

Brain-midgut cross-talk and autocrine metabolastat via the sNPF/CCAP negative feed-back loop in the American cockroach, *Periplaneta americana*

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Abstract Immunohistochemical reactivities against short neuropeptide F (sNPF-ir) and crustacean cardioactive peptide (CCAP-ir) were detected in both the brain–subesophageal ganglion (Br-SOG) and midgut epithelial cells of the male American cockroach, *Periplaneta americana*. Four weeks of starvation increased the number of sNPF-ir cells and decreased the CCAP-ir cells in the Br-SOG, whereas refeeding reversed these effects. The contents of sNPF in the Br-SOG, midgut and hemolymph titer decreased in response to an injection of CCAP into the hemocoel of normally fed male cockroaches, while CCAP titers/contents decreased in response to an injection of sNPF. The results of a double-labeling experiment demonstrated that sNPF-ir co-existed in CCAP-ir cells in the pars intercerebralis (PI), dorsolateral region of protocerebrum (DL), deutocerebrum (De) and SOG. sNPF-ir and CCAP-ir were also colocalized in the midgut. sNPF and CCAP are neuropeptides and midgut factors that interact with each other. Since the two peptides are known to be secreted by identical cells that affect each other, this constitutes autocrine negative feedback regulation for a quick response to food accessibility/inaccessibility. These peptides not only constitute the switch in the digestive mechanism but

also couple digestive adaptation with behavior. A CCAP injection suppressed locomotor activity when cockroaches were starved, whereas sNPF activated it when they were fed.

Keywords Short neuropeptide F · Crustacean cardioactive peptide · Locomotor activity · Metabolastat · *Periplaneta americana*

Introduction

Feeding is most efficient when coupled with upregulated digestive activity. Several neuropeptides such as cholecystokinin, orexin, and neuropeptide Y (NPY) are involved in the regulation of feeding behavior in mammals (Strand 1999). Short neuropeptide F (sNPF) has been identified as a counterpart to mammalian NPY in invertebrates (Chen and Pietrantonio 2006). Several sNPFs and NPFs are known to be present in the central nervous system (CNS) of *Drosophila* and regulate various physiological functions such as clock, feeding, learning/memory and intestinal functions. The functions of these peptides were found to be similar to those of vertebrate NPYs (Nässel and Wegener 2011). In the silk worm, *Bombyx*, it has been suggested that sNPF regulates hormone release (Yamanaka et al. 2008). This may also be the case in the locust, in which an injection of sNPF may have indirectly affected the synthesis of vitellogenin via juvenile hormone (JH) synthesis (Schoofs et al. 2001). In *B. mori*, sNPF has been shown to regulate JH synthesis along with allatotropin (Nagata et al. 2012). sNPF has also been implicated in the regulation of feeding behavior in *D. melanogaster* (Garczynski et al. 2006). A large number of neurons in the *D. melanogaster* CNS as well as “endocrine cells” or “paraneurons” in the larval anterior midgut have been shown to express sNPF. This diverse population of sNPF-expressing

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neurons and paraneurons suggests that sNPF may serve multiple functions as a neuropeptide and midgut factor that is released into the circulation (Garczynski et al. 2006). We recently demonstrated that sNPF functioned as both a neuropeptide and midgut factor to suppress α -amylase, protease and lipase activities during starvation in American cockroach *Periplaneta americana*. Our findings revealed an intricate interplay between the brain and midgut to regulate digestive activities (Mikani et al. 2012; Sakai et al. 2006).

CCAP was originally isolated from neurohemal structures and pericardial organs of the shore crab *Carcinus maenas* (Stagier et al. 1987). To date, this peptide has been identified in the neuro-endocrinal system of *P. americana* (Sakai et al. 2004), *Tenebrio molitor* (Wasielewski and Skonieczna 2008) and several other insects such as *D. melanogaster*, *Locusta migratoria*, and *Manduca sexta* (Loi et al. 2001). It has been shown to modulate oviduct contractions in *L. migratoria* (Wasielewski and Skonieczna 2008). In adult *M. sexta*, cells that exhibited immunohistochemical reactivity against CCAP were observed in the brain and acted in an excitatory manner in the heart during adult wing inflation and adult flight. In larvae, it has been suggested that CCAP increases the frequency of gut contractions (Loi et al. 2001), as has been demonstrated in adult *P. americana* (Sakai et al. 2004).

In *P. americana*, CCAP has been shown to not only regulate autonomous contractions of the fore-, mid-, and hindgut but also to up-regulate α -amylase and protease activities in the midgut when these insects were fed. CCAP was detected not only in the midgut but also in the Br-SOG and stomatogastric nervous system, therefore suggesting a multifunctional peptide (Sakai et al. 2006). Removal or bisectomy of the pars intercerebralis (PIC) was found to up-regulate CCAP-ir in the midgut epithelium as well as the stomatogastric nervous system, implying brain–midgut cross-talk (Matsui et al. 2009). Our previous findings demonstrated that, although sNPF and CCAP regulated the same function, their direction of regulation was in opposition (Sakai et al. 2006; Mikani et al. 2012).

Materials and methods

Animals

Laboratory cultures of *P. americana* were maintained at 25 °C under a light:dark regimen of 12:12 h. Each nymph was kept individually in a clear plastic cup (10.0 cm diameter, 4.5 cm in height) with an artificial diet (MF; Oriental Yeast, Tokyo, Japan) and water ad libitum. By the time they became adult roaches and were equipped with wings, they were kept for a further 5 days, then experimental animals were kept for 4 weeks more, individually and without food but with water, while control animals were still fed with food and water. After 4 weeks of starvation, the experimental animals were re-fed

for 3 h, then both they and the normally fed cockroaches were injected with either sNPF or CCAP, 3 h before being dissected. The brain–subesophageal ganglion complex (Br-SOG) was dissected in phosphate-buffered saline (PBS, pH 7.4) at 4 °C under CO₂ anesthesia (Park et al. 2009).

Antigen

Automated Edman degradation revealed the following sequence for the C-terminal of putative cockroach sNPF in *P. americana*: Ala-Asn-Arg-Ser-Pro-Ser-Leu-Arg-Leu-Arg-Phe (Veenstra and Lambrou 1995). Strong homology between this peptide and other sNPF-like sequences has been identified in other insects (Mikani et al. 2012). We conjugated peptide with KLH to produce antibodies in two rabbits (Genemed Synthesis, San Antonio, TX, USA).

Chromatographic isolation revealed that the primary structure of the CCAP in *P. americana* was PFCNAFTGC (Sakai et al. 2004). We produced an antibody against this sequence as described above.

Specificity of the primary antibodies

The antisera (anti-sNPF and anti-CCAP) were replaced with normal serum for the control. Their specificity were further confirmed by a preabsorption test for immunohistochemistry (IHC). Each antiserum was added to the synthetic antigen (1 μ g/ml) in dilution buffer, and incubated overnight at 4 °C before use (Fouda et al. 2010). Neither normal nor preabsorbed serum showed immunoreactivity.

Immunohistochemistry

Cockroaches were anesthetized by cooling on ice before being dissected. The Br-SOG was dissected out from adult male *P. americana* in ice-cold phosphate-buffered saline (PBS; 145 mM NaCl, 1.45 mM NaH₂PO₄, 8.55 mM Na₂HPO₄, pH 7.5) and fixed in Bouin's solution (15 vol. picric acid, 5 vol. formalin, 1 vol. acetic acid) at 4 °C overnight. Standard histochemical techniques were employed for tissue dehydration, embedding in paraffin, sectioning (8 μ m), deparaffinization, and rehydration as described by Shao et al. (2006). The sections were blocked with 1.5 % normal goat serum diluted in Tris-buffered saline (TBS; 135 mM NaCl, 2.6 mM KCl, 25 mM Tris-HCl, pH 7.6) for 30 min at room temperature (RT). The sections were then incubated overnight with the primary antibody diluted with the blocking serum (sNPF 1:2000 and CCAP 1:1500) 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 diluted with blocking serum (1:200) was applied for 1.5 h at room temperature. The sections were rinsed with TBS (10 min \times 3) and incubated for 30 min with Vectastain ABC reagent (Vectastain ABC KIT PK-6101). After 3 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 % H₂O₂, 30 µL) for 10 min. The sections were dehydrated through an ethanol–xylene series and then mounted in Bioleit medium (Kouken Rika, Osaka, Japan).

Immunoreactivity was visualized under a BX50F4 microscope (Olympus, Tokyo, Japan). A double-labeling experiment was performed according to the method of Shao et al. (2010). This experiment employed antibodies derived from the same animal (rabbit), and was conducted as follows. The sNPF antibody was diluted (1:2000), and sections were incubated overnight at 4 °C with the antibody. After rinsing with PBS (5 min × 3), the slides were incubated with Cy3-conjugated (red) goat anti-rabbit IgG for 60 min at RT. After rinsing with PBS (5 min × 3), the sections were blocked with goat normal serum (3 % diluted with PBS). The goat polyclonal antibody to the rabbit IgG H&L-Fab fragment (Rhodamine, Abcam) was subsequently added to retrieve the extra-binding sites of the first secondary antibody. After these retrieval steps, the slides were incubated with anti-CCAP (1:1500) overnight at 4 °C. After rinsing with PBS (5 min × 3), Alexa488-conjugated (green) goat anti-rabbit IgG was added and incubated for 60 min at RT. The slides were then rinsed with PBS, and the sections mounted in Aqua Polymount and visualized using a BX50F4 microscope (Olympus).

Competitive ELISA

Competitive ELISA was performed according to the method of Sakai et al. (2006). Cockroaches were anesthetized by cooling on ice before dissection. The Br-SOG was dissected out from adult male *P. americana* in ice-cold TBS. The midgut was also dissected in ice-cold TBS and the luminal content was removed. After homogenization and centrifugation (4000g, 4 °C, 15 min), the supernatants were used to measure the contents of sNPF and CCAP using competitive ELISA. A sNPF-BSA or CCAP-BSA conjugate was prepared by coupling sNPF or CCAP to BSA with dimethyl suberimidate (Sigma-Aldrich, Switzerland). The coating of plates (96 wells) (Corning, USA) was made with antigen-BSA (0.6 µg/ml per well) in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.0) for 3 h, and plates were then blocked with 250 µL of 2 % skimmed milk for 1 h at RT for the analysis. Standard peptide solutions (0.01–100 nmol/well) or the supernatant of the Br-SOG homogenate was added in a volume of 50 µL/well. Fifty microliters of the antibody diluted in TBS with 2 % skimmed milk (sNPF 1:12000, CCAP 1:10000) was subsequently added to each well and the plate was incubated overnight at 4 °C with gentle shaking. The plates were rinsed three times with TBS containing 0.5 % Tween-20 (TBS-Tw) after incubation and were then incubated with 100 µL of the secondary

antibody solution containing goat immunoglobulin anti-rabbit IgG labeled with alkaline phosphatase at 1:1000 in TBS at RT for 1 h. After the plate was rinsed three times, 100 µL of substrate solution [1 mg/ml *p*-nitrophenylphosphate disodium salt hexahydrate (Sigma, UK) in 10 mM diethanolamine buffer (Sigma-Aldrich, USA), pH 9.5] was added to each well and incubated for 1 h at RT. The reaction was stopped by the addition of 50 µL of 4 M NaOH and the absorbance was read at 405 nm using a microplate reader (SH-9000; Corona Electric, Ibaraki, Japan).

Neuropeptide injection into the hemolymph

Synthetic sNPF and synthetic CCAP were purchased from Invitrogen (CA, U.S.A.) and Bachem (Bubendorf, Switzerland), respectively. A total of 10⁻⁷ or 10⁻⁸ moles of sNPF or CCAP in 10 µL of PBS was injected using a Hamilton syringe (Hamilton, NV, U.S.A.) 3 h before dissection into the following three groups: normally fed insects with head intact, decapitated insects, and adult males in which a hemostat (YDM, Japan) was placed between foregut and midgut. The puncture made by the injection was sealed with the instant adhesive, Aron Alpha (Toagosei, Tokyo, Japan).

Recording of locomotor activity

Locomotor activity was recorded in the monitoring chamber (plastic box of 193 × 104 × 27 mm) flanked by an infrared light emitter and detector (GT2; Takenaka Electronic Industrial, Kyoto, Japan). Adult males of 3–5 days after emergence were transferred to the monitoring chamber that contained a bottle of water (35 mm diameter × 50 mm) with a wire-meshed opening. Cockroaches were kept individually for 2 weeks without food. After 2 weeks of starvation, they were re-fed with an artificial diet for 1 week. Another group of adult males 3–5 days after emergence were transferred to the monitoring chamber containing food and water. Two weeks later, they were anaesthetized by ice after re-feeding. The cockroaches were injected either with 5 µL of different concentrations of CCAP or sNPF into the hemocoel using a Hamilton syringe. Five microliters of PBS was injected for the control.

All cockroaches were transferred back to the monitoring chamber that contained water and artificial food. When a cockroach crossed the infrared beam, a signal was fed to a computer (PC98; NEC, Tokyo, Japan) every 6 min. The locomotor rhythms of individuals were recorded at 25 °C under 12:12 L:D with a fluorescent lamp providing more than 400 lux (Muraleedharan and Prabhu 1979; Matsui et al. 2009).

Statistical analysis

Results are expressed as the mean ± SEM. Differences were accepted as significant for *p* < 0.05. Student's *t* test was used

for pair-wise comparisons between the mean contents of sNPF or CCAP in the Br-SOG of starved, re-fed or injected cockroaches with CCAP or sNPF, respectively. It also was used in pair-wise comparison of the mean contents of sNPF or CCAP in the midgut before and after the injection of CCAP or sNPF, respectively. Locomotor activity was analyzed by Student's *t* test.

Results

sNPF-ir in the Br-SOG of normally fed adult male cockroaches

sNPF-ir was detected in the anterior PI of adult male *P. americana* in four neural cells of considerable size (Fig. 1b, j). The dorsolateral region of the protocerebrum (DL) in each hemisphere contained two moderate-sized and one considerably stained cells (Fig. 1c, d). The nerve bundle projected from the cells in the optic lobe and ran anteriorly into the anterior optic commissure (AOC) of the contralateral hemisphere (Fig. 1e). Neuronal bundles originated from the pars intercerebralis (Fig. 1j) and ran into the tritocerebrum and ramified there (Fig. 1k). Varicose fibers were detected within the upper division of the central body (Fig. 1f). One considerably stained cell was present in the posterior PI (Fig. 1g). In the optic lobe (OL), two moderate-sized cells were present in the proximal frontodorsal region (Pfd), while one considerably stained cell was observed in the frontoventral parts (Pfv) (Fig. 1h). Three weakly (Fig. 1i), and one strongly stained neurons were observed in the De (Fig. 1i). Two single sNPF-ir neurons in the ventral tritocerebrum (Tr) (Fig. 1l, m) widely arborized there (Fig. 1n) and projected through the circumesophageal connective into the SOG, in which they ramified (Fig. 1p). The SOG harbored one weakly stained cell in the mandibular neuromere (Mdb) (Fig. 1n), one considerably stained cell (Fig. 1q) in the ventromedial region of the maxillary neuromere (Mxl-Vm), one strongly stained neuron in the lateral region of the maxillary neuromere (Mxl-L) (Fig. 1r) and one weakly stained neuron in the ventromedial region of the labial neuromere (Lb-Vm) (Fig. 1s). sNPF-ir in fiber arborization occurred within the corpora cardiaca (Fig. 1o).

sNPF-ir in the Br-SOG of adult male cockroaches starved for 4 weeks

The Br-SOG of adult male cockroaches starved for 4 weeks possessed an additional strongly stained perikaryon in the posterior PI (Fig. 2b), and one considerably stained (Fig. 2c), 3–6 weakly stained (Fig. 2d) and three strongly stained perikarya in the anterior PI (Fig. 2e, f). Each DL region contained an additional moderately-stained perikaryon

(Fig. 2g). The De of cockroaches starved for 4 weeks contained one additional strongly stained cell (Fig. 2h) and the Tr contained one additional moderately stained cell (Fig. 2i) compared with normally fed cockroaches. The SOG harbored 3 strongly stained neurons in the Mxl-Vm (Fig. 2j) and 4 moderately stained cells in the Lb-Vm (Fig. 2k). Starvation induced sNPF-ir cells mostly in the PI (Table 1).

sNPF-ir in the Br-SOG of adult male cockroaches re-fed after starvation

Re-feeding almost restored the staining typical for normally fed individuals (Fig. 3a). Compared with sNPF-ir in the brain-SOG of normally fed adult male cockroaches, the immunoreactivity of one strongly stained perikaryon in the anterior PI (Fig. 3b), and the immunoreactivity of two moderately stained cells in the Mxl-Vm of SOG disappeared (Fig. 3c; also see Table 1).

sNPF-ir in the Br-SOG of normally fed cockroaches in which CCAP was injected into the hemocoel

The CCAP-injection induced the disappearance of sNPF-ir neuron immunoreactivity, marked as crosses in Fig. 4a. It induced a strong effect particularly in neurons of the OL, PI, and SOG (Fig. 4, arrowheads showing missing positive immunoreactivity of neurons). Compared with sNPF-ir neurons (marked as crosses in Fig. 4a) in the Br-SOG of normally fed cockroaches, just one strongly (Fig. 4b) and one weakly stained sNPF-ir cell (Fig. 4c) were detected in the PI of cockroaches injected with CCAP. sNPF-ir was detected in the OL, but only in one weakly stained cell in the Pfd and not in the Pfv (Fig. 4d).

Immunoreactivity of the cells that disappeared after the injection of CCAP are marked as crosses in Fig. 4a.

CCAP-ir in the Br-SOG of normally fed cockroaches

Figure 5a shows a schematic diagram of CCAP-ir in the Br-SOG of normally fed cockroaches. CCAP-ir was prominently and consistently observed in one strongly and 3–4 weakly stained cells (Fig. 5b), and 5 moderately stained cells (Fig. 5e) were detected in the anterior PI. Three strongly stained cells were observed in the posterior PI (Fig. 5f). One strongly stained CCAP-ir cell was detected in the anterior PI (Fig. 5g). The DL cells of each hemisphere contained three weakly and one considerably stained cells (Fig. 5h, i, arrowheads). CCAP-ir was only detected in two weak Pfd (Fig. 5i, arrowheads) and Pfv cells (Fig. 5j). The projections of the large neurons arborized within the protocerebrum and formed a nerve bundle that ran in the DL and OL (Fig. 5d, k). One strongly stained cell was located in the dorsolateral region of

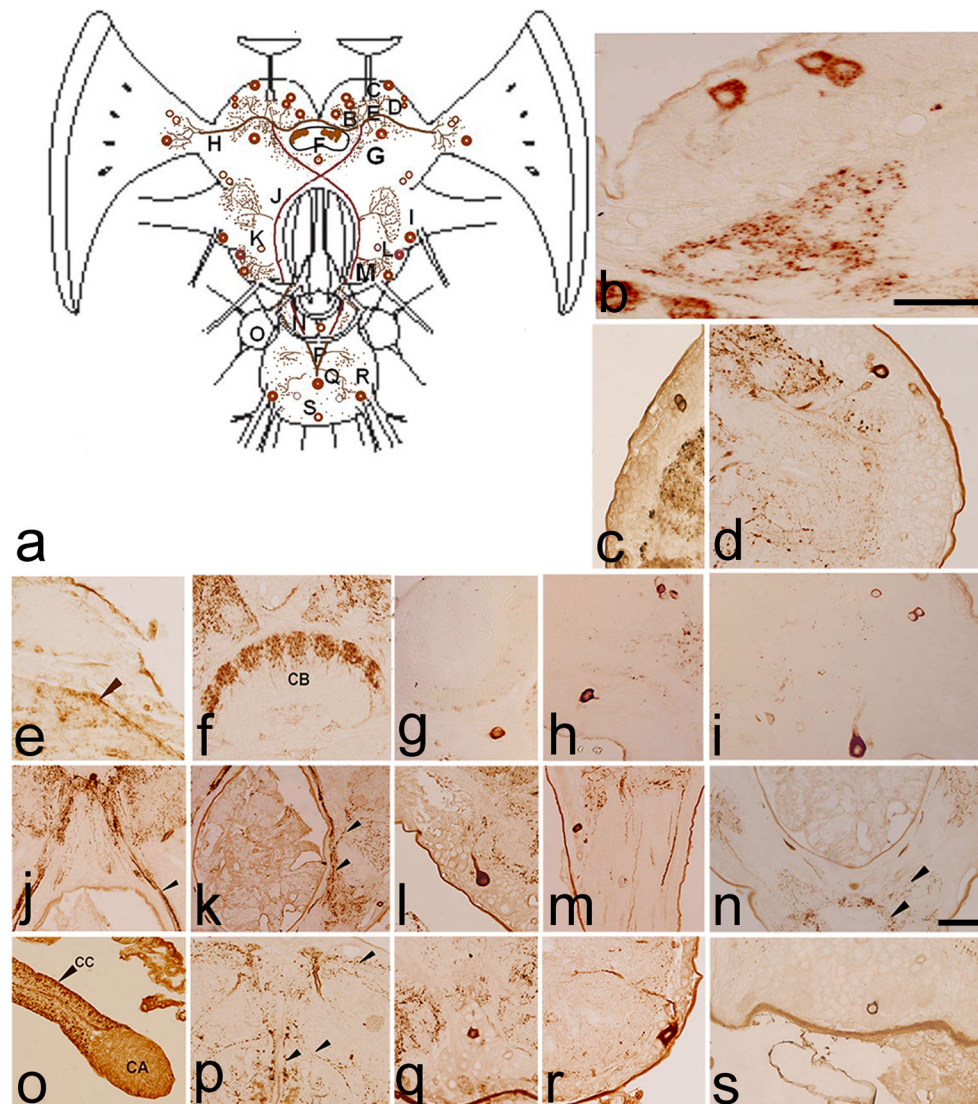


Fig. 1 sNPF-ir in the cephalic ganglia of normally fed adult male American cockroach, *Periplaneta americana*. **a** The numbers and topography of sNPF-ir cells and the pathways of their projections (**b–s** correspond to the respective micrographs). **b** Three considerably stained cells in the anterior PI. **c** Two moderately stained cells in DL. **d** One considerably stained neuron in the DL. **e** A fiber (*arrowhead*) ran anteriorly into the contralateral hemisphere. **f** Varicose fibers were detected within the upper division of the central body. **g** One considerably stained cell in the posterior PI. **h** Two moderately stained cells in Pfd and one considerably stained cell in the Pfv. **i** One strongly and three weakly stained neurons in the De. **j** One considerably stained cell in the anterior PI. **k** A varicose fiber (*arrowheads*) ran posteriorly into the De. **l** One

strongly stained neuron in the ventral tritocerebrum. **m** One moderately stained cell in the ventral tritocerebrum. **n** One weakly stained cell and an arborization (*arrowhead*) in the mandibular neuromere (Mdb). **o** Nerve bundle arborizing in the CC. **p** Arborization (*arrowhead*) projected through the circumesophageal connective into the SOG resulting in ramification (*arrowheads*). **q** One considerably stained cell in ventromedial region of the maxillary neuromere (Mxl-Vm). **r** One strongly stained neuron in the lateral region of the maxillary neuromere (Mxl-L). **s** One weakly stained neuron in the ventromedial region of the labial neuromere (Lb-Vm). *CB* central body, *CC* corpora cardiaca, *CA* corpus allatum. *Bar* 48 μ m

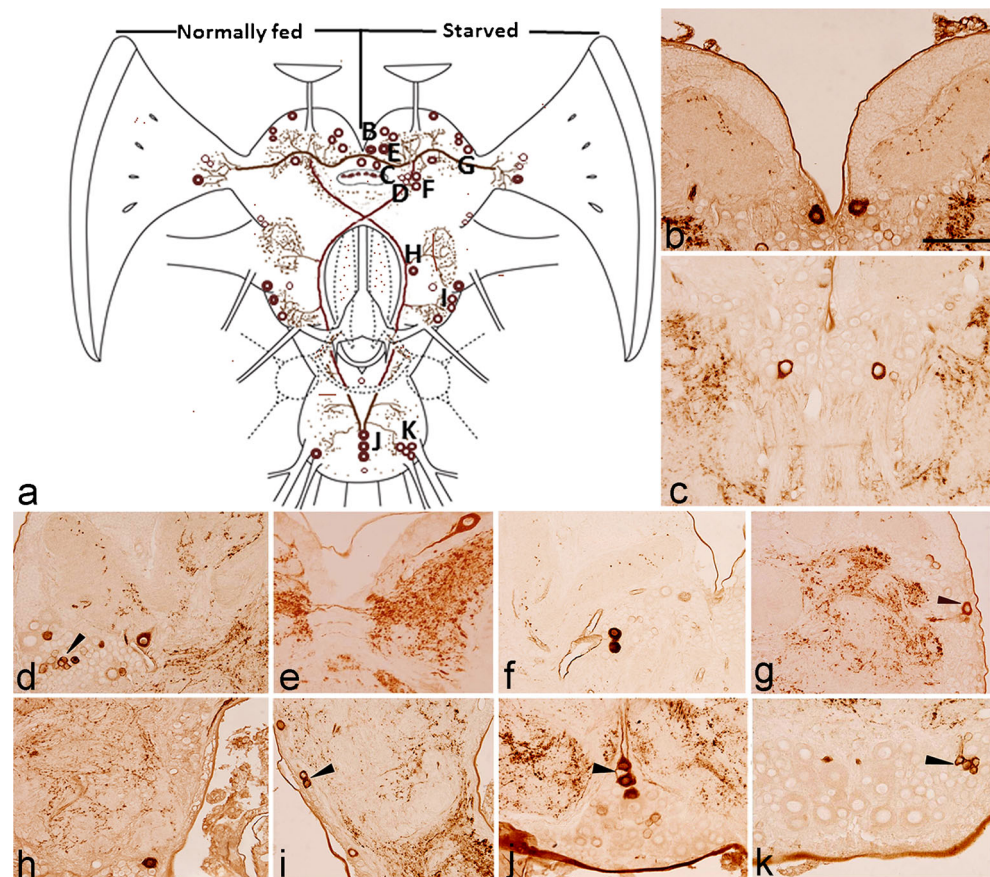
De (Fig. 5l) and one moderately stained cell was observed near the lateral region of the ventral Tr (Fig. 5m). One moderately and one strongly stained cell were observed in the Lb-Vm (Fig. 5n, *arrowhead*). One strongly stained cell was observed in the Mxl-L (Fig. 5o). Two moderately stained cells were observed in the Mdb-L (Fig. 5p). One moderately stained CCAP-ir cell was detected in the Mdb (Fig. 5q, *arrowhead*). Five moderately stained cells made a cluster in the Mxl-Vm

(Fig. 5r, *arrowhead*). CCAP-ir was detected in fiber arborization within the corpora cardiaca (Fig. 5c).

CCAP-ir in the Br-SOG of cockroaches starved for 4 weeks

All the immunoreactivity of CCAP-ir cells detected in the anterior PI of normally fed cockroaches (Fig. 5b, e, g) were

Fig. 2 sNPF-ir in the cephalic ganglia of adult male *P. americana* starved for 4 weeks. **a** The numbers and topography of sNPF-ir cells and the pathways of their projections. **b** One strongly stained perikaryon in the posterior PI. **c** One considerably stained cell in the anterior PI. **d** Three–six weakly stained neurons in the anterior PI. **e** One strongly stained perikaryon in the anterior PI. **f** Two strongly stained cells in the anterior PI. **g** One moderately stained perikaryon in the DL. **h** One strongly stained cell in the De. **i** One moderately stained cell in the Tr. **j** Three strongly stained neurons in the Mx1-Vm. **k** Four moderately stained cells in the Lb-Vm. *Bar* 48 μ m. *Arrowheads* indicate cells that became reactive to the sNPF antibody after starvation



absent in adult male cockroaches starved for 4 weeks except for one moderately sized cell (Fig. 6b, arrowheads). Three strongly stained neurons were observed in the posterior PI of normally fed cockroaches (Fig. 5f); however, the immunoreactivity of one of these cells was absent in cockroaches starved for 4 weeks (Fig. 6c, arrowhead). All signals disappeared in the DL and a marked reduction was observed in the number of PI neurons showing CCAP-ir (Table 2).

CCAP-ir in the Br-SOG of cockroaches re-fed after starvation

Re-feeding generally restores the normally fed condition in terms of the number and intensity of CCAP-ir. However, only one strongly and three moderately immunoreactive CCAP-ir neurons detected in the anterior PI of normally fed cockroaches (Fig. 5e) were absent in re-fed cockroaches (Fig. 7b, arrowheads).

CCAP-ir in the Br-SOG of normally fed cockroaches in which sNPF was injected into the hemocoel

The sNPF injection induced a strong effect in the Br-SOG of normally fed cockroaches. The immunoreactivity

of all CCAP-ir cells in the anterior PI of normally fed cockroaches (Fig. 5b, e, g), disappeared in sNPF-injected cockroaches except for two moderately stained cells (Fig. 8b). Three strongly immunoreactive CCAP-ir neurons were observed in the posterior PI of normally fed cockroaches (Fig. 5f), whereas two of these were absent in sNPF-injected cockroaches (Fig. 8c). No signal was detected in the DL (Fig. 8a; Table 2), and the reactivity did not change in the SOG. Although the Lb-Vm contained one strongly stained cell (Fig. 5n) in normally fed cockroaches, its immunoreactivity was not detected in sNPF-injected cockroaches (Fig. 8d). One strongly stained cell in the dorsolateral region of the De in normally fed cockroaches (Fig. 5l) lost reactivity in sNPF-injected cockroaches (Fig. 8a, Table 2). The DL showed the clearest response and no CCAP-ir cells remained positive, whereas 50 % of the positive neurons remained positive (Table 2).

Co-localization of sNPF-ir and CCAP-ir in the Br-SOG and midgut of normally fed *P. americana*

Since both sNPF-ir and CCAP-ir occurred in the PI, DL, De and SOG, double-labeling using both anti-sNPF and anti-

Table 1 Numbers of sNPF-ir cells and their intensity in the brain-SOG of American cockroach, *Periplaneta americana*

Brain subdivision	Locus	Normally fed	Starved for 4 weeks	Re-fed	Injected with CCAP
Optic lobe (OL)	Proximal frontoventral cluster (Pfv)	1+++	1+++	1+++	–
	Proximal frontodorsal cluster (Pfd)	2++	2++	2++	1+
	Total	3	3	3	1
Protocerebrum (Pr)	Dorsolateral region of the protocerebrum (DL)	2++	3++	2++	2++
	Anterior	1+++	1+++	1+++	1+++
		4+++	3–6+	4+++	
			5+++	1++++	
	Pars Intercerebralis (PI)		3++++		1++++
	Posterior	1+++	1+++	1+++	1+
			1++++		
	Total	8	17–20	9	6
Deutocerebrum (De)		3+	3+	3+	3+
		1++++	2++++	1++++	
	Total	4	5	4	3
Tritocerebrum (Tr)		1++	2++	1++	1++
		1++++	1++++	1++++	1++++
	Total	2	3	2	2
Subesophageal ganglion (SOG)	Mdb	1+	1+	1+	–
	Mxl-Vm	1++ +	3++++	2++	–
				1++++	
	Mxl-L	1++++	–	1++++	1++++
	Lb-Vm	1+	1+	1+	1+
			4++		
	Total	4	9	6	2

A summary of the sNPF-ir in normal fed, 4 weeks of starvation, re-fed and injected with 10^{-8} moles of CCAP into the hemolymph of adult male *P. americana*. Immunoreactivity was quantified as absent (–), weak (+), moderate (++), considerable (+++) and strong (++++)

Fig. 3 sNPF immunoreactivity in re-fed adult male, *P. americana*. **a** Schematic representation of immunostaining patterns. **b** One strongly stained perikaryon (arrowhead) in the anterior PI. **c** Two more moderately stained cells (arrowhead) in the Mxl-Vm. Bar 48 μ m. Arrowheads indicate cells that were still reactive to the sNPF antibody after re-feeding versus normally fed cockroaches

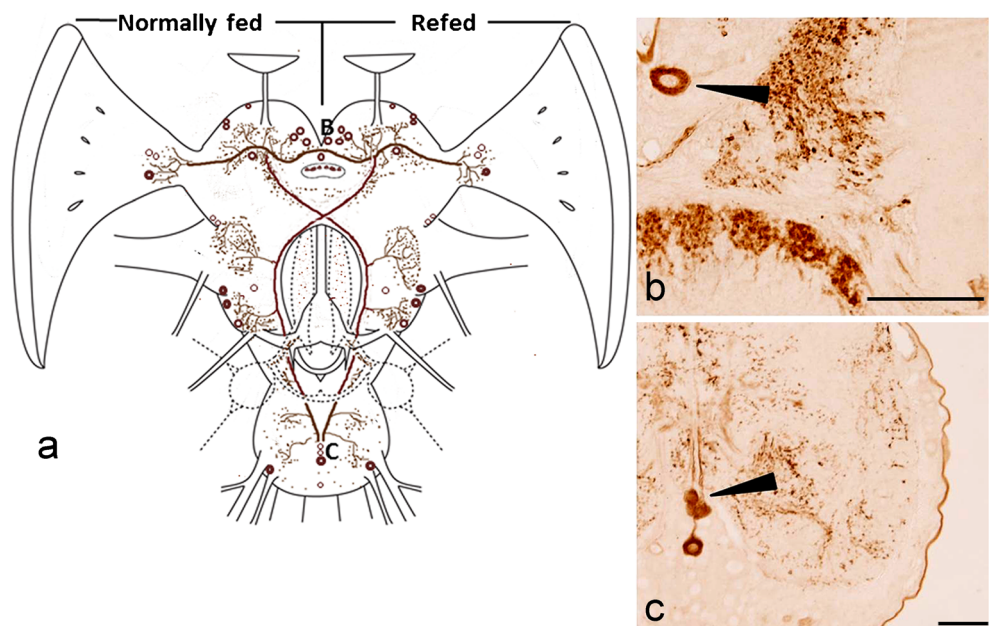
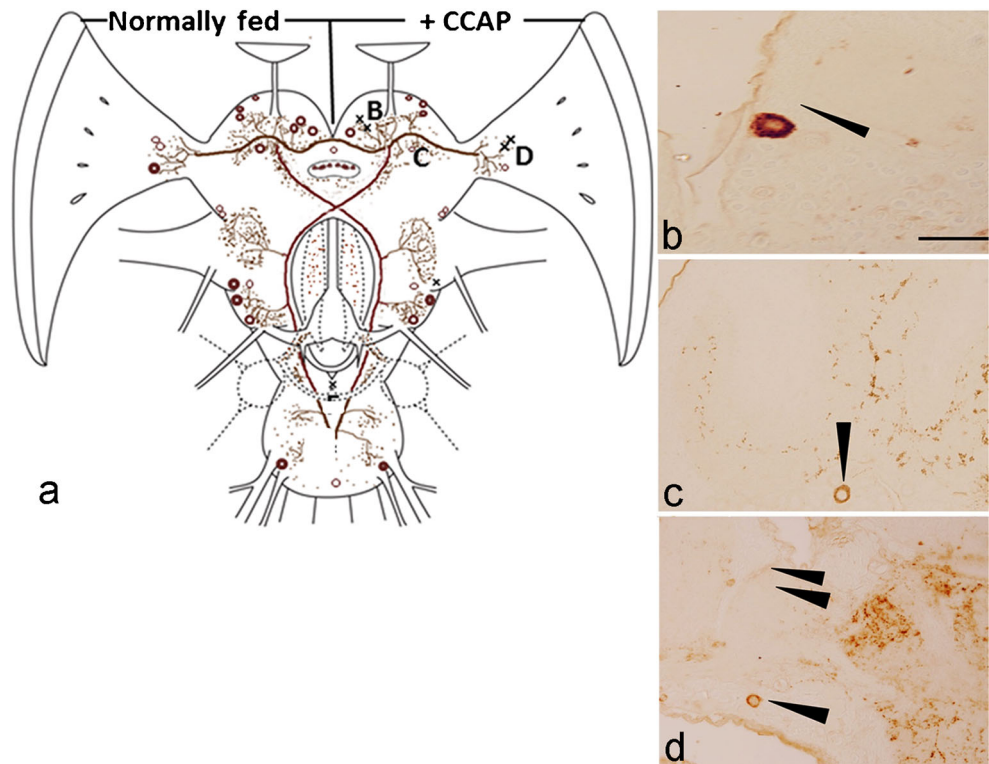


Fig. 4 sNPF-ir in normally fed adult male *P. americana* injected with CCAP into the hemolymph. **a** Schematic representation of immunostaining patterns. Left hemisphere represents sNPF-ir in normally fed animals, while the right represents that in CCAP-injected animals. Crosses (×) represent cell immunoreactivity that was present in the fed animals but absent in the injected. **b** One strongly stained cell in the anterior PI. **c** One weakly stained cell (arrowhead) in the posterior PI. **d** The immunoreactivity of two moderately stained cells (arrowheads) in the Pfd disappeared and one considerably stained cell (arrowhead) in the Pfv changed to weakly stained. Bar 48 μm



CCAP antibodies was conducted (Fig. 9). The results revealed that sNPF-ir (Fig. 9a) and CCAP-ir (Fig. 9b) were colocalized in one pair of neurosecretory cells in the PI (Fig. 9c), two cells in the DL (Fig. 9f), one pair of strongly stained cells in the De (Fig. 9i), and one pair of strongly (Fig. 9l) and moderately stained cells in the SOG (Fig. 9o). The results of the double-labeling experiment indicated that CCAP-ir (Fig. 10a) and sNPF-ir (Fig. 10b) were also colocalized in the epithelial cells and muscle layer (Fig. 10c) of the midgut in normally fed cockroaches.

DL neurons reacted most strongly to starvation, re-feeding and the sNPF injection (Table 2) while SOG neurons did not respond to starvation, re-feeding and the CCAP injection as strongly as the CCAP injection in the case of sNPF-ir (Table 2). Therefore, the DL neurons in this species may constitute a center of satiety.

Locomotor activity

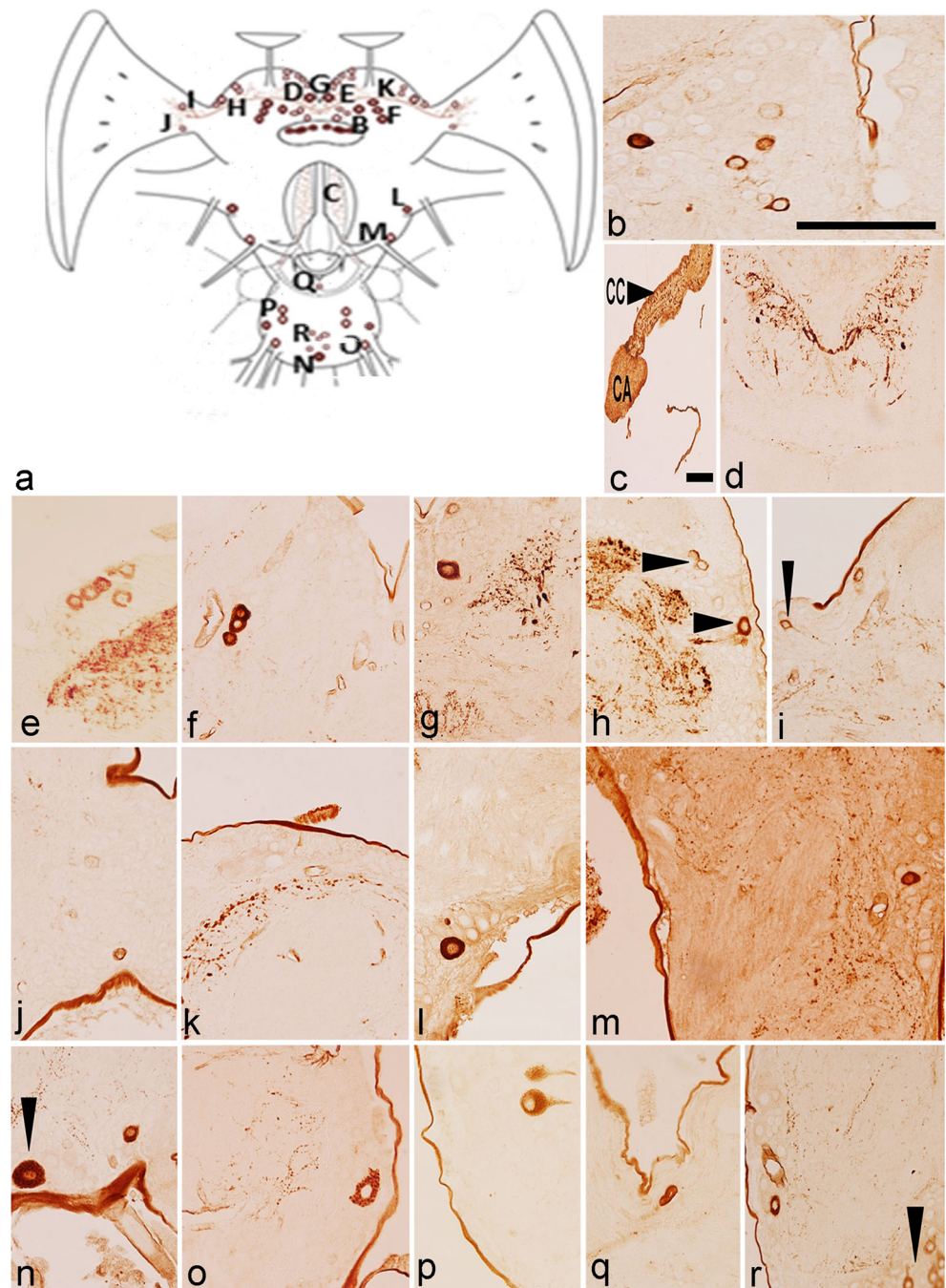
Cockroaches moved freely in the monitoring chamber. The locomotor activity of starved cockroaches was approximately 2.4-fold of the fed ones, but decreased 1 week after re-feeding (Fig. 11a). The sNPF injection stimulated locomotor activity (Fig. 11a), whereas the CCAP injection suppressed it (Fig. 11b).

sNPF and CCAP contents in the Br-SOG and midgut of the normally fed cockroaches with an intact head, decapitated and those placed with a hemostat between the foregut and midgut

The results of the competitive ELISA showed that the content of sNPF and CCAP in the brain-SOG homogenate of cockroaches starved for 4 weeks, 3 h after re-feeding, and also 3 h after an injection of CCAP or sNPF, respectively, was consistent with the profile of immunohistochemical reactivity. The content of sNPF in the Br-SOG of cockroaches starved for 4 weeks was significantly higher (2.5-fold) than that of normally fed cockroaches. The content of sNPF markedly decreased 3 h after re-feeding. The content of sNPF clearly decreased 3 h after the injection of CCAP into the hemocoel of normally fed cockroaches (Fig. 12a). After 4 weeks of starvation the contents of CCAP in Br-SOG extracts decreased to 66.3 % and increased 3 h after re-feeding. The content of CCAP rapidly decreased 3 h after the injection of sNPF into the hemocoel of normally fed cockroaches (Fig. 12b).

The results of competitive ELISA showed that the sNPF content in the midgut markedly decreased 3 h after the injection of CCAP into the hemocoel of normally fed cockroaches (Fig. 13a). The content of CCAP in the midgut decreased to 38.1 %, 3 h after the injection of sNPF into normally fed

Fig. 5 CCAP-ir in normally fed adult male *P. americana*. **a** Schematic representation of immunostaining patterns. **b** One strongly and three–four weakly stained cells in the anterior PI. **c** Immunoreactivity in fiber arborization within the corpora cardiaca. **d** The projections of the large neurons arborized within the Pr. **e** Five moderately stained CCAP-ir in the anterior PI. **f** Three strongly stained cells in the posterior PI. **g** One strongly stained cell in the anterior PI. **h** Two weakly (*arrowheads*) and one considerably stained cells (*arrowhead*) in the DL. **i** Two weakly stained cells (*arrowheads*) in the Pfd and DL. **j** One weakly stained cell in the Pfv. **k** The nerve bundle that ran posteriorly in the DL and OL. **l** One strong cell in the dorsolateral region of the De. **m** One moderately stained cell in the ventral Tr. **n** One moderately and one strongly stained cell (*arrowhead*) in the posterior midline of the Lb-Vm. **o** One strongly stained cell in the Mxl-L. **p** Two moderately stained cells in the lateral mandibular region. **q** One moderately stained CCAP-ir (*arrowhead*) in the Mdb. **r** Cell cluster (*arrowhead*) in the maxillary neuromere (Mxl-Vm). Bar 48 μ m



cockroaches (Fig. 13b). These results also showed that sNPF and CCAP contents in the midgut decreased to 33.9 and 42.5 %, respectively, 3 h after an injection of CCAP or sNPF into normally fed adult male cockroaches in which a hemostat was placed between the foregut and midgut (Fig. 14a, b). Moreover, sNPF and CCAP contents in the midgut decreased 3 h after the injection of CCAP or sNPF into decapitated cockroaches (Fig. 14c, d). These results indicated that this particular response does not require feedback from cephalic ganglia.

Discussion

The regulation of feeding in insects is very complex, and involves interactions between a number of mechanisms, one of which is the release, either centrally or locally, of neuropeptides. However, the role of neuropeptides, their mechanisms of action, interactions with each other, and their release are still poorly understood (Audesley and Weaver 2009). CCAP is a multifunctional midgut factor as well as a neuropeptide. CCAP-related peptides play a role in the generation and

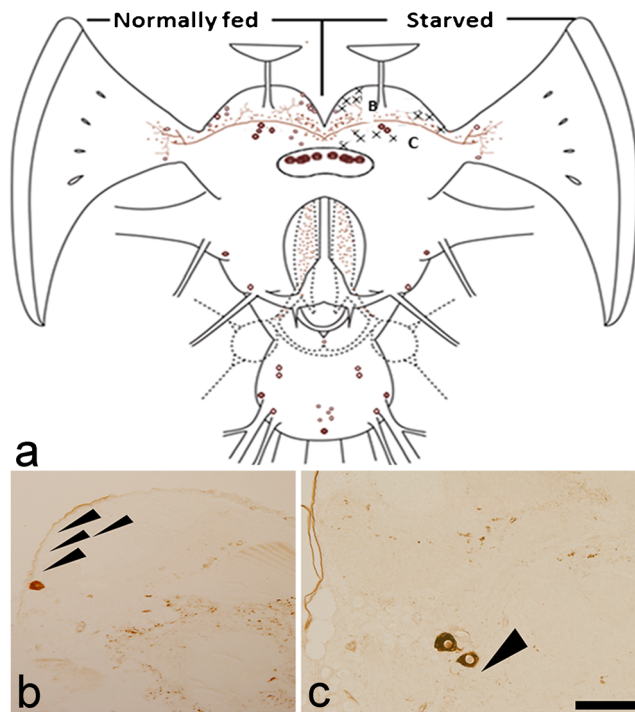


Fig. 6 CCAP-ir in adult male *P. americana* starved for 4 weeks. **a** Schematic representation of immunostaining patterns. Crosses (×) represent cell immunoreactivity that was present in the fed while absent in the starved cockroach. **b** One moderately stained cell in the anterior PI. **c** The immunoreactivity of one strongly stained cell was missing in the posterior PI. Arrowheads show the location of the missing cells. The immunoreactivity of CCAP-ir cells that disappeared after starvation were marked by crosses. Bar 48 μm

modulation of rhythmic feeding movements in the pond snail, *Lymnaea stagnalis* (Vehovszky et al. 2005). CCAP participates in the regulation of feeding behavior by concerted functions as a paracrine substance for the induction of carbohydrate, lipid and protein digestion by the midgut epithelium and myostimulatory neuropeptide of the gut tissues (Sakai et al. 2004; Mikani et al. 2012).

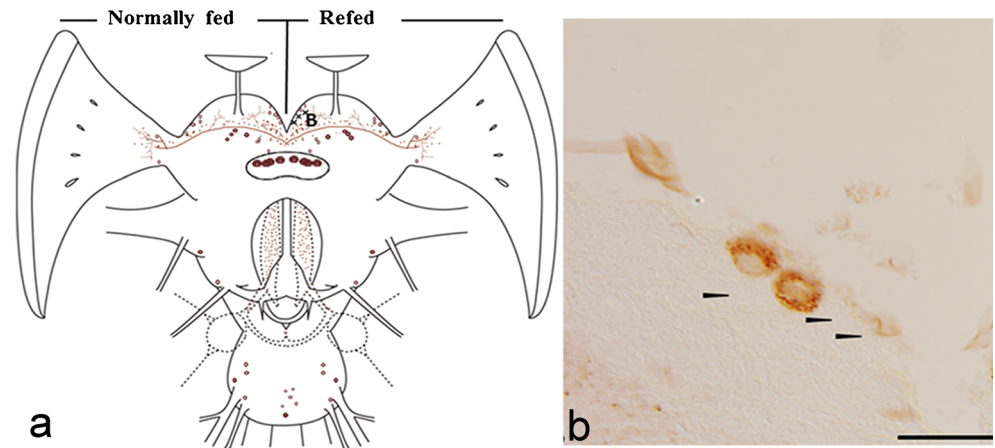
Some of the proposed functions of sNPF include the regulation of feeding, as was previously suggested for NPY in mammals (Nässel and Wegener 2011). Short neuropeptide F (sNPF) has been shown to modulate feeding behavior in a wide variety of insect species. While this peptide stimulates feeding and food-searching behavior in *Drosophila melanogaster* (Hong et al. 2012) and *Apis mellifera* (Hummon et al. 2006), an opposite effect has recently been demonstrated in the desert locust, *Schistocerca gregaria* (Dillen et al. 2014) and *P. americana* (Mikani et al. 2012). In *D. melanogaster*, sNPF-ir cells were detected in both the nervous system and the epithelium of midgut (Brown et al. 1999). We have also reported the presence of sNPF-ir cells in the midgut of *P. americana* (Mikani et al. 2012). Our results indicated that the number of sNPF-ir paraneuronal cells increased during 4 weeks of starvation and decreased after 3 h of re-feeding. We also found that starvation reduced α-amylase and protease activities in the midgut and digestive enzyme levels increased after 3 h of re-feeding. This quick response was also observed in the case of CCAP (Sakai et al. 2006). We previously demonstrated that incubation of

Table 2 The number and intensity of CCAP-ir cells in normal fed, 4 weeks of starvation, re-fed and injected with 10^{-8} mole of sNPF into the hemolymph of adult male *P. americana*

Brain subdivision	Locus	Normally fed	Starved for 4 weeks	Re-fed	Injected with sNPF	
Optic lobe (OL)	Proximal frontoventral cluster (Pfv)	1+	1+	1+	1+	
	Proximal frontodorsal cluster (Pfd)	1+	1+	1+	1+	
	Total	2	2	2	2	
Protocerebrum (Pr)	Dorsolateral region of the protocerebrum (DL)	3+	–	3+	–	
	Total	4	0	4	0	
	Pars Intercerebralis (PI)	Anterior	3–4+	5++	3+	2++
		Posterior	2++++	3++++	1++++	1++++
Total	13–14	3	9	3		
Deutocerebrum (De)		1++++	1++++	1++++	–	
Tritocerebrum (Tr)		1++	1++	1++	1++	
Subesophageal ganglion (SOG)	Mdb	3++	3++	3++	3++	
	Mxl-Vm	5++	5++	5++	–	
	Mxl-L	1++++	1++++	1++++	1++++	
	Lb-Vm	1++	1++	1++	1++	
	Total	11	11	11	10	

Immunoreactivity was quantified as absent (–), weak (+), moderate (++) and strong (++++)

Fig. 7 CCAP-ir in re-fed adult male *P. americana*. **a** Schematic representation of immunostaining patterns. Crosses (×) represent the immunoreactivity of CCAP-ir cells that were present in the fed animals while absent in the re-fed. **b** Three moderately stained cells immunoreactivity that were detected in the anterior PI of normally fed cockroaches were absent. Arrowheads show the location of the immunoreactivity of the missing cells. Bar 48 μm



a dissected midgut in the medium containing sNPF suppressed α -amylase, protease and lipase activities. This finding indicated that enzyme activities were down-regulated by sNPF (Mikani et al. 2012). Therefore, the midgut itself appears to regulate the secretion of enzymes. This was supported by the hematostat experiment. This particular regulation did not require the presence of Br-SOG or stomatogastric nervous system (SGNS), though feedback regulations from the cephalic elements do exist, since the removal of the pars

intercerebralis induced hyperphagy supported by intensification or suppression of the midgut peptide system both in the epithelium and muscle layer in the Hemocoel in free-running conditions (Matsui et al. 2013). The injection of nutrients in the hemocoel suppressed digestive enzyme secretion in the head-intact starved animal but this was not observed with decapitated animals (Mikani et al. 2012). The digestive enzyme secretion was upregulated in 3 h but the incubation of midgut with CCAP only took 2 h to up-regulate digestive

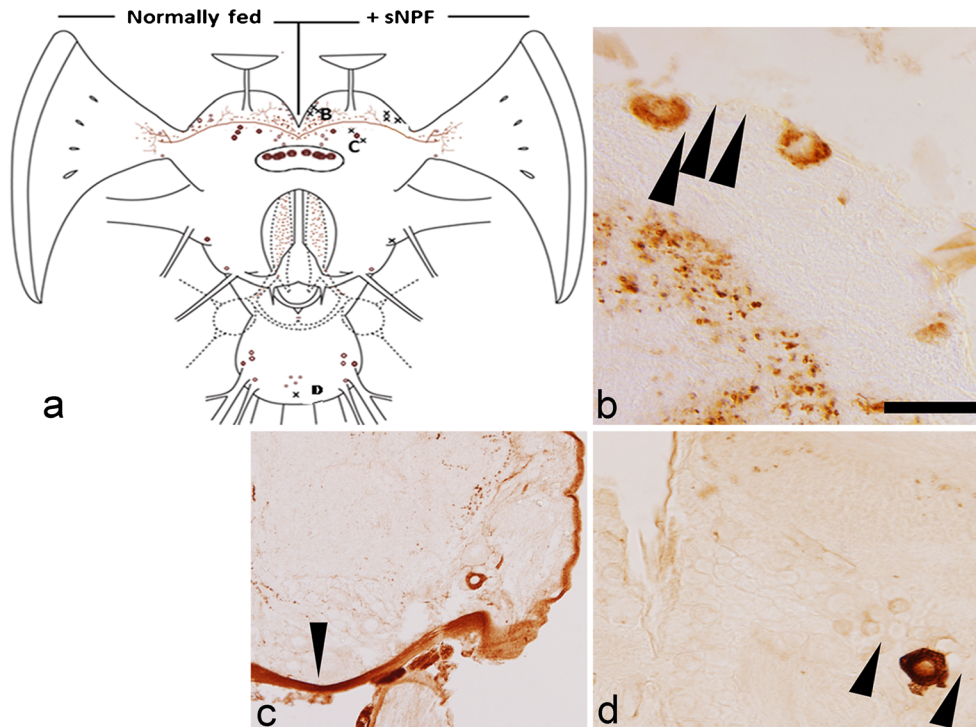
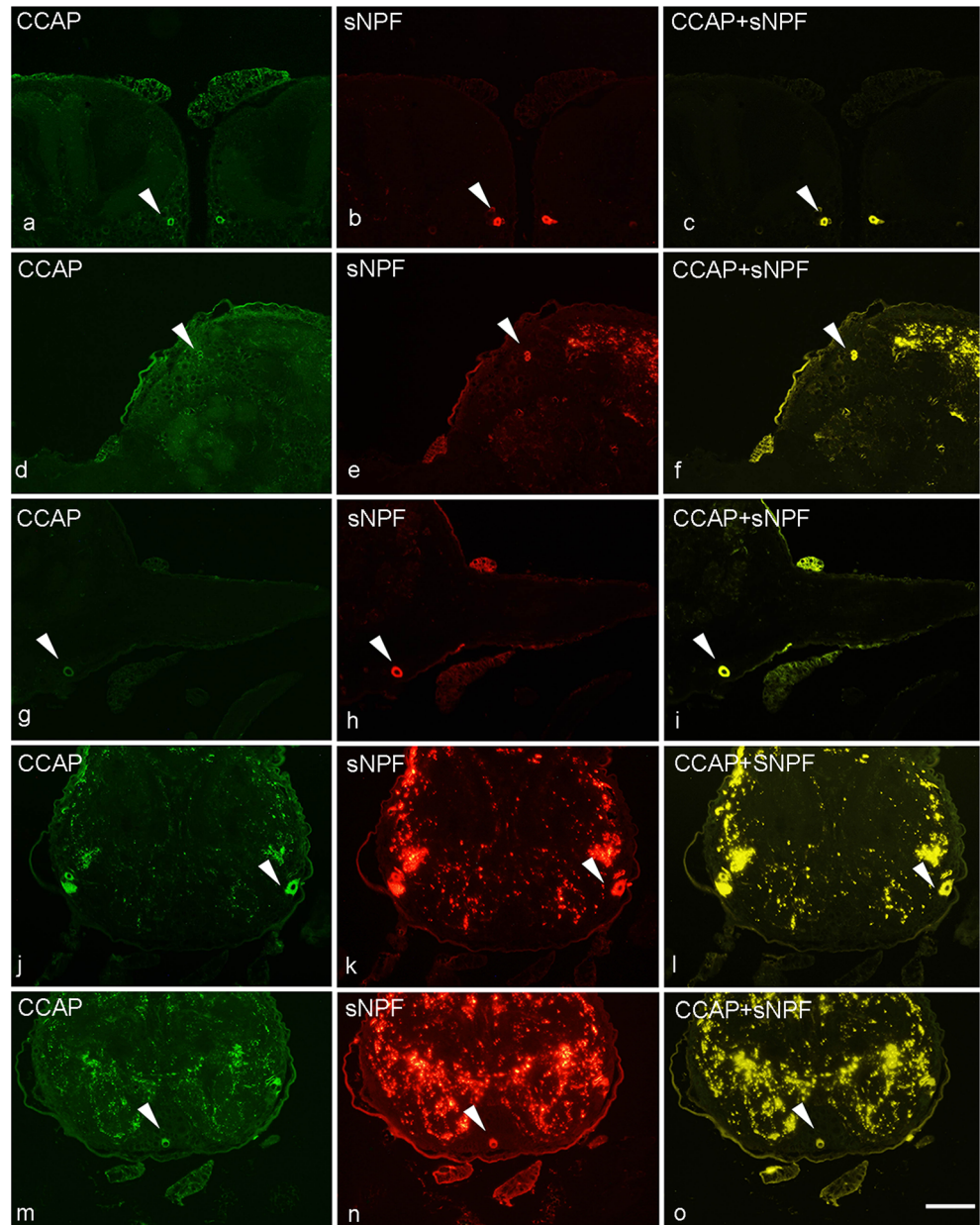


Fig. 8 CCAP-ir in normal fed adult male *P. americana* injected with sNPF into the hemolymph. **a** Representation of immunostaining patterns. Crosses (×) represent the absent immunoreactivity of cells versus the normally fed cockroach. **b** The immunoreactivity of all CCAP-ir cells was missing in the anterior PI of sNPF injected cockroaches except for two moderately stained cells (arrowhead shows the

location of the immunoreactivity of missing cells). **c** Two signals were missing in the posterior PI (arrowheads). **d** The immunoreactivity of one strongly stained cell was missing in the Lb-Vm. Arrows show the location of the missing immunoreactivity of the cell. CCAP-ir cells that disappeared due to the injection of sNPF were marked as crosses. Bar 48 μm

Fig. 9 Double-labeling using anti-sNPF (red) and anti-CCAP (green) antibodies in the brain-SOG of normally fed adult male *P. americana*. **a** CCAP-ir in the posterior PI. **b** sNPF-ir in the posterior PI. **c** Merged image of CCAP-ir and sNPF-ir in the posterior PI. **d** CCAP-ir in the DL. **e** sNPF-ir in the DL. **f** Merged image of CCAP-ir and sNPF-ir in the DL. **g** CCAP-ir in the De. **h** sNPF-ir in the De. **i** Merged image of CCAP-ir and sNPF-ir in the De. **j** sNPF-ir in the lateral part of the Lb. **k** CCAP-ir in the lateral part of the Lb. **l** Merged image of CCAP-ir and sNPF-ir in the lateral part of the Lb. **m** sNPF-ir in the Lb. **n** CCAP-ir in the Lb. **o** Merged image of CCAP-ir and sNPF-ir in the Lb. Bar 100 μm



enzyme secretion (Sakai et al. 2006). These indicate that midgut digestive activity is under the regulation of cephalic

elements such as brain neural activities, SGNS and neuropeptide secretion as well as midgut epithelial paraneurons. Within

Fig. 10 Double-labeling using anti-sNPF (red) and anti-CCAP (green) antibodies in the midgut of normally fed adult male *P. americana*. **a** CCAP-ir in the midgut. **b** sNPF-ir in the midgut. **c** Merged image of CCAP-ir and sNPF-ir in the midgut. Bar 100 μm

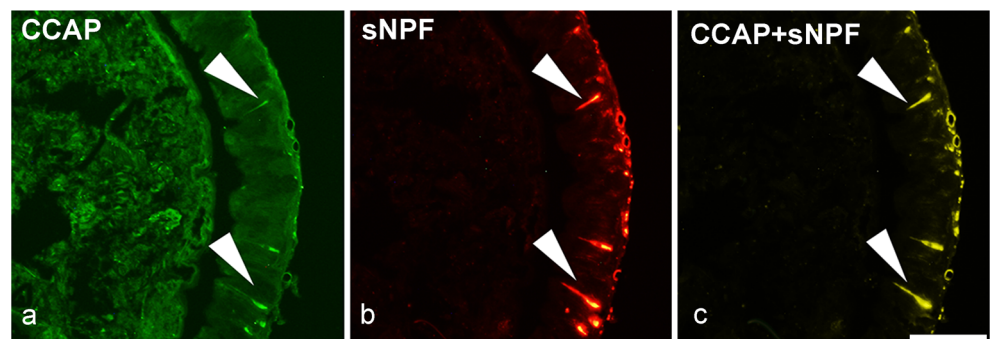
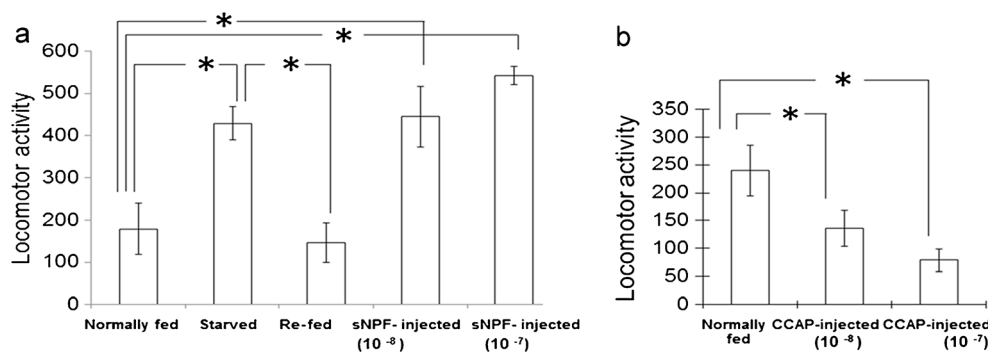


Fig. 11 Locomotor activity of normally fed cockroaches injected with sNPF (a) or CCAP (b) at 10^{-7} and 10^{-8} moles



the midgut epithelium, negative feedbacks between CCAP and sNPF are mutually and directly coupled each other. The food or nutrient is sensed by the microvilli of the midgut epithelium and this triggers the secretion of CCAP from the brain. The peptide secreted into the hemocoel further triggers a massive secretion of CCAP from the epithelial paraneurons. This induces digestive enzymes, a positive feedback. Starvation may exert a much slower switch action but the response to re-feeding is rapid and the secreted sNPF induces a quick response to suppress CCAP in 3 h. The contribution of SGNS is not clear at this moment because of technical difficulties during the surgical operation but the brain regulates this system in a peptide-specific manner (Matsui et al. 2013). The removal of the frontal ganglion is an easy operation but this does not sufficiently sustain the life of the cockroach.

The midgut contains paraneuronal cells, some of which may monitor nutrient contents and stretch the gut (Fuse et al. 1999). The passage of food through the gut lumen triggers the release of digestive enzymes. Paraneurons serve as a switch mechanism in this circuit. However, digestive activities must be integrated in harmony with other activities such as peristalsis or throughout regulation and food searching behavior. We

previously demonstrated such functional interplay between the midgut, brain and SGNS (Mikani et al. 2012). We recently reported that the number of CCAP-ir cells markedly increased in the epithelium after the removal or bisectomy of the PI, indicating that the PI suppresses the expression of CCAP in the midgut epithelium of *P. americana*. This could also be due to the removal of the sNPF system in the PI (Matsui et al. 2009; Matsui et al. 2013).

Jankovic-Hladni et al. (1978) showed that the brain regulated midgut α -amylase activity in *Tenebrio molitor*. Thomsen and Moller (1963) also found that the brain controlled protease activity in *Calliphora erythrocephala* and *Dysdercus cingulatus* (Muraleedharan and Prabhu 1979). The results of the present study clearly demonstrated that the number of sNPF-ir cells in the Br-SOG of *P. americana* increased after 4 weeks of starvation and decreased after 3 h of re-feeding (Figs. 1, 2, 3; Table 1). The PI in the protocerebrum reacted most strongly to sNPF-ir, followed by SOG neurons (Table 1). Thus, the pars may be the locus of the center of appetite in this species, whereas neurons in the DI

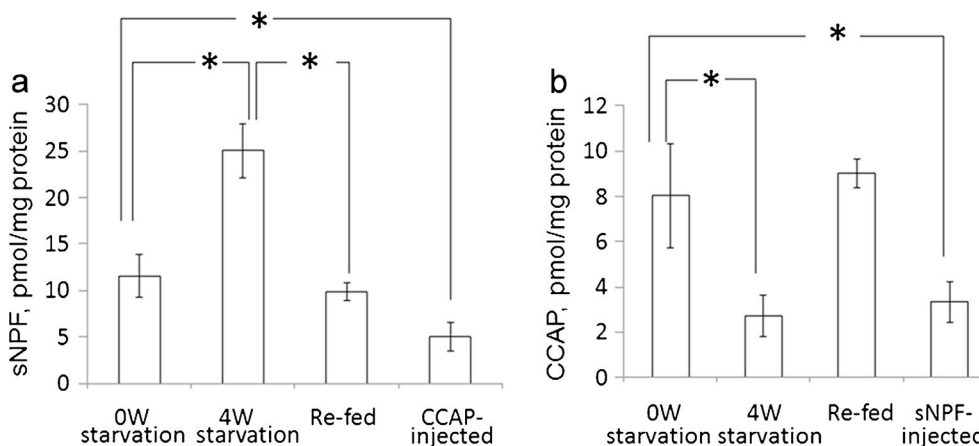
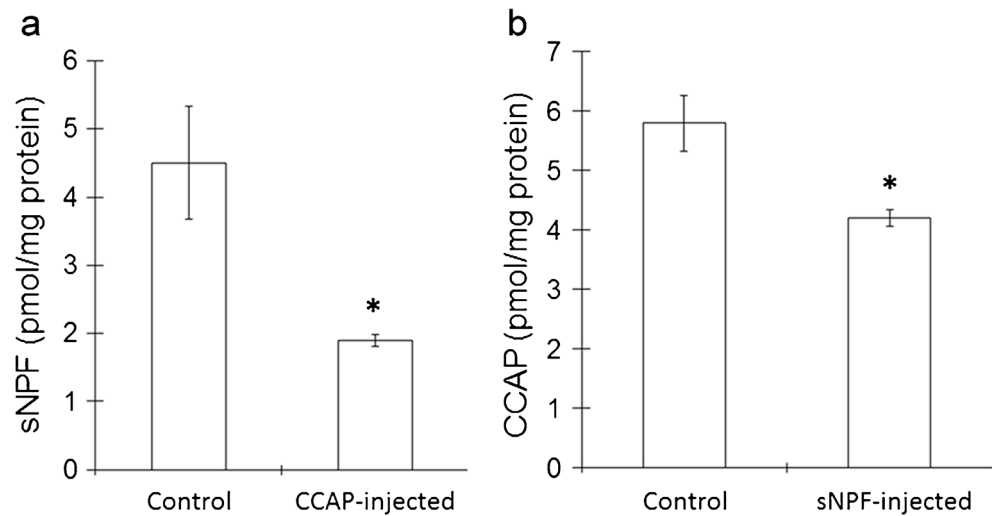


Fig. 12 Competitive ELISA-detected sNPF (a) and CCAP (b) concentrations in the supernatant of the Br-SOG of normally fed adult male *P. americana* that were normally fed, starved for 4 weeks, re-fed for 3 h, or injected with CCAP and sNPF (10^{-8} moles of sNPF or CCAP in

10 μ L of PBS) into the hemolymph, respectively. Values are expressed as the average sNPF or CCAP concentrations of 8 replicates. Each point represents the mean \pm SEM. * $p < 0.05$ (Student's *t* test)

Fig. 13 Competitive ELISA detected sNPF (a) and CCAP (b) concentrations in the supernatant of the midgut of adult male *P. americana* that were normally fed or injected with CCAP and sNPF (10^{-8} moles of sNPF or CCAP in 10 μ L of PBS) into the hemolymph, respectively. Values are expressed as the average sNPF or CCAP concentrations of 8 replicates. Each point represents the mean \pm SEM. * $p < 0.05$, significantly different from normally fed cockroaches (Student's *t* test)



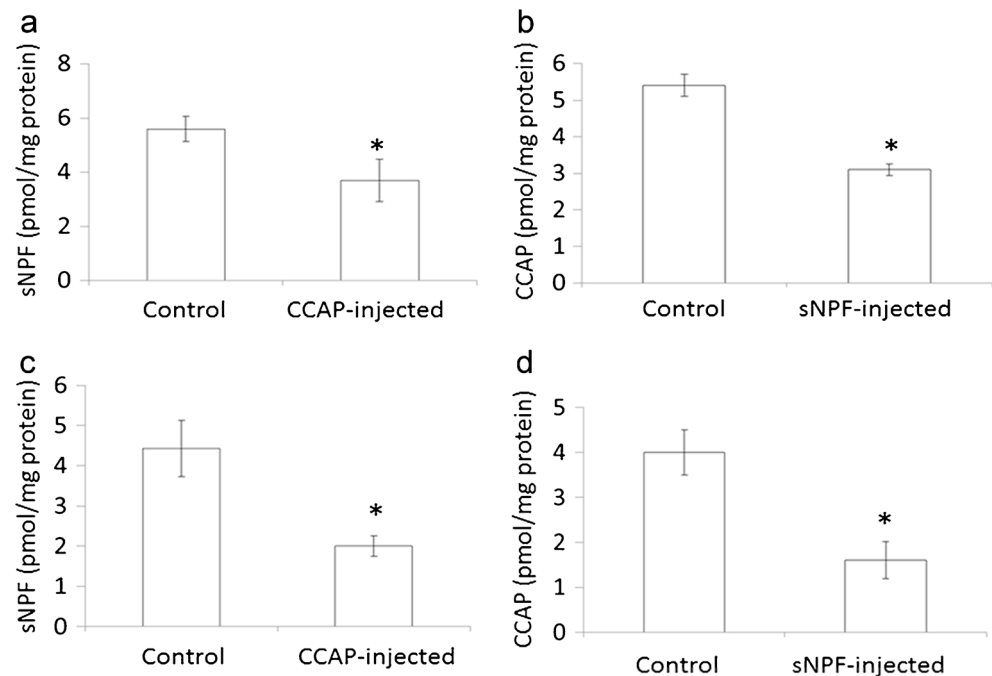
region reacted most strongly to CCAP-ir and SOG did not react at all. An asymmetry may exist in the control of appetite and satiety.

The competitive ELISA results of the present study were consistent with those of IHC (Fig. 12a). sNPF-ir was intense in the CC (Fig. 10). Brain–midgut coupling can be either neural via the stomatogastric nervous system or neurosecreted via the corpora cardiaca (CC). CCAP- and sNPF-ir were detected on both routes. Competitive ELISA revealed that sNPF was released into the circulation via the corpora cardiaca (Mikani et al. 2012). Our results showed an intricate interplay between the brain and midgut for regulation of digestive activities. Previous results demonstrated that the number of CCAP-ir cells

increased in the epithelium 3 h after re-feeding cockroaches that had been starved for 3 weeks. Incubation of the dissected midgut with CCAP led to an increase in α -amylase and protease activities (Sakai et al. 2006). We have shown here that the number of CCAP-ir cells in the Br-SOG of *P. americana* decreased after 4 weeks of starvation and increased after 3 h of re-feeding (Figs. 5, 6, 8; Table 2). The ELISA results were in good agreement with those of IHC (Fig. 12b). CCAP-ir was stained intensely in the CC (Fig. 5c).

Our co-localization study revealed that sNPF-ir cells in the PI (Fig. 9a), DL (Fig. 9d), De (Fig. 9g) and SOG (Fig. 9j, m), also exhibited CCAP-ir. This results strongly suggest that these sNPF-ir cells are also CCAP-ir cells, although unique

Fig. 14 Competitive ELISA detected sNPF and CCAP concentrations in the supernatant of the midgut of adult male *P. americana* in which a hemostat was placed between the foregut and midgut (a, b), and also decapitated adult male cockroaches (c, d) injected with CCAP and sNPF (10^{-8} moles of sNPF or CCAP in 10 μ L of PBS) into the hemolymph, respectively. Values are expressed as the average sNPF or CCAP concentrations of 8 replicates. Each point represents the mean \pm SEM. * $p < 0.05$, significantly different from normally fed cockroaches (Student's *t* test)



sNPF-ir or CCAP-ir has also been detected. Cross-reactivity was excluded because the CCAP-ir and sNPF-ir changed to opposite directions when the insect was starved, re-fed and injected with the other peptide.

Our study showed that the content of CCAP in the Br-SOG markedly decreased 3 h after the injection of sNPF into normally fed cockroaches (Fig. 12a). The content of sNPF in the Br-SOG also decreased after the injection of CCAP (Fig. 12b). We obtained the same result in the midgut (Fig. 12a, b). These results strongly suggest that CCAP and sNPF mutually suppress each other.

sNPF and CCAP titers decreased in the midgut 3 h after the injection of CCAP or sNPF into normally fed cockroaches in which a hemostat was placed between the foregut and midgut (Fig. 14a, b). This treatment isolated the midgut from the Br-SOG and SGNS elements. sNPF and CCAP titers also decreased in the midgut 3 h after the injection of CCAP or sNPF into the isolated abdomen (Fig. 14c, d), suggesting that sNPF or CCAP in the hemolymph may have directly affected CCAP and sNPF concentrations in the midgut, respectively.

Both sNPF-ir and CCAP-ir are widely distributed in the Br-SOG but depend on nutritional and hormonal conditions, and marked changes in the number of positive cell bodies were only observed in the PI, DL, Pfd and SOG. These parts express the circadian protein period (Matsui et al. 2009; Ichihara 2000). The circadian control of feeding has already been described by Matsui et al. (2009) who reported that hyperphagy was induced by the removal of the PI, but only under constant dark and not under a L:D 12:12 cycle.

Locomotor activity in starved cockroaches was approximately 2.4 times that of fed cockroaches (Fig. 11a). Furthermore, different concentrations of sNPF changed locomotor activity (Fig. 11a). We previously reported that the number of sNPF-ir cells increased during starvation in the midgut (Mikani et al. 2012). The sNPF injection increased locomotor activity (Fig. 11). We have demonstrated here that CCAP decreased locomotor activity (Fig. 11b). The number of CCAP-ir cells is known to decrease during starvation in the midgut (Sakai et al. 2006) and the brain (Fig. 6). Thus, both sNPF and CCAP regulate a diversity of physiological functions and behaviors in *P. americana* such as feeding, digestion, peristalsis, locomotion, and circadian rhythms in such a way that the two peptide system operates as a switch based on a negative feedback loop (Mikani et al. 2012; Sakai et al. 2004, 2006), similar to *D. melanogaster* (Nässel and Wegener 2011).

Conclusions

Various physiological functions are integrated by interactions between some critical peptide factors. sNPF and CCAP constitute a central switch by antagonizing each other. These

peptides have a wide tissue distribution and thus are multifunctional. They are not only released into the circulation but also function as neurotransmitters and serve as paracrine factors for midgut paraneurons. These negative feedback loops serve as an autocrine switch. Shifts to both sides require a relatively short time at least for the initial reaction. This regulatory mechanism is of immense interest and requires future study. This feedback loop serves as an autocrine metabolastat integrating feeding with other physiological and behavioral functions.

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