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Brain-midgut short neuropeptide F mechanism that inhibits digestive activity of the American cockroach, *Periplaneta americana* upon starvation

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ABSTRACT

Immunohistochemical reactivity against short neuropeptide F (sNPF) was observed in the brain-corpus cardiacum and midgut paraneurons of the American cockroach, *Periplaneta americana*. Four weeks of starvation increased the number of sNPF-ir cells in the midgut epithelium but the refeeding decreased the number in 3 h. Dramatic rises in sNPF contents in the midgut epithelium and hemolymph of roaches starved for 4 weeks were confirmed by ELISA. Starvation for 4 weeks reduced α-amylase, protease and lipase activities in the midgut of *P. americana* but refeeding restored these to high levels. Co-incubation of dissected midgut with sNPF at physiological concentrations inhibited α-amylase, protease and lipase activities. sNPF injection into the hemocoel led to a decrease in α-amylase, protease and lipase activities, whereas PBS injection had no effects. The injection of D-(+)-trehalose and L-proline into the hemocoel of decapitated adult male cockroaches that had been starved for 4 weeks had no effect on these digestive enzymes. However, injection into the hemocoel of head-intact starved cockroaches stimulated digestive activity. Injection of D-(+)-trehalose and L-proline into the lumen of decapitated cockroaches that had been starved for 4 weeks increased enzymes activities and suppressed sNPF in the midgut. Our data indicate that sNPF from the midgut paraneurons suppresses α-amylase, protease and lipase activities during starvation. Injection of D-(+)-trehalose/L-proline into the hemocoel of head-intact starved cockroach decreased the hemolymph sNPF content, which suggests that sNPF could be one of the brain factors, demonstrating brain-midgut interplay in the regulation of digestive activities and possibly nutrition-associated behavioral modifications.

1. Introduction

Multicellular organisms have methods of communication among cells and tissues that integrate physiological functions. The nervous system contains neurons that coordinate the physiology of an animal and transmit signals between different parts of the body. Peptides belonging to the neuropeptide Y family function not only as neurotransmitters but could also be released as hormones, such as pancreatic polypeptides from the pancreas in mammals [27].

In vertebrates, neuropeptide Y (NPY) regulates food consumption, circadian rhythms, and other physiological processes. In invertebrates, neuropeptide F (NPF) shares structural similarity with vertebrate NPY [10]. In *D. melanogaster*, in addition to the 36 amino acid NPF (long NPF), the genome also contains a gene encoding four shorter peptides (6–11 amino acids) that are designated as short NPFs [30].

Analysis of the sequences of the peptides and prepropeptides suggest that NPFs and sNPF are not closely related. Experimental data suggest a role in *Drosophila* in feeding, regulation of insulin signaling, metabolic homeostasis and in modulation of locomotor control circuits in the central complex. Based on sNPF distribution in *Drosophila*, it may play additional roles in the biological clock, in learning and memory and in modulation of chemosensory inputs to the antennal lobe. It can be suggested that sNPF is multifunctional and may be employed as a contransmitter in numerous small neurons operating with fast neurotransmitters. In moth *Bombyx* and probably in *Drosophila* sNPF is likely to regulate hormone release [17].

Short NPFs (sNPFs) regulate physiological processes in insects such as ovarian development. Administration of sNPF to *Locusta migratoria* stimulates ovarian development [5].

Starvation exerts fatal stress on animals and affects various aspects of life such as behavior, development, reproduction and metabolism [18,19]. Starvation for 10 days reduced α-amylase and protease activities by about 60% and 67% in the midgut of *Gryllus rubens*, respectively. Starvation reduced lipase activity in a manner similar to its effect upon α-amylase and protease in the midgut [28].

In this study, we identified sNPF as a novel regulatory factor of the midgut functions in the cockroach, *Periplaneta americana*,

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Table 1
Some insect sNPF sequences.

Species	Sequence	Reference
Periplaneta americana	-ANRSPSLRLRF	Veenstra and Lambrou [32]
Locusta migratoria	-SNRSPSLRLRF	Clyen et al. [3]
Leptinotarsa decemlineata	–ARGPQLRLRF	Spittaels (1996)[34]
Drosophila melanogaster	-AQRSPSLRLRF	Vanden Broeck [30]
Nezara viridula	FAPRSPQLRLRF	Predel et al. (2008)[35]
Banasa dimiata	FAPRSPQLRLRF	Predel et al. (2008)[35]
Acrosternum hilare	FAPRSPQLRLRF	Predel et al. (2008)[35]
Euschistus servus	FAPRSPQLRLRF	Predel et al. (2008)[35]

focusing on its inhibition of digestive enzyme (α-amylase, protease and lipase) activities.

2. Material and methods

2.1. Insect

A culture of the American cockroach, *P. americana*, has been maintained in the laboratory for more than 30 years, at 25 °C under a light/dark cycle of 12:12 h, fed with an artificial diet (MF; ORIENTAL YEAST, Tokyo, Japan) and water ad libitum. Adult males at 3–5 days after emergence were transferred to clear plastic cups (10.0 cm diameter, 4.5 cm high) containing a bottle filled with water and kept individually for 0, 1, 2 and 4 weeks without food. After 4 weeks of starvation, they were refed for 3 h. The midgut from each cockroach was collected for subsequent experiments. Cockroaches were anesthetized by cooling on ice before dissection.

2.2. Antigen

Automated Edman degradation revealed the following sequence for the C-terminal of the peptide in *P. americana*: Ala-Asn-Arg-Ser-Pro-Ser-Leu-Arg-Leu-Arg-Phe [32]. Strong homology between this peptide and sNPF-like sequences has been identified in other insects (Table 1) [3]. We used this peptide with KLH conjugation for antibody production in two rabbits (Genemed Synthesis, Inc., USA).

2.3. Specificity of the primary antibody

For control, the antiserum was replaced with normal serum. Its specificity was further confirmed by a preabsorption test for immunohistochemistry (IHC). The antiserum was added to synthetic antigen (1 μg/mL) in dilution buffer, and incubated overnight at 4 °C before use [7]. Neither normal nor preabsorbed serum showed immunoreactivity.

2.4. Immunohistochemistry

The midgut dissected from anesthetized young adult males in cool phosphate-buffered saline (PBS; 145 mM NaCl, 1.45 mM NaH_2PO_4, 8.55 mM Na_2HPO_4, pH 7.5) was fixed at 4 °C overnight in Bouin solution (15 vol. picric acid, 5 vol. formalin, 1 vol. acetic acid). Standard histochemical techniques were employed to prepare tissue sections (8 μm) in paraffin after dehydration [23,24]. Following dewaxing and rehydration, sections were rinsed in Tris-buffered saline (TBS; 135 mM NaCl, 2.6 mM KCl, 25 mM Tris–HCl, pH 7.6) for 5 min and then 1.5% normal goat serum diluted in TBS for 30 min at room temperature was overlayed to block non-specific binding sites. Thereafter, they were incubated with primary antibody diluted with blocking serum (1:2000) in a humidified chamber overnight at 4 °C. After the slides were rinsed with TBS, 3 times for 10 min each, biotinylated anti-rabbit IgG was diluted with blocking serum (1:200), which was applied for 1.5 h at room temperature. They were rinsed with TBS (10 min × 3) and incubated for 30 min with VECTASTAIN ABC reagent (Vectastain ABC KIT PK-6101). After three final rinses with TBS (10 min each time) and once with 0.1 M Tris–HCl, pH 7.5, for 10 min, the sections developed a brown color in 144 mL of diaminobenzidine tetrahydrochloride (DAB) solution (DAB; 0.25 mM in 0.1 M Tris–HCl, pH 7.5, 144 mL, 30% H_2O_2, 30 μL) for 10 min. Finally, the slides were dehydrated through an alcohol-xylene series, mounted in Bioleit mounting medium (Kouken Rika, Osaka, Japan) and observed using a BX50F4 microscope (Olympus, Tokyo, Japan).

2.5. Morphometric analysis

The immunoreactive cells were assessed using the point counting method [13]. Seven sections of the midgut from each cockroach were randomly selected from 50 sections. A grid lattice of 1000 (25 × 40) points was put on the image of immunostained midgut sections and then the number of points covering sNPF-ir cells and total number of points covering the epithelium of the part within the quadrats were counted. Finally the number of sNPF-ir cells per 100 points of covering the midgut epithelium was counted [22].

2.6. sNPF injection into the hemolymph

Synthetic sNPF was purchased from Invitrogen (California, USA). Different amounts of sNPF in 10 μL of PBS were injected using a Hamilton syringe (Hamilton Company, Nevada, USA) into each animal 3 h before dissection. The puncture made by injection was sealed with an instant adhesive, Aron-alpha (TOAGOSEI Co. Ltd., Tokyo, Japan).

2.7. D-(+)-Trehalose and L-proline injection into the hemolymph

The head of each adult male cockroach that has been starved for 4 weeks was cut off after the neck ligature and different amounts of D-(+)-trehalose (Sigma–Aldrich, USA) and L-proline (Sigma–Aldrich, USA) in 10 μL of PBS were injected using a Hamilton syringe (Hamilton Company, Nevada, USA) into each cockroach in the hemocoel, 3 h before dissection. The same experiment was conducted for adult male cockroaches with head-intact that had been starved for 4 weeks.

2.8. D-(+)-Trehalose and L-proline injection into the lumen

Different amounts of D-(+)-trehalose (Sigma–Aldrich, USA) and L-proline (Sigma–Aldrich, USA) in 100 μL of PBS were injected using a syringe (Terumo, Tokyo, Japan) into the lumen of each decapitated adult male cockroach that had been starved for 4 weeks, 3 h before dissection. A small amount (15 mg/L) of phenol red (Wako Pure Chemical Industries, Osaka, Japan) was added to injected solution to have pink – red color to recognize the syringe reached to the midgut.

2.9. Measurement of lipase, α-amylase, maltase and protease activities

Lipase activity of the cockroach midgut was measured using a lipase measuring kit (Quantichrom™ Lipase Assay Kit, BioAssay System, USA). The midgut was dissected in PBS and food particles were removed. It was incubated individually in cold PBS for 30 min in the presence or absence of sNPF. Lipase activity released into the medium was quantified. For preparing working reagent, 5 mg of color reagent was mixed in 140 μL of assay buffer and 8 μL of dimercaptopropanol tributyrate (BALB) reagent. The working reagent should be prepared freshly and used within 1 h. 140 μL of working reagent was added to 10 μL of each sample. The assay was based