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Cloning, localization, and physiological effects of sulfakinin in the kissing bug, *Rhodnius prolixus*

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ABSTRACT

Sulfakinins (SKs) are a family of multifunctional neuropeptides that have been shown to have myotropic activity on muscles of the digestive system and to function as feeding satiety factors. Here, we confirm via cloning the presence of two sulfakinins (Rhopr-SK-1 and Rhopr-SK-2) in *Rhodnius prolixus*. Reverse transcriptase quantitative PCR demonstrates that the *Rhopr-SK* transcript is highly expressed in the central nervous system (CNS) of unfed fifth-instar *R. prolixus*. Fluorescent *in situ* hybridization shows transcript expression only in neurons in the brain. Immunohistochemical staining of SK-like peptides was observed in the same neurons in the brain and in processes extending throughout the CNS, as well as over the posterior midgut and anterior hindgut. Rhopr-SK-1 (sulfated form) induces contractions of the hindgut in a dose-dependent manner. Injection Rhopr-SK-1 (sulfated form) significantly decreases the overall weight of the blood meal consumed, suggesting SK's role as a satiety factor in *R. prolixus*.

1. Introduction

Blood meals are required by *Rhodnius prolixus,* the vector of Chagas' disease that is commonly known as the kissing bug, for growth and development. Indeed, an unfed *R. prolixus* remains in an arrested state of development until blood gorging, which then initiates a variety of physiological and endocrinological changes that stimulate the development of *R. prolixus* into the subsequent instar or into adulthood. Thus, blood feeding initiates crucial developmental processes, including growth, metamorphosis, and reproduction. In addition, there are short-term changes associated with blood feeding, including the elimination of excess water and salts (diuresis) [33,34]. Various tissues and processes are associated with diuresis, including the muscular contractions of the midgut, hindgut, and dorsal vessel, as well as the movement of ions and water through the epithelium of the anterior midgut and the Malpighian tubules [14,33].

Neuropeptides regulate a number of the short-term physiological processes involved in feeding, digestion, and satiety [1,9,15,25,28,29,33]. These processes have been linked to some of the neuropeptides associated with the brain and/or gastrointestinal tract [29,40]. One such neuropeptide family is the sulfakinins (SKs), which are multifunctional neuropeptides that have been shown to have myotropic activity on muscles of the digestive system and to function as feeding satiety factors in various insects, including *Schistocerca gregaria*, *Blattella germanica*, and *Phormia regina* [7,13,21,26,27,43]. Most SKs possess a sulfated tyrosyl residue in their characteristic *C*-terminal heptapeptide core sequence D/EYGHMRFamide, although nonsulfated SKs have also been shown to occur *in vivo* [32,35,39]. Using a cell-based receptor assay system, Yu et al. [45] have demonstrated that sulfated SK is 1000–10,000 times more potent than the nonsulfated SK in activating the SK receptors of *Tribolium castaneum*. Moreover, the sulfated SK was 3000 fold more potent than the nonsulfated counterpart in stimulating the *Drosophila* sulfakinin receptor 1 (DSK-R1) in *Drosophila melanogaster* [18]. Recently, two sulfakinins were *de novo* sequenced from *Rhodnius prolixus* central nervous system (CNS) using MS/MS and bioinformatics tools but it was not stated whether these SKs had sulfated tyrosyls [31,32].

Sulfakinin-like immunoreactive neurons are restricted to the brain in flies, crickets, and crustaceans [7,10,12,17,30,40]. For example, an immunocytochemical study, complemented by *in situ* hybridization, of SKs and their transcript in *Calliphora vomitoria* revealed that SK is only localized to four pairs of caudo-dorsal neurons in the brain, with the cells possessing axons that project into the thoracic and abdominal ganglia [10].

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0196-9781/

Little is known about the role of SKs in controlling feeding and satiety in *R. prolixus*. Here we have cloned the SK open reading frame (ORF), confirming previous work that indicated the presence of two SKs in *R. prolixus*, namely Rhopr-SK-1 and Rhopr-SK-2 [31,32]. Reverse-transcriptase quantitative PCR (RT-qPCR) and fluorescent *in situ* hybridization were performed to determine the spatial expression profile of the *Rhopr-SK* transcript in *R. prolixus*. Immunohistochemistry was utilized to determine the location of SK in the CNS and peripheral tissues in *R. prolixus*, and finally the effects of Rhopr-SK-1 on hindgut and dorsal vessel contraction, and on the blood meal consumed, was examined.

2. Materials and methods

2.1. Animals

Male and female 5th instar *R. prolixus* were obtained from a colony raised at the University of Toronto Mississauga. Insects were reared at 50% humidity, 25 °C in incubators and fed defibrinated rabbit blood (Cedarlane Laboratories Inc., Burlington, ON, Canada). All tissues were dissected from 5th instars, 2–4 weeks after ecdysing into 5th instars.

2.2. Chemicals

Rhopr-SK-1 (pQFNEY(SO_3H)GHMRFamide) was custom synthesized by SynPeptide (Shanghai, China), and the sequence including the sulfation was confirmed by the Advanced Protein Technology Centre (Hospital for Sick Children, Toronto, ON). Rhopr-FMRFamide (GNDNFMRFamide), Rhopr-FIRFamide (AKDNFIRFamide), and Rhopr-Kinin 2 (AKFSSWGamide) were custom synthesized by GenScript (Piscataway, NJ, USA). All peptides were reconstituted in double distilled water into stock solutions at 10^{-3} M. Stock solutions were stored at −20 °C as 10 μl aliquots until working solutions were made using physiological saline (KCl 8.6 mM, NaCl 150 mM, $CaCl_2$ 2 mM, $NaHCO_3$ 4 mM, glucose 34 mM, $MgCl_2$ 8.5 mM, HEPES 5 mM [pH 7.0]).

An anti-perisulfakinin antibody was provided by Jan Veenstra (Bordeaux, France), with its specificity described in Veenstra et al. [41]. Goat cyanine dye 3 (Cy3) anti-rabbit (IgG) secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Both antibodies were stored at −20 °C.

2.3. Screening of 5th instar CNS cDNA

Rhopr-SK amino acid sequences [32] were used to search the *R. prolixus* genome assembly via BLAST [3]. The remaining *in silico* procedures were performed in Geneious v. 4.7.6 (http://www.geneious.com). Using these sequences, a putative SK sequence was highlighted in the *R. prolixus* genome (supercontig: KQ034228). Gene-specific primers were then designed and used to amplify the *Rhopr-SK* ORF, Rhopr-SK-FOR (5′ ATAATGGGTAGCAGCTTCC 3′) and Rhopr-SK-REV (5′ GGAATTATAAGTGGAGTGTATGC 3′), from CNS cDNA. All reactions were performed using an s1000 thermal cycler (Bio-Rad Laboratories, Mississauga, ON, Canada). The PCR thermal cycler conditions were maintained at an initial 3 min at 94 °C, which was followed by 35 cycles of 30 s at 94 °C, 30 s at 53 °C, and 60 s at 72 °C. The final step consisted of running the samples for 10 min at 72 °C. The gene-specific primers were used to amplify specific products using a nested PCR approach. Here, the amplicons of each PCR were column purified via the EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc., Markham, ON, Canada), then used for subsequent PCR reactions. Agarose gel electrophoresis was performed, whereby the desired amplicon was run on 1% agarose gel. The cDNA was then gel purified using the EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Inc.) and ligated onto a pGEM T Vector System (Promega Corporation, Madison, WI, USA). The amplified cDNA-containing vectors were extracted from the cells via the EZ-10 Spin Column Plasmid DNA Miniprep Kit (Bio Basic Inc.), and sequenced at The Hospital for Sick Children's DNA sequencing facility (Toronto, ON, Canada). To ensure the accuracy of the obtained sequences, sequencing results were confirmed via several independent clones.

2.4. Sequence analysis of cDNA

Intron-exon boundaries were found using a BLAST search using the *R. prolixus* genome assembly, and confirmed using the online splice site prediction software Genie [36]. The online software ProP 1.0 was used to predict the mono- and dibasic arginine and/or lysine cleavage sites [8]. Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to align the Rhopr-SK prepropeptide (ACS45388.1) with its homologs from *Habropoda laboriosa* (KOC62825.1), *Nilaparvata lugens* (AFW19802.1), *Psacothea hilaris* (BAH11170.1), *Gryllus bimaculatus* (CAL48349.1), *Anopheles maculatus* (AAW82713.1), *Thermobia domestica* (ALG35949.1), *Anopheles sinensis* (KFB42310.1), *Anopheles gambiae* (AAR03495.1), and *Drosophila simulans* (XP_002102055.1). Boxshade 3.21 server (http://www.ch.embnet.org/software/BOX_form.html) was used to obtain an alignment figure.

2.5. RT-qPCR tissue profiling

The following tissues were used for the spatial expression analysis: brain and suboesophageal ganglion (SOG), prothoracic ganglion (PRO), mesothoracic ganglionic mass (MTGM), midgut, and hindgut. RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) was used to extract RNA from the tissues. At least 20 ng of total RNA was used as a template for the corresponding cDNA synthesis via the iScript Reverse Transcription Supermix for RT-qPCR (BioRad Laboratories Ltd., Mississauga, ON, Canada). The RT-qPCR analyses were performed using an Mx4000 Multiplex Quantitative PCR System (Stratagene, Mississauga, ON, Canada), using SsoFast EvaGreen Supermix with Low ROX (BioRad Laboratories Ltd.). The temperature-cycling profile consisted of an initial denaturation step at 95 °C for 30 s, followed by 40 cycles of denaturation (5 s at 95 °C) and annealing/extension (24 s at 58 °C), which was followed by a melt curve analysis (58 °C-to-95 °C). The melt curve analysis as well as gel electrophoresis were performed to confirm the specificity of the obtained products. A 207 base-pair *Rhopr-SK* cDNA fragment was amplified using the forward primer, 5′ AGCCAGCTGAAAGGAGATCA 3′, and the reverse primer, 5′ GGTAAAGAATTGGCCATGGTCT 3′. Primer efficiencies were calculated for the target, *Rhopr-SK*. Three reference genes, namely ribosomal protein 49, β-actin, and α-tubulin, were also amplified via the primers described previously [46]. The levels of expressed transcripts were quantified via the standard curve method. The delta–delta C_t ($\Delta\Delta$CT) method was applied to calculate the relative expression of the transcripts. The expression levels for *Rhopr-SK* transcripts were normalized via geometric averaging of the transcript levels of the housekeeping/reference genes. Each reaction contained two technical replicates as well as a no template control and a no reverse transcriptase control. Three biological replicates were performed.

2.6. Cell-specific expression of rhopr-SK mRNA in R. prolixus CNS

To determine the cell-specific expression pattern of the *Rhopr-SK* transcript, a fluorescent *in situ* hybridization (FISH) technique was performed as previously described, with the following modifications [19]. Sense and antisense gene-specific primers were used to amplify a 430 base-pair *Rhopr-SK* cDNA fragment, which was used to synthesize the respective RNA probes used for *in situ* hybridization (Table 1). The digoxigenin (DIG)-labelled

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Table 1
Rhopr-SK Fluorescent *in situ* Hybridization (FISH) primers.

Primer Type	Sequence of Primer
T7 SK-Forward	5′ TAATACGACTCACTATAGGGAGAATAATGGGTAGCAGCTTCC 3′
SK FISH REV 1	5′ GCATACACTCCACTTATAATTCCTCTAATTTAT 3′
T7 SK-Reverse	5′ TAATACGACTCACTATAGGGAGAGCATACACTCCACTTATAATTCC 3′
SK FISH FOR 2	5′ ATAATGGGTAGCAGCTTCCTAATCAC 3′

sense RNA probe was synthesized as a negative control. CNS tissues were dissected in phosphate buffered saline (PBS) (2.1 mM $NaH_2PO_4{\cdot}H_2O$, 8.3 mM Na_2HPO_4, 150 mM NaCl; pH 7.2) placed in a 4% paraformaldehyde (in PBS) solution, and left overnight at 4 °C. The tissues were then placed in 4% Triton X-100 (Sigma Aldrich, Oakville, ON, Canada) in PBT (1xPBS and 0.1% Tween-20: BioShop Canada Inc., Burlington, ON, Canada) at room temperature for one hour. Pre-hybridization was performed at 56 °C for 2 h. Hybridization was then performed using a 100 μl hybridization solution containing antisense, or sense (as control), DIG-labelled Rhopr-SK RNA probes at a concentration of approximately 1.0 ng/μl. Biotin-SP-conjugated IgG fraction monoclonal mouse antidigoxigenin (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) was utilized to detect the DIG signal. Tissues were then incubated in an Alexa Flour 568 tyramide working solution, diluted 1:200 in amplification buffer (containing 0.0015% H_2O_2), at room temperature for one hour. Tissues were then mounted onto slides in glycerol then viewed using a Zeiss LSM 510 confocal laser microscope (Carl Zeiss, Jena, Germany). ImageJ (https://imagej.nih.gov/ij/) was used to analyse the images [6].

2.7. Immunohistochemistry

Immunohistochemistry was performed on whole mount preparations of CNS, gut, dorsal vessel, and salivary glands of male and female 5th instar, as well as adult, *R. prolixus*. The tissues were dissected under physiological saline, then directly transferred to 2% paraformaldehyde in Millonig's buffer (130 mM $NaH_2PO_4{\cdot}H_2O$, 100 mM NaOH, 1.2 g glucose, 0.3 mM $CaCl_2{\cdot}2H_2O$; pH 7.0) for 24 h at 4 °C. The immunohistochemical staining procedure was performed as previously described with the following modifications [20]. The tissues were incubated with the SK primary antiserum (1:10,000 rabbit anti-Y(SO_3H)GHMRFamide), which was combined with 2% normal goat serum (NGS) and 0.4% Triton X-100 in PBS, for 48 h at 4 °C. The tissues were washed with PBS every 15 min for 5 h and then incubated in a secondary goat anti-rabbit antibody (1:600 in 10% NGS in PBS), which was conjugated to Cy3. The samples were incubated overnight at 4 °C. Samples were then washed using PBS every 15 min for 5 h, and run through a series of increasing glycerol concentrations. The tissues were then mounted, and viewed using a Zeiss LSM 510 confocal laser microscope (Carl Zeiss, Jena, Germany). To control for the specificity of the primary antiserum, the primary antiserum was pre-absorbed with either 10^{-5} M Rhopr-FMRFamide or 10^{-5} M Rhopr-SK-1.

2.8. Contraction assays

R. prolixus hindguts were dissected in physiological saline, and the contraction assay was performed as previously described, with the following modifications [20]. Rhopr-SK-1 was applied to the tissues by replacing 100 μl of the 200 μl saline bath in the wells containing the hindgut with an equal volume of the experimental solution. A Grass strain gauge amplifier (Grass Instruments, Warwick, RI, USA) was used to amplify the contractions monitored by a Grass FT03 force displacement transducer (Grass Instruments, Warwick, RI, USA), which were subsequently analysed using PicoScope 2200 (Pico Technology, St. Neots, UK) software. Rhopr-SK-1-induced contractions were compared to those induced by Rhopr-FIRFamide, Rhopr-Kinin 2, as well as Rhopr-FMRFamide. A heartbeat rate assay was also performed as previously described [20]. Heartbeat rate was monitored after the application of varying concentrations of Rhopr-SK-1 (10^{-12} to 10^{-5} M) using an impedance converter (UFI model 2991, Morro Bay, CA, USA).

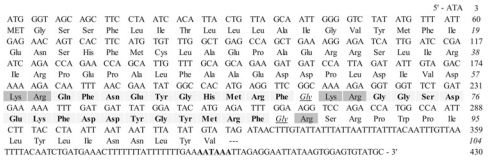

Fig. 1. *Rhodnius prolixus* sulfakinin (SK) cDNA and the corresponding amino acid sequence. The numbering for each sequence is on the right. Rhopr-SK-1 and Rhopr-SK-2 amino acid sequences are bolded. The capitalized methionine is the translation start site. The mono- and dibasic lysine and arginine post-translational cleavage sites are shaded in gray. The glycine residues required for amidation are italicized and underlined. The polyadenylation consensus sequence is bolded and underlined.

2.9. Feeding assay

Rhopr-SK-1 (1 μl of 10^{-4} M) was injected into 25 5th instar *R. prolixus* (both males and females) using a 5 μl Hamilton syringe. As a control, 25 5th instar *R. prolixus* were injected with 1 μl of saline. Injected *R. prolixus* were left for approx. 24 h at 25 °C, then fed defibrinated rabbit blood. The insects fed to satiation, which took approx. 20 min, and diuresis began at the end of feeding. The animals were weighed before feeding, following feeding, then at 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, and 24 h post-feeding.

2.10. Statistical analyses

Graphical representations and statistical analyses were generated using Graph Pad Prism (www.graphpad.com). Two way ANOVA followed by a Bonferroni's test was performed to analyze the dose-response curve, calculate the differences in effect between the examined peptides on hindgut contraction, and compare the post-feeding weights of animals injected with Rhopr-SK-1 to saline. Heartbeat frequencies were analysed by one way ANOVA.

3. Results

3.1. Rhopr-SK transcript and prepropeptide

The cDNA encoding *Rhopr-SK* was cloned and sequenced (Fig. 1). The ORF is 312 base pairs long, encoding 104 amino acids. No exon–exon boundaries were found in the ORF. A polyadenylation consensus sequence (AATAAA) is found 78 base pairs downstream of the stop codon. The *Rhopr-SK* prepropeptide, predicted by the transcript, contains a signal peptide 17 amino acids long and contains 2 predicted Rhopr-SKs that are produced by post-translational cleavage at the monobasic and dibasic lysine and arginine residues (Table 2). The glycine residue at the *C*-termini of both peptides predicts amidation.

Cloned SK prepropeptide amino acid sequences from other insect species were aligned in order to analyse the conservation of the *Rhopr-SK* prepropeptide sequence (Fig. 2). Identical and sim-

Table 2
The amino acid sequences for the *R. prolixus* sulfakinins.

Peptide	Amino Acid Sequence
Rhopr-SK-1	pQFNEY(SO_3H)GHMRFamide
Rhopr-SK-2	GGSDEKFDDY(SO_3H)GYMRFamide

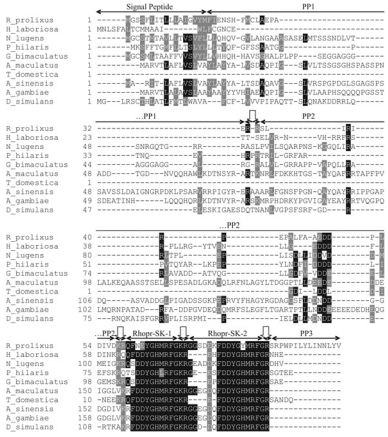

Fig. 2. Amino acid sequence alignment for the sulfakinin transcript of nine species, aligned via Clustal Omega. Following the 50% majority rule, identical amino acids are highlighted in black, whilst conserved amino acids are shaded in grey. The sequences used consisted of *Rhodnius prolixus, Habropoda laboriosa* (KOC62825.1), *Nilaparvata lugens* (AFW19802.1), *Psacothea hilaris* (BAH11170.1), *Gryllus bimaculatus* (CAL48349.1), *Anopheles maculatus* (AAW82713.1), *Thermobia domestica* (ALG35949.1), *Anopheles sinensis* (KFB42310.1), *Anopheles gambiae* (AAR03495.1), *Drosophila simulans* (XP_002102055.1). For *R. prolixus*, the two mature peptides, Rhopr-SK-1 and Rhopr-SK-2, as well as the signal peptide and precursor peptide (PP) regions, are also displayed. Arrows indicate arginine and/or lysine cleavage sites.

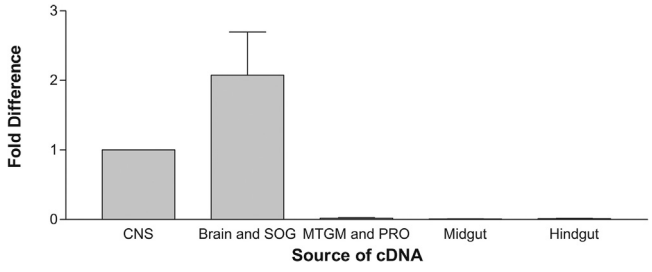

Fig. 3. Spatial expression of *Rhopr-SK* transcript in *R. prolixus* (5th instar) via reverse transcriptase quantitative PCR (RT-qPCR). Expression was examined in the following tissues: CNS, brain and SOG (suboesophageal ganglion), MTGM (mesothoracic ganglionic mass) and PRO (prothoracic ganglion), midgut, and hindgut. Data points are mean ± standard error of the mean for 3 independent samples.

ilar amino acids are highlighted via a 50% conservation cut-off. The resulting alignment shows that the sequences and lengths of the SK prepropeptides are well conserved across insect species. The number of SKs is also conserved with 2 SKs predicted for each species. At the carboxyl-terminus, Rhopr-SK-2 is unusual in that it contains a tyrosyl residue in place of the histidine amino acid that is present in all other SK sequences.

3.2. *Rhopr-SK transcript spatial expression*

RT-qPCR was used to measure transcript expression of *Rhopr-SK*. Expression was found in the CNS, with transcript expression levels highest in the Brain and SOG, with little or no expression in the MTGM and PRO, or in the midgut and hindgut (Fig. 3).

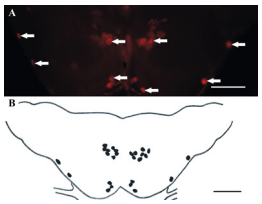

Fig. 4. (A) Fluorescent *in situ* hybridization (FISH) showing the neurons expressing the *Rhopr-SK* transcript (arrows) in 5th instar *Rhodnius prolixus* brain. (B) Diagrammatic representation of the cells revealed by FISH. Scale bars: 100 μm.

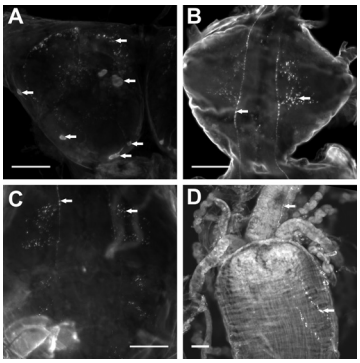

Fig. 5. Immunohistochemical staining in cells and processes of 5th instar *Rhodnius prolixus* (A) brain, (B) prothoracic ganglion, (C) mesothoracic ganglionic mass. Stained processes are seen on the posterior midgut and hindgut (D). Arrows indicate the immunostained cells and processes in the CNS, as well as the posterior midgut (upper) and hindgut (lower). Two stained ventral axons extend from the brain to the mesothoracic ganglionic mass. Scale bars: 100 μm.

3.3. *Cell specific expression of rhopr-SK transcript in the CNS*

Using peroxidase-based tyramide signal amplification, FISH was performed on 5th instar *R. prolixus* CNS to determine the cell-specific expression for *Rhopr-SK* (Fig. 4A). On the dorsal side of the brain, *Rhopr-SK* transcript expression was observed in 13 bilaterally-paired neurons, 8 of which were medial, 3 posterior, and 2 lateral (Fig. 4B). No neurons expressing the *Rhopr-SK* transcript were observed in the rest of the CNS. The sense probe was used as a control in order to verify the specificity of the antisense RNA probe in binding to the Rhopr-SK mRNA. No staining was observed in CNS tissues hybridized with the control (sense) DIG-labelled Rhopr-SK RNA probe.

3.4. *Sulfakinin-like immunoreactivity*

Immunoreactivity was observed in cells and processes throughout the CNS of males and females, as well as processes over the posterior midgut and anterior hindgut (Fig. 5). Immunoreactive cell bodies were observed in the brain, SOG, as well as the MTGM. There are multiple neuropeptide families that terminate with RFamide, and so the antiserum utilized may recognise multiple peptides. The *C*-terminal hexapeptide –Y(SO_3H)GHMRFamide, which is recognized by the antiserum used in this study, is identical in almost all insect SKs; however, cross-reactivity with other peptides sharing similar *C*-terminal sequences is a possibility, essentially the other RFamide families [10]. Consequently, as a way to improve specificity, the SK antiserum was preabsorbed with a *R. prolixus* extended FMRFamide (Figs. 5 and 6B). Here, immunostaining was eliminated from all neurons except those in the brain, matching the neurons that stained positively using *in situ* hybridization (Figs. 5 and 6). Two ventral axons, containing SK-like immunoreactivity, extended from the brain throughout the entire CNS to the MTGM (Figs. 5A–C and 6B). No sulfakinin-like immunoreactive processes were found

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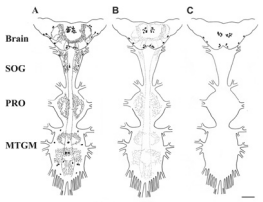

Fig. 6. Diagrammatic representations of cells and processes in 5th instar *Rhodnius prolixus* central nervous system (CNS) via (A) immunohistochemical staining using an SK antiserum, (B) immunohistochemical staining using an SK antiserum preabsorbed with FMRFamide (GNDNFMRFamide), and (C) fluorescent *in situ* hybridization of the cells expressing the *Rhopr-SK* transcript. The BR (Brain), SOG (suboesophageal ganglion), PRO (prothoracic ganglion), and MTGM (mesothoracic ganglionic mass) are displayed. Scale bar: 250 μm.

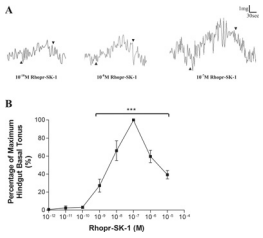

Fig. 7. (A) Rhopr-SK-1 increases basal tension of *R. prolixus* hindgut; Rhopr-SK-1 was applied at the upward arrowheads and saline washes were started at the downward arrowheads. (B) Dose-response curve of Rhopr-SK-1–induced tension (mg) on hindgut contraction. A statistically significant increase in basal tonus is observed at 10^{-9} M to 10^{-5} M (two way ANOVA, $P<0.0001$; Bonferroni's post-test, $P<0.001$). Data points are mean ± standard error of the mean for 5 replicates.

on the dorsal vessel, salivary glands, or adult male and female reproductive systems. Pre-absorbing the antiserum with Rhopr-SK-1 eliminated all staining (not shown).

3.5. Effects of rhopr-SK-1 on hindgut muscle contraction and heartbeat frequency

Rhopr-SK-1 increased both the frequency of spontaneous contractions and basal tension in hindguts of 5th instars in a dose-dependent manner with a threshold between 10^{-10} M and 10^{-9} M (Fig. 7). The contractions observed following the addition of Rhopr-SK-1 at 10^{-9} M to 10^{-5} M were significant in relation to the ones observed in physiological saline (two way ANOVA, $P<0.0001$; Bonferroni's post-test, $P<0.001$). The maximal change in basal tension (3.5 mg) was observed at 10^{-7} M Rhopr-SK-1 with desensitization occurring at concentrations equal to 10^{-6} M and greater (Fig. 7B). Physiological saline washes effectively reversed the effects of Rhopr-SK-1. Rhopr-FMRFamide also led to a dose-dependent increase in basal tonus of the hindgut, with the maximal tension being observed at 10^{-7} M, which was approximately equivalent to 10 mg of force and approximately three-times higher in magnitude than the tension caused by Rhopr-SK-1 (two way ANOVA, $P<0.0001$; Bonferroni's post-test, $P<0.001$) (Figs. 8 and 9). Overall, when compared to Rhopr-FMRFamide, the effect of Rhopr-SK-1 on hindgut contractions was significantly lower at all concentrations (Fig. 8). A second extended-FMRFamide was also tested on this preparation and the magnitude of the tensions observed for Rhopr-FIRFamide at 10^{-7} M were also significantly higher than that observed for Rhopr-SK-1 (two way ANOVA, $P<0.0001$; Bonferroni's post-test, $P<0.05$) (Figs. 8 and 9). As previously shown by Bhatt et al. [4], the addition of Rhopr-Kinin 2 dramatically increased the basal tension of the hindgut. The increase in basal tonus at 10^{-7} M for Rhopr-Kinin 2 was also significantly higher than that of Rhopr-SK-1 (two way ANOVA, $P<0.0001$; Bonferroni's post-test, $P<0.001$)

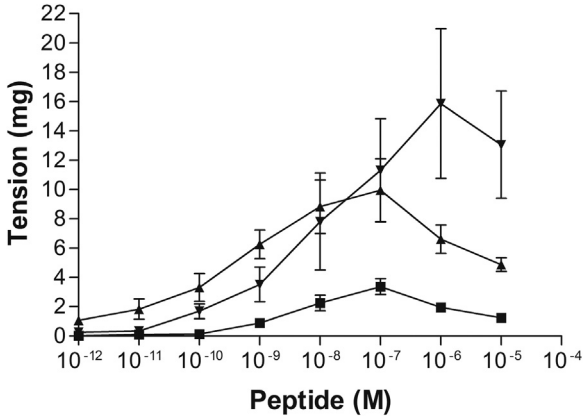

Fig. 8. Dose-response curve of Rhopr-SK-1 (pQFNEY(SO_3H)GHMRFamide) (■), Rhopr-FMRFamide (GNDNFMRFamide) (▲), and Rhopr-FIRFamide (AKDNFIRFamide) (▼) –induced tensions (mg) in relation to concentration (M). Data points are mean ± standard error of the mean for 5 replicates (Rhopr-SK-1), 4 replicates (Rhopr-FMRFamide), and 8 replicates (Rhopr-FIRFamide).

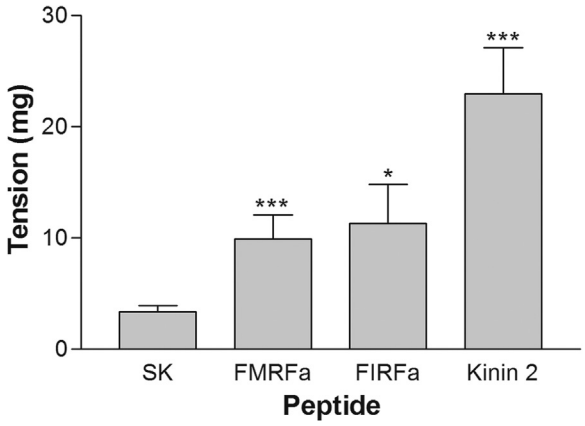

Fig. 9. Tension (mg) induced by 10^{-7} M Rhopr-SK-1 (pQFNEY(SO_3H)GHMRFamide), Rhopr-FMRFamide (GNDNFMRFamide), Rhopr-FIRFamide (AKDNFIRFamide), and Rhopr-Kinin 2 (AKFSSWGamide). Tensions observed for Rhopr-SK-1 at 10^{-7} M were significantly lower than those observed for Rhopr-FMRFamide (two way ANOVA, $P<0.0001$; Bonferroni's post-test, $P<0.001$) (***), Rhopr-FIRFamide (two way ANOVA, $P<0.0001$; Bonferroni's post-test, $P<0.05$) (*), or Rhopr-Kinin 2 (two way ANOVA, $P<0.0001$; Bonferroni's post-test, $P<0.001$) (***). Data points are mean ± standard error of the mean for 5 replicates.

(Fig. 9). Heartbeat frequency was not significantly altered by Rhopr-SK-1 (one way ANOVA, $P=0.44$) (Fig. 10).

3.6. Effect of Rhopr-SK-1 on feeding

Injection of Rhopr-SK-1 24 h prior to feeding did not alter the time spent feeding, approx. 20 min, but significantly decreased the overall weight of the blood meal consumed by 5th instars, when compared to control insects injected with saline (two way ANOVA, $P<0.0001$; Bonferroni's post-test, $P<0.01$). Diuresis began at the end of feeding and the rate of diuresis was higher in *R. prolixus* injected with saline over the first two hours post-feeding when compared to animals injected with Rhopr-SK-1; however, this difference was eliminated by 3 h post-feeding (Fig. 11).

4. Discussion

The complete *Rhopr-SK* cDNA sequence from *R. prolixus* was isolated and cloned, matching the previously identified SK mRNA sequence (GQ162784) of Ons et al. [31]. The 104 amino acid Rhopr-SK prepropeptide contains 2 predicted SKs (Rhopr-SK-1 and

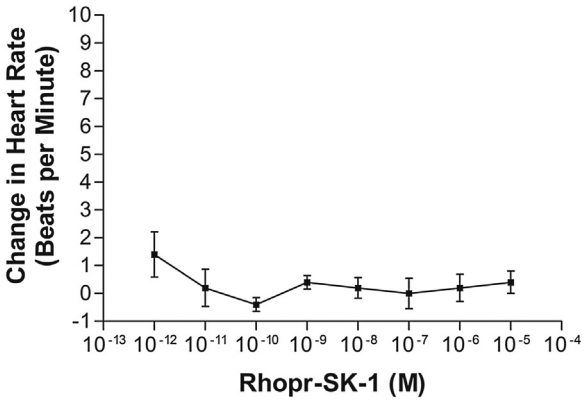

Fig. 10. Effects of Rhopr-SK-1 on heartbeat rate (beats per minute) in 5th instar *R. prolixus*. Change in heartbeat rate is calculated as the difference in the heartbeat frequency (over 60 s) in saline and following peptide addition. Heartbeat frequency was not significantly altered by Rhopr-SK-1 (one way ANOVA, $P = 0.44$). Data points are mean ± standard error of the mean for 5 replicates.

Rhopr-SK-2). Both of these peptides have been *de novo* sequenced using MS/MS from *R. prolixus* [31] although it is not clear as to whether they exist in the sulfated or non-sulfated forms or both. In our study, we used the sulfated form of Rhopr-SK-1 since this was the first form identified in insects and hence the family was given the name sulfakinins [26,27]. SKs are highly conserved in arthropods, sharing the carboxyl-terminal amino acid sequence YGHMRFamide [38], with a unique variation in Rhopr-SK-2, whereby the histidine is replaced with a tyrosyl residue [31]. Whether this tyrosyl is sulfated or not still remains to be studied.

Expression of *Rhopr-SK* transcript was found in neurons of the brain of male and female insects. Previous studies have shown the presence of the SK transcript in the brains of the beetle *T. castaneum*, as well as the cricket *G. bimaculatus* [24,44]. In *T. castaneum,* SKs have been shown to be expressed in all developmental stages, mainly in the head, with significantly lower expression in the gut region [44].

To localize the SK transcript within the CNS, FISH was performed. The results were consistent with the RT-qPCR analysis, showing SK expression in 13 bilaterally-paired neurons in the brain. Sulfakinin-like immunoreactivity (as revealed by preabsorption of the antiserum with Rhopr-FMRFamide) was also found to stain 13 pairs of neurons in the brain along with axonal processes projecting posteriorly throughout the ventral nerve cord. Processes were also stained over the posterior midgut and the anterior hindgut. The number of SK-positive neurons in the brain varies considerably between insect species. For example, in *C. vomitoria*, SK-like immunoreactivity was only observed in four bilaterally-paired neurons in the brain, and in a similar manner to *R. prolixus,* the SK-positive neurons in the brain of *C. vomitoria* project axons into the thoracic and abdominal ganglia [10,11]. In these studies, the SK antiserum was preabsorbed with an extended FMRFamide and the resulting immunolabeled pattern also matched that obtained via *in situ* hybridization. Similar numbers of immunoreactive neurons were seen in *Helicoverpa armigera*; however, in *Periplaneta americana,* thirteen pairs of brain neurons expressing SK-like immunoreactivity were found, whilst only two pairs of neurons were observed in *Teleogryllus commodus* [12]. Downer et al. [7] found four pairs of brain neurons in *P. regina*, which they considered to be specific for SKs. Similar staining in the brain was also observed in the fruitfly *D. melanogaster* and the prawn *Penaeus monodon* [17,30]. Despite the differences observed in the number of immunoreactive neurons, their distribution throughout the medial and posterior brain regions are similar [10–12]. Immunoreactivity has also been observed in midgut endocrine cells of the cockroach *B. germanica* and the mosquito *Aedes aegypti*, but not in the *D. melanogaster* midgut [21,41,42]. While SK-like immunoreactive neurons were observed in the CNS of the cricket *T. commodus* and the cockroach *P. americana*, no SK-like immunoreactivity was observed in the midguts or hindguts of either species [12]. No SK-like immunostaining was observed in endocrine cells in the midgut of *R. prolixus*.

Gut contractions are important for mixing the ingested food with enzymes, and have also been suggested to play a role in reducing the unstirred layers, as well as increasing hormone circulation

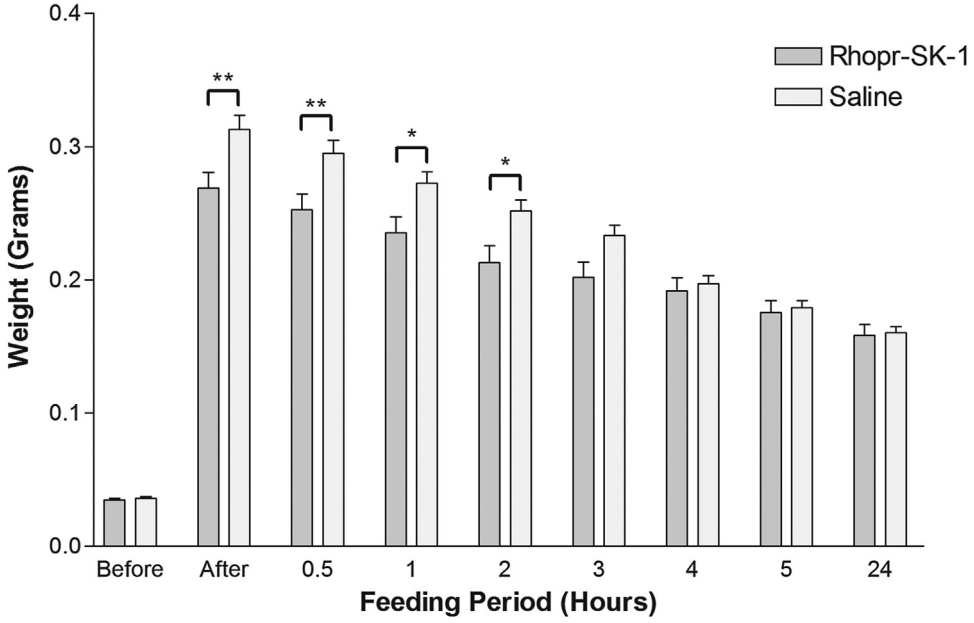

Fig. 11. Post-feeding weight of 25 5th instar *R.* prolixus previously injected (24 h earlier) with either Rhopr-SK-1 (1 μl of 10^{-4} M) or saline (1 μl). Post-feeding weights were significantly lower in animals injected with Rhopr-SK-1 in comparison to saline after feeding and 30 min post-feeding (two way ANOVA, $P < 0.0001$; Bonferroni's post-test, $P < 0.01$) (**), as well as one and two hours post-feeding (two way ANOVA, $P < 0.0001$; Bonferroni's post-test, $P < 0.05$) (*). Histograms are mean + standard error of the mean for 25 insects.

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within the haemolymph [33,37]. SKs (sulfated form) were originally identified in *Leucophaea maderae* based on their ability to stimulate hindgut contractions [26,27]. Similar effects were found in *Zophobas atratus* and *Locusta migratoria* [22,40]; however, no effects on hindgut contractions were observed in *P. regina* or *C. vomitoria* [11,16]. In *R. prolixus*, SKs were postulated to play a role in myomodulation of the gut since SK-like immunoreactive processes are distributed over the posterior midgut and anterior hindgut. The frequency of *R. prolixus* hindgut contractions was increased following the addition of Rhopr-SK-1, with receptor desensitization occurring at concentrations equal to 10^{-6} M or higher. The magnitude of the effect, however, is quite weak when compared to other peptides from *R. prolixus* including Rhopr-FMRFamide, Rhopr-FIRFamide, and Rhopr-Kinin 2. The data do show the potential for Rhopr-SK-1 to influence gut contractions, although the data also revealed the inability of Rhopr-SK-1 to influence heartbeat rate.

SKs have been suggested to act as satiety factors in insects [7,26,27]. SK injections inhibited food intake in *S. gregaria* and *B. germanica* [21,43]. Similar effects were also observed in *P. regina*, whereby flies injected with SK had a reduced food intake of up to 44% [7]. In *R. prolixus*, the weight of the blood meal gorged was significantly reduced in insects previously injected with Rhopr-SK-1 in comparison to saline-injected insects. RNA interference has also linked SK with the regulation of food intake in *G. bimaculatus* [23]. Adult and last instar larval food intake was stimulated by the systemic silencing of the SK gene via injection of SK double stranded RNA [23]. The SK-signalling pathway appears to play a vital physiological role in regulating the intake of food in *R. prolixus*, as well as in other insects. The rate of diuresis in saline injected *R. prolixus* was greater than in Rhopr-SK-1 injected insects for two hours following feeding, probably because of the larger meal consumed. At three hours, however, the post-feeding weights were not different. SKs are not themselves associated with diuresis in *R. prolixus*.

Aguilar et al. [2] compared the effects on feeding of perisulfakinin (PSK) and leucomyosuppressin (LMS) in *B. germanica*. Both peptides inhibited food intake, although LMS is myoinhibitory on gut contractions and PSK is myostimulatory. LMS led to the accumulation of food in the foregut and a decrease in the hindgut, whilst no differences in food accumulation were observed in animals injected with PSK. As a result, the authors postulated that PSK and LMS inhibit feeding via different mechanisms. LMS inhibits feeding due to the persistence of signals from gut stretch receptors, whilst PSK leads to satiety via primarily acting on the CNS.

It is likely that a variety of neuropeptides, including SK, act additively alongside mechanisms such as neural feedback from abdominal stretch receptors in order to produce satiation in insects [5]. Although SK has been shown to have a myostimulatory effect on hindgut contractions, as well as decrease the weight of the blood meal during gorging, is it unlikely that this family of neuropeptides acts alone to induce satiety in *R. prolixus*.

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