-Triton containing 10% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories Inc., West Grove, PA; catalog #017-000-121). After incubation in primary antibody, tissues were rinsed five times at 1-hr intervals in P-Triton and then incubated for 12-24 hours in DyLight 488-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch; catalog 711-485-152) diluted to 1:300 in P-Triton containing 10% NDS. Following incubation in secondary antibody, tissues were rinsed five times at 1-hr intervals in P and then mounted between a glass microscope slide and coverslip in Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA; catalog #H-1000). Fixation and incubations in both primary and secondary antibody were conducted at 4°C, while all rinses were conducted at room temperature (approximately 22°C). Incubation in secondary antibody and subsequent processing was conducted in the dark and slides were stored at 4°C in the dark until examined for labeling.

**Specificity controls**

To strengthen our confidence that the immunolabeling reported here is due to the presence of pyrokinin-related peptides, we conducted two sets of specificity controls. In the first set of experiments, the two CoGs from a lobster were isolated after fixation; one ganglion was then processed using R3 pre-immune serum (cocs left long), while the other CoG (cocs cut short) was processed with serum R3-30 as described above. After incubation in primary antibody, the two CoGs were rinsed separately and then incubated together in secondary antibody solution (see above). All subsequent processing and imaging were done...
simultaneously.

For the second set of specificity controls, we pre-adsorbed the antibody with a number of peptides, including both pyrokinins and other crustacean peptides, particularly those that have significant sequence similarity to the pyrokinins. For these experiments, serum R3-30 was incubated with a $10^{-3}$ M concentration of FSPRLamide (the *H. americanus* peptide against which the antibody was directed; Ma et al., 2008), CabPK2 (Saideman et al., 2007), PevPK1 (Torfs et al., 2001), Dappu-ETH (Gard et al., 2009), Homam-sNPF (Ma et al., 2008) or SGRNFLRFamide (Ma et al., 2008) for 3 hr at room temperature. Simultaneously, aliquots of serum R3-30 were held at room temperature for 3 hr without peptide. As was the case for the preimmune controls, CoG pairs were used here, with one CoG from each lobster incubated with control antibody and the other incubated in pre-adsorbed antibody; they were processed simultaneously as described above.

Epifluorescence and confocal microscopy

Data were collected and digital images were generated using an Olympus BX-51 upright compound microscope (Olympus America, Center Valley, PA, USA) outfitted with epifluorescence and an OPRONICS MacroFire digital camera (OPRONICS, Goleta, CA, USA) or an Olympus Fluoview 1000 confocal system that utilizes an Olympus IX-81 inverted microscope and blue diode, multi-argon, green HeNe and red HeNe lasers. For the production of figures, digital images were exported from the Olympus confocal system as tiff files and then arranged using Photoshop software (version 7.0; Adobe Systems Inc., San Jose, CA, USA). It should be noted that the contrast and brightness of the final figures were adjusted as needed to optimize the clarity of the printed images.

**Extracellular Recordings**

For recordings of neuronal activity, the STNS was pinned out in a clear Sylgard184 (KR Anderson, Santa Clara, CA, USA)-lined Petri dish. The STG was desheathed to provide the pyrokinins with direct access to the ganglion. Neuronal activity was recorded using monopolar pin electrodes inserted in petroleum jelly wells surrounding and isolating portions of each nerve of interest [medial ventricular nerve (*mvn*), inferior cardiac nerve (*icn*), *lvn*, dorsal gastric nerve (*dgn*), *dlvn*, ventral lateral ventricular nerve (*vlvn*), ventral pyloric dilator nerve (*vpdn*)], with ground electrodes in the bath. Electrical activity was amplified using an A-M Systems Differential AC Amplifier (Model 1700) and a Brownlee Precision
Instrumentation Amplifier (Model 210 A). A Cambridge Electronic Design Power 1401 and Spike2 were used for data recording into a computer with a sampling rate of 10kHz. The STNS was constantly superfused with saline kept at 10-13°C with a Peltier cooling system (CL-100 bipolar temperature controller and SC-20 solution heater/cooler; Warner Instruments, Hamden, CT, USA) via a peristaltic pump, with a flow rate of approximately 5 ml/minute. Peptides were applied through the perfusion system.

**Peptides**

Five pyrokinins, FSPRLamide, PevPK1, PevPK2, CabPK1 and CabPK2, were used for physiological studies (Table 1). PevPK1, PevPK2, and FSPRLamide were custom synthesized by GenScript Corporation, while the initial CabPK1 and CabPK2 used here were kind gifts from Dr. Michael Nusbaum (University of Pennsylvania School of Medicine (Philadelphia, PA, USA)), with additional samples of these peptides custom synthesized by GenScript. Regardless of source, all peptides were stored at -20°C as a 10^-3 M solution in deionized water. Pyrokinins were diluted to 10^-6 M in saline immediately before use. The pyrokinin solution was then superfused over the STG for approximately ten minutes, followed by a saline wash for at least 50 minutes to ensure that neuronal activity returned to baseline before another pyrokinin solution was applied.

**Data Analysis**

Data were analyzed in Spike2 using the built in functions and custom written scripts available at http://stg.rutgers.edu/Resources.html. Quantitative analyses of extracellular recordings were used to examine cycle frequency, burst duration, and spike frequency of two gastric neurons, the LG and DG neurons and of two pyloric neurons, the LP and PD neurons. To compare the effects of the peptides to control activity, the average of measurements taken from 10 bursts just before pyrokinin application was compared to the averages of 10 bursts taken during the time at which the peptide had its maximum effect. Data were graphed and statistical comparisons made using Prism 6 (GraphPad Software, San Diego, CA). For some parameters, data were normalized by determining percent change from control; one-sample t-tests (two tailed) were used to determine whether these changes were significantly different from zero. Gastric cycle frequency, as well as burst parameters of individual neurons in saline vs percent change during pyrokinin superfusion, were fit with a one-phase exponential decay function, from which the R^2 was analyzed to assess the fit. A one-way ANOVA was
run to test for differences in mean residuals between the different crustacean pyrokinins. The model residuals did not systematically vary across the independent variables for any peptide.

Physiology figures were made from Spike2 files incorporated into CorelDraw (Corel, Inc., Mountain View, CA).

**LIST OF ABBREVIATIONS**

BSA, bovine serum albumin
C, carboxyl
CabPK1, *Cancer borealis* pyrokinin 1
CabPK2, *Cancer borealis* pyrokinin 2
CG, cardiac ganglion
coc, circumoesophageal connective
CoG, commissural ganglion
Dappu-ETH, *Daphnia pulex* ecdysis-triggering hormone
DG, dorsal gastric neuron
dgn, dorsal gastric nerve
dlvn, dorsal lateral ventricular nerve
dpon, dorsal posterior oesophageal nerve
dvn, dorsal ventricular nerve
ETH, ecdysis-triggering hormone
Homam-sNPF, *Homarus americanus* short neuropeptide F
IC, inferior cardiac neuron
icn, inferior cardiac nerve
ion, inferior oesophageal nerve
LG, lateral gastric neuron
ln, labral nerve
LP, lateral pyloric
LPG, lateral posterior gastric neuron
lvn, lateral ventricular nerve
mvn, medial ventricular nerve
N, amino
NDS, normal donkey serum
OG, oesophageal ganglion
on, oesophageal nerve
$P$, 0.1 M sodium phosphate buffer

PBAN, pheromone biosynthesis activating neuropeptide

PD, pyloric dilator neuron

PevPK1, *Penaeus vannamei* pyrokinin 1

PevPK2, *Penaeus vannamei* pyrokinin 2

$P$-Triton, 0.1 M sodium phosphate buffer containing 0.3% Triton-X 100

son, superior oesophageal nerve

STG, stomatogastric ganglion

STKR, *Stomoxys calcitrans* tachykinin-related peptide receptor

ston, stomatogastric nerve

STNS, stomatogastric nervous system

VD, ventricular dilator

vlvn, ventral lateral ventricular nerve

vpdn, ventral pyloric dilator nerve
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AUTHOR CONTRIBUTIONS

P.S.D. and A.E.C conceived and designed this study. Experiments were executed and data were analyzed by P.S.D., S.C.K., X.Q., A.S., M.A.K., A.H.W., A.B.Y., B.P. and A.E.C. The manuscript was written by P.S.D., S.C.K. and A.E.C.

AUTHOR COMPETING INTERESTS

The authors declare no competing or financial interests.
REFERENCES


Figures

Figure 1. Schematic representation of the stomatogastric nervous system (STNS) of Homarus americanus. The STNS consists of 4 ganglia as well as the nerves that connect them and motor nerves that innervate the muscles of the gastric mill and pylorus. *coc*, circumoesophageal connective; CoG, commissural ganglion; *dgn*, dorsal gastric nerve; *dlvn*, dorsal lateral ventricular nerve; *dvn*, dorsal ventricular nerve; *dpon*, dorsal posterior oesophageal nerve; *icn*, inferior cardiac nerve; *ion*, inferior oesophageal nerve; *ln*, labral nerve; *mvn*, medial ventricular nerve; OG, oesophageal ganglion; *on*, oesophageal nerve; *pdn*, pyloric dilator nerve; *son*, superior oesophageal nerve; *stn*, stomatogastric nerve; *vlvn*, ventral lateral ventricular nerve.
Figure 2. Distribution of pyrokinin-like labeling in the stomatogastric ganglion (STG) and commissural ganglion (CoG) of *Homarus americanus*. (A) In the STG, several fascicles of immunopositive axons projecting from the stomatogastric nerve (*stn*) arborize within the ganglion, producing a neuropile composed of a dense network of fine fibers studded with bead-like varicosities; most if not all of the axons producing this neuropile appear to exit the ganglion via the dorsal ventricular nerve (*dvn*). No pyrokinin-like immunoreactivity was seen in any of the 30 or so intrinsic STG somata. This image is a
brightest pixel projection of 43 optical sections taken at approximately 2 μm intervals. Scale bar = 100 μm. (B) Pyrokinin-like labeling in the CoG consisted of approximately 14 immunopositive somata (two groups indicated by arrows) and an extensive neuropile. This neuropile is likely derived from processes originating from both the intrinsic immunopositive cell bodies and from immunoreactive axons projecting into the ganglion from the circumoesophageal connective (coc). Immunopositive axons exiting the CoG via the superior oesophageal nerve (son) are the source of the pyrokinin labeling in the STG. No pyrokinin-like immunoreactive axons were seen exiting the CoG via the inferior oesophageal nerve (ion). This image is a brightest pixel projection of 38 optical sections taken at approximately 2 μm intervals, and is shown at the same scale as A.
Figure 3. An extensive pyrokinin immunoreactive plexus covers the surface of much of the anterior portion of the *Homarus americanus* stomatogastric nervous system. An extensive neuroendocrine-like plexus covers much of the anterior portion of the STNS. This structure is located within or just below the sheath that covers the STNS and has a distinctly bark-like appearance. This putative neuroendocrine release structure extends from the branch point of the dorsal posterior oesophageal nerve on each superior oesophageal nerve (*son*) to the junction of the *sons* and stomatogastric nerve (*stn*), down the *stn* approximately half way to the stomatogastric ganglion, and up the oesophageal nerve and inferior oesophageal nerves (*ions*) to the branch point of the labral nerve on each *ion*. The images shown were collected
from the *stn*, just below the *son/stn* junction (see Fig. 1). Panel A is a brightest pixel projection of 68 optical sections taken at approximately 1 μm intervals through this region, while panels B-D are single optical sections taken at roughly the top (section 14), middle (section 35) and bottom (section 58) portions of the nerve, and illustrate the superficial nature of the staining. All images are shown at the same magnification. Scale bar = 100 μm.
Figure 4. **Superfusion of PevPK1 at 10^{-6} M excited gastric activity within the STNS of the lobster; activity in 3 gastric neurons is shown in (A), with a fourth gastric neuron shown in (B) in another preparation. Pyloric activity was not altered by PevPK1 superfusion.** (A) The LPG, LG, and DG motor neurons, whose axons were recorded in extracellular recordings of activity in the lateral posterior gastric nerve (*lpgn*), the medial ventral nerve (*mvn*), and the dorsal gastric nerve (*dgn*), all fired with short, high frequency bursts or modulation, which corresponds to the pyloric motor in saline (left panel). In the presence of pyrokinins (PevPK1 shown here), they began to fire with typical gastric timing (longer bursts at lower frequency; right panel). The pyloric VD neuron (*mvn*) continued to fire in pyloric time in PevPK1. (B) In control saline (left panel), the inferior cardiac (IC) neuron (*icn*) fired largely in pyloric-timed bursts (similar to the pyloric-timed VD bursts recorded on the *mvn*); in the presence of PevPK1 (10^{-6} M; right panel), the IC neuron began to fire more strongly during gastric bursts, coordinated with the LG neuron (*mvn*).
Figure 5. Superfusion of the five pyrokinins elicited similar effects on gastric activity in the STNS of the lobster, suggesting that receptors for pyrokinins within the STG bind to pyrokinins indiscriminately. (A) FSPRLamide, (B) PevPKI, (C) PevPK2, (D) CabPK1 and (E) CabPK2. In each panel, the top extracellular recording (mvn) shows LG becoming activated during pyrokinin application, while the VD neuron continues to fire in pyloric time.
The middle recording of the \textit{dgn} shows DG changing from firing less-defined gastric bursts with some pyloric characteristics in saline to firing in defined gastric bursts during pyrokinin application. In the recording of the dorsal lateral ventricular nerve (\textit{dlvn}), the bursts in the LPG neuron became more focused and intense. Recordings are from a single preparation in which the five pyrokinins were applied sequentially, with 1-hour washes in saline between each peptide application.
Figure 6. At $10^{-6}$ M, all crustacean pyrokinins altered gastric activity, with the extent of change being dependent on the initial state of the preparation. All five pyrokinins elicited similar changes in the STNS of the lobster. Generally, change in cycle frequency recorded during pyrokinin application was inversely correlated with the gastric cycle frequency before application of the peptides, which was best fit to a two-phase exponential decay, $R^2=0.59$. Data pooled from 20 lobsters.
Figure 7. All five pyrokinins (superfused at $10^{-6}$M) enhanced activity in the lateral gastric (LG) neuron, with state dependent increases in both burst duration (A,B) and spike frequency within the bursts (C,D). (A) LG burst duration increased in most cases in all five pyrokinins, as seen by the fact that most of the points fall above the equality line. However, when burst durations were initially long (>~5 sec), burst duration often decreased. (B) To see the extent to which the effects of the pyrokinins were dependent on the initial parameters, we graphed the % change in pyrokinin as a function of the initial burst duration. Although there is some scatter, the % change clearly decreased as baseline duration increased, with $R^2 = 0.45$. (C) Like burst duration, spike frequency in the LG neuron increased in the presence of all five pyrokinins. (D) Although changes in spike frequency were inversely correlated with initial spike frequency, there was considerable variability between preparations, with $R^2 = 0.26$. 
Figure 8. All five pyrokinins had similar effects on the firing of the DG motor neuron: they did not consistently alter burst duration, but they all increased spike frequency within DG neuron bursts. (A) Burst duration did not consistently change in the presence of the pyrokinins, as seen by the even distribution of points above and below the equality line. (B) Changes in burst duration were highly variable between preparations, as seen by the wide scatter of points; the average change in burst duration was thus not significantly different from 0 (p=0.99, one sample t-test, n=56), although some individual changes were quite large. Consequently, the R² values for a one-phase exponential decay or linear regression was only 0.19. However, the slope of a linear regression (not shown) was significantly non-zero (p=0.01), suggesting that the extent of the change is nonetheless dependent on the starting spike frequency. (C) Spike frequency consistently increased in all of the pyrokinins, with no obvious differences between isoforms. In nearly all cases, the points fall above the equality line. (D) As was the case with burst duration, the extent of the increase in DG spike frequency was highly variable between preparations. Nonetheless, a single value t-test for pooled data showed that spike frequency on average did increase (t-test, p<0.001, n =53).
Although $R^2$ for the non-linear regression was only 0.12, the slope of a linear fit was significantly different from 0 ($p=0.02$), indicating that the change was dependent on the starting conditions, but that other factors are likely also important in determining the extent of the response to pyrokinins in the DG neuron. Moreover, there were no differences between peptides in the extent to which spike frequency increased (ANOVA, $p=0.66$, df = 4,48), nor in the residuals from the non-linear fit ($p=0.9$).
Figure 9. **Superfusion of pevpyrokinin-1 (10^{-6} M) over the STNS of the lobster did not alter the pyloric motor pattern.** (A) Control recordings show the typical triphasic pyloric pattern, with bursts in the pyloric dilator (PD) neurons (pdn), followed by bursts in the lateral pyloric (LP) and pyloric (PY) motor neurons (vlvn). Pyloric-timed bursts in the ventricular dilator (VD) neuron (mvn) are largely in phase with the PY neuron bursts. A single burst in the gastric motor neuron is also visible on the mvn recording. (B) When PevPK1 was superfused across the stomatogastric system, no changes in the firing of any of the pyloric neurons was evident, although the duration of the single burst seen here in the lateral gastric (LG) motor neuron increased.
Figure 10. **Crustacean pyrokinins (10^{-6}M) superfused over the stomatogastric ganglion did not alter the pyloric motor pattern.** Scatterplots show the effects of crustacean pyrokinins on (A) pyloric cycle frequency, (B) burst duration in the PD neuron, (C) spike frequency in the PD neuron, (D) burst duration in the LP neuron, and (E) spike frequency in the LP neuron. Each data point represents the mean value of 10 consecutive cycles in a single preparation during both saline and pyrokinin superfusion. All points fell on or near the equality line, indicating that the pyrokinins did not quantitatively alter the pyloric pattern.
Data are pooled from 6 preparations that were treated consecutively with the five pyrokinins, separated by 1-hour saline washes.
### Table 1. Known decapod crustacean pyrokinin isoforms

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<th>Peptide structure</th>
<th>Acronyms used in text</th>
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*The reference provided is the first identification of the peptide in the species in question.

Abbreviations used in peptide structures: a, amide; +, a partial peptide with additional unidentified amino-terminal residues likely present.

Peptides shown in bold font have been identified in multiple species.