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Morphological Characterization of Neurons Projecting to the Ring Gland in the Larval Blow Fly, *Protophormia terraenovae*

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The cephalic neuroendocrine system of the larval blow fly, *Protophormia terraenovae* was studied by backfills using either horseradish peroxidase or $NiCl_2$, and peptide immunocytochemistry. Backfills through a proximal part of the ring gland mainly revealed three groups of neurons: (1) neurons with somata in the pars intercerebralis (PI) of the protocerebrum, (2) neurons with somata in the pars lateralis (PL) of the protocerebrum, and (3) neurons with somata in the subesophageal ganglion (SEG). Dense arborization was found mainly in the superior protocerebral, tritocerebral and SEG neuropils. Backfills through a distal part of the ring gland exclusively revealed two types of neurons with somata in the PL, viz., those with ipsilateral projections and those with contralateral projections to the ring gland. Antisera against cholecystokinin-8, FMRFamide, and *Gryllus bimaculatus* pigment-dispersing factor labeled cells in the PI and PL as well as fibers in the ring gland and aorta. Anti-cholecystokinin-8 and anti-FMRFamide antisera also labeled cells in the SEG. These results suggest that cephalic neurons projecting to the ring gland and aorta receive information at restricted regions in the superior protocerebral, tritocerebral and SEG neuropils, and that they release cholecystokinin-8, FMRFamide, and pigment-dispersing factor-like peptides from the ring gland and aorta into the hemolymph as neurohormones, or locally in the ring gland and aorta to regulate the production and/or release of hormones by the gland cells.

Key words: neurosecretory cell, pars intercerebralis, pars lateralis, subesophageal ganglion, backfill, immunocytochemistry

INTRODUCTION

Various physiological processes, including development and homeostasis, in insects are under the control of cephalic neurons projecting to the retrocerebral complex, consisting of endocrine organs, viz., the corpora cardiaca (CC) and corpora allata (CA), and neurohemal sites (Raabe, 1989; Nijhout, 1994). Their somata are located in distinctive regions, viz., the pars intercerebralis (PI) and pars lateralis (PL) of the protocerebrum in the brain, and a medioventral region of the subesophageal ganglion (SEG) (Orchard and Loughton, 1985; Raabe, 1989). Most of the cephalic neurons with projections to the retrocerebral complex are neurosecretory and release neurohormones into the hemolymph from the aorta, CC, and CA, but some serve to control the release of hormones from intrinsic endocrine cells in the CC and CA.

In the tobacco hornworm, *Manduca sexta*, prothoracicotropic hormone (PTTH) is produced in two pairs of neurons with somata in the PL and contralateral fibers to the CA, at which PTTH is released into the hemolymph (Agui et al., 1980; O'Brien et al., 1988). PTTH activates biosynthesis of ecdysteroids in the prothoracic gland (PG). In the cockroach, *Diploptera punctata*, neurons with somata in the PL and ipsilateral fibers to the CC and CA are immunolabeled with a monoclonal antibody against an allatostatin (Stay et al., 1992). Allatostatins are released within and from the CA and act through both neural and humoral pathways to inhibit production of juvenile hormone (JH) by the CA (Yu et al., 1993; Lloyd et al., 2000). In the silkworm, *Bombyx mori*, an insulin-like peptide, bombyxin, which is produced in four pairs of neurons with somata in the PI and released from the CA, reduces the amounts of the storage carbohydrates trehalose and glycogen in the larvae (Mizoguchi et al., 1987; Satake et al., 1997).

In fly larvae, the CC and CA constitute the ring gland, with another endocrine organ producing ecdysteroids, and

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ABBREVIATIONS

Bom-PTTH, *Bombyx mori*-prothoracicotropic hormone; β-**PDH**, β-pigment-dispersing hormone; **CA**, corpus allatum; **CC**, corpus cardiacum; **CCK-8**, cholecystokinin-8; **Ef**, esophageal foramen; ***Gryllus*-PDF**, *Gryllus bimaculatus* pigment-dispersing factor; **JH**, juvenile hormone; **NCC**, nervi corporis cardiaci; **PDF**, pigment-dispersing factor; **PG**, prothoracic gland; **PI**, pars intercerebralis; **PL**, pars lateralis; **PTTH**, prothoracicotropic hormone; **SEG**, subesophageal ganglion.

this gland is located posteriorly and dorsally to the brain (Thomsen, 1951; Bollenbacher et al., 1976). Several important roles in developmental and physiological phenomena have recently been reported for neurons projecting to the ring gland. In the flesh fly, *Sarcophaga bullata*, a pupariation factor designated Neb-pyrokinin-2, which accelerates puparium formation, has been purified. Immunocytochemical study has suggested that Neb-pyrokinin-2 is produced in neurons with somata in the SEG and released into the hemolymph from the ring gland and aorta (Verleyen et al., 2004). In larvae of *Drosophila melanogaster*, about 20 somata in the SEG express the gene *hugin* encoding two putative neuropeptides, pyrokinin-2 and *hug-γ* (Meng et al., 2002; Melcher and Pankratz, 2005). The *hugin*-expressing neurons, a subset of which projects fibers to the CC and expresses pyrokinin-2, modulate taste-mediated feeding behavior (Melcher and Pankratz, 2005; Bader et al., 2007; Neupert et al., 2007). Furthermore, a cluster of PI cells with axons to the CC in larvae of *D. melanogaster* produces insulin-like peptides and functions in carbohydrate metabolism and growth regulation (Rulifson et al., 2002).

Although these findings indicate the importance of neurons projecting to the ring gland in various developmental and physiological processes, dendritic fields, where these neurons receive internal and external information, are not well known. Morphological characterization of these neurons is an important step to understanding regulatory mechanisms of the peptidergic neurosecretory cells involved in developmental or physiological phenomena. In *D. melanogaster* larvae, Siegmund and Korge (2001) mapped cephalic neurons innervating the ring gland by using the GAL4 enhancer trap system, and connections with circadian pacemaker neurons were suggested. However, dendritic projections from the neurosecretory cells in the brain have not been thoroughly analyzed. Also for a general understanding of dendritic fields and projection patterns of neurons innervating the ring gland, more species should be studied.

In the current study, we morphologically investigated cephalic neurons projecting to the ring gland in the blow fly, *Protophormia terraenovae*, using backfills and immunocytochemistry with eight antisera against different neuropeptides, and then also inferred their likely peptidergic phenotypes.

MATERIALS AND METHODS

Insects

The blow fly *P. terraenovae* (Diptera: Calliphoridae) was kept as a laboratory culture under diapause-averting conditions of 18:6 hours LD at 25±1°C, as previously reported (Numata and Shiga, 1995). In our experiments, third-instar larvae (4–6 days after hatching) of either sex were used.

Backfills

For backfills, either 2% horseradish peroxidase (HRP) in 0.1 M KCl containing 3% lysolecithin, or 500 mM $NiCl_2$ containing 1% bovine serum albumin, was used according to the methods of Nässel (1982) and Shiga et al. (2000), respectively. To fill entire sets of neurons projecting to the ring gland, the ring was cut at one-quarter to one-third away from the posterior proximal region (Fig. 1), and then the HRP was introduced through both cut ends by using a sharpened glass capillary. To fill neurons projecting to a distal part of the ring gland, the ring was cut at the most anterior distal end in order to open it, and then either HRP or $NiCl_2$ was introduced through either of the cut ends by using the capillary. Backfills were performed for 2–3 h at about 22°C. After backfilling, the entire central nervous system was dissected out in 0.1 M phosphate-buffered saline (PBS, pH 7.4).

Tissues backfilled with HRP were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 3 h at about 22°C or overnight at 4°C. After washing in PBS, the tissue was desheathed, pre-incubated with 0.03% 3, 3'-diaminobenzidine (DAB, Sigma, MO) in 0.1 M Tris-HCl (pH 7.4) for 40 min at about 22°C, and finally reacted with 0.03% DAB in 0.1 M Tris-HCl containing 0.01% H_2O_2 for 10–15 min at about 22°C. Tissues backfilled with $NiCl_2$ were, after a brief wash in PBS, incubated for 10–15 min in PBS containing a drop of saturated rubeanic acid solution in 70% ethanol. After washing in PBS, the preparation was fixed in aqueous Bouin's solution for 24 h at about 22°C, washed with 70% ethanol, and subsequently processed for silver intensification according to the method of Shiga et al. (2000). After DAB reaction of HRP-labeled preparations, or silver intensification of Ni^{2+}-filled preparations, the tissues were dehydrated in an ethanol series and cleared in methyl salicylate for observation.

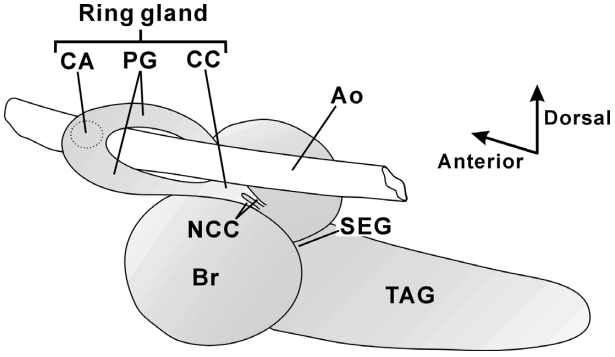

Fig. 1. Schematic illustration of the larval central nervous system and ring gland of the blow fly, *Protophormia terraenovae*. The central nervous system consists of the brain (Br), subesophageal ganglion (SEG), and thoracico-abdominal ganglion (TAG). Bilateral nervi corporis cardiaci (NCC) exit from the dorso-medial region of the brain and extend to the ring gland. The ring gland surrounding the aorta (Ao) is composed of the corpus allatum (CA), corpus cardiacum (CC), and prothoracic gland (PG).

Immunocytochemistry

Antibodies listed in Table 1 were used in the current study. Immunocytochemistry was performed by the ABC technique (Vectastain ABC Standard Kit PK-4001, PK-4002, Vector Laboratories, Burlingame, CA) or fluorescence immunocytochemistry according to the method of Hamanaka et al. (2007). The entire central nervous system dissected out in PBS was fixed in 4% paraformaldehyde for 3 h at about 22°C or overnight at 4°C. Two fixatives, either a Bouin-Hollande solution without acetic acid or a formaldehyde-picric acid solution (Eckert and Ude, 1983), were also used for immunolabeling with an antibody against *Bombyx mori*-prothoracicotropic hormone (Bom-PTTH). After washing with PBS, the tissues were incubated in a primary antiserum at a working dilution, as listed in Table 1, for 2 days at 4°C. This was followed by incubation in a secondary antiserum of either goat anti-rabbit or horse anti-mouse immunoglobulin conjugated with biotin (for the ABC technique), or swine anti-rabbit immunoglobulin conjugated with tetramethylrhodamine isomer R (TRITC, R0156, Dako, Denmark) (for fluorescence immunocytochemistry), used at a dilution of 1:200 for 2 days at 4°C. The

Table 1. Source or first use of antisera.

Antisera	Working dilutions	Sources of antisera
Anti-bombyxin	1:1000	Mizoguchi et al., 1987
Anti-*Bombyx mori*-prothoracicotropic hormone	1:1000	Mizoguchi et al., 1990
Anti-β-pigment-dispersing hormone	1:5000	Dircksen et al., 1987
Anti-cholecystokinin-8	1:500 or 1:1000*	IncStar Inc., MN
Anti-FMRFamide	1:1000	Peninsula Laboratories Inc., CA
Anti-*Gryllus bimaculatus*-pigment-dispersing factor	1:5000	Dr. K. Tomioka, Okayama University
Anti-*Manduca sexta*-allatotropin	1:500	Veenstra et al., 1994
Anti-ovary ecdysteroidogenic hormone I	1:500	Brown et al., 1998

*, 1:500 dilution for fluorescence immunocytochemistry; 1:1000 for the ABC technique.

primary and secondary antisera were diluted in PBS with 0.5% Triton X (PBST) containing 10% normal goat serum. For the ABC technique, after washing in PBS, the DAB reaction was performed as described in the method of backfills. Preparations were washed in PBS, subsequently dehydrated in an ethanol series, and cleared in methyl salicylate for observation.

Antibody information and specificity

An anti-cholecystokinin-8 (CCK-8) antiserum, which was raised in a rabbit using sulfated CCK-8 coupled to bovine thyroglobulin with glutaraldehyde, was purchased from IncStar (Cat. No. 20078, Stillwater, MN, USA). The specificity test was performed by the pharmacological company: pre-adsorption of the antiserum with CCK-8 peptide completely abolished CCK-8 immunolabeling in the rabbit spinal code and hypothalamus, whereas pre-adsorption of the anti-CCK-8 antiserum with FMRFamide resulted in no reduction of immunolabeling (from the antiserum information sheet). The rabbit anti-FMRFamide antiserum was purchased from Peninsula Laboratories (San Carlos, CA, USA). The specificity against FMRFamide was examined by the pharmacological company by radioimmunoassay, and they also determined that the anti-FMRFamide antiserum does not cross-react with substance P and gastrin peptides (from the antiserum information sheet). A rabbit anti-*Gryllus bimaculatus*-pigment-dispersing factor (*Gryllus*-PDF) antiserum was kindly provided by Dr. K. Tomioka (Okayama University, Okayama, Japan). The epitope structures recognized by the anti-*Gryllus*-PDF have been well characterized by enzyme-linked immunosorbent assay (Honda et al., 2006). The anti-*Gryllus*-PDF antiserum recognizes consensus residues in the PDF peptides of many insect species, such as *D. melanogaster*, the cockroach *Periplaneta americana*, and the cricket *Gryllus bimaculatus* (Honda et al., 2006). In addition, the specificity of the anti-CCK-8, anti-FMRFamide, and anti-*Gryllus*-PDF antisera had been confirmed in adult females of *P. terraenovae* by the lack of immunolabeling after pre-absorption of primary antisera with antigen peptides (Hamanaka et al., 2005, 2007).

Microscopy and measurements

Bright-field images were examined with a compound microscope (BX50-33DIC, Olympus, Tokyo) and fluorescent images with a compound photomicroscope (BX50-34FLA-3, Olympus, Tokyo). Microscopic images were captured with a CCD camera (CoolSNAP, Nippon Roper Co., Chiba). The fluorescence-labeled preparations were also studied with a confocal laser microscope (FluoView, Olympus, Tokyo). TRITC was excited by a green HeNe laser (543 nm), and the fluorescent emission viewed through a 565-nm long-pass filter. The brightness, contrast, and size of images were adjusted using the software Adobe Photoshop 6.0 (Adobe Systems, Tokyo) and Corel Draw 9.0 (Corel, Ottawa). The diameters of somata were measured by using Image J software (NIH), and expressed as the mean±standard deviation (S.D.).

RESULTS

Neurons projecting to a proximal part of the ring gland

HRP was used to reveal neurons projecting to a proximal part of the ring gland. Axon bundles in the proximal part of the ring gland possibly contain fibers projecting to the ring gland (CC, CA, and PG), aorta, frontal ganglion, and stomatogastric system (Possompès, 1953). When HRP was introduced into the proximal part, somata in the PI, PL, and SEG, and distinct fiber bundles, were revealed mainly in the brain and SEG. In our preparations, neurons with somata in the PI and PL (here designated PI and PL neurons, respectively) were successfully labeled in seven larvae, and SEG neurons were labeled in five of the seven larvae. In the PI, 13 to 17 somata were stained per hemisphere (PI neurons, Figs. 2A, 3A). The cell diameters were 15.7±1.5 μm (mean±

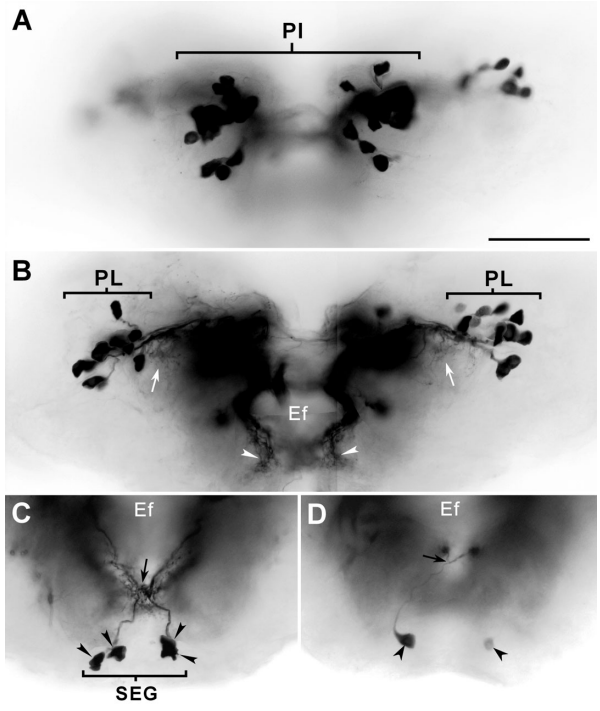

Fig. 2. Frontal views of neurons in the brain and subesophageal ganglion (SEG) backfilled with HRP through a proximal part of the ring gland in a wholemount preparation of the larval blow fly, *P. terraenovae*. Dorsal is to the top. All micrographs are from the same brain and SEG preparation. **(A)** Somata labeled in the PI (PI neurons). **(B)** Somata labeled in the PL (PL neurons) are in focus. Axons of PL neurons bear fine branches in the superior protocerebral neuropil (arrows). Many fine fibers surrounding the esophagus were labeled in the tritocerebral neuropil (long arrowheads). **(C, D)** Somata of SEG neurons are in focus (C is anterior to D). SEG neurons are divided into two subgroups according to their cell locations; two pairs in the anterior region of the ventral cortex of the SEG (long arrowheads in C) and one pair in the posterior region (arrowheads in D). Axons of SEG neurons project dorsally and appear to decussate in the dorsal region of the SEG neuropil (arrows), where the anterior group bears fine branches (arrow in C). Ef, esophageal foramen. Scale bar, 100 μm in A (also applies to B–D).

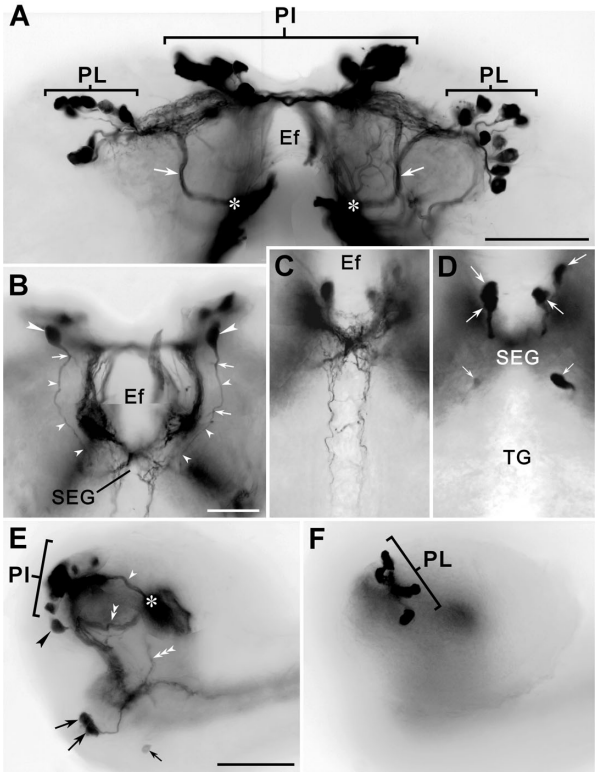

Fig. 3. Dorsal and sagittal views of neurons in the brain and subesophageal ganglion (SEG) backfilled with HRP through a proximal part of the ring gland in a whole-mount preparation of the larval blow fly, *P. terraenovae*. All micrographs are from the same preparation. **(A–D)** Dorsal views with anterior to the top. **(A)** Somata in the PI and PL are seen in the dorsal region of the brain. Somata in the PI are located anteriorly and medially to those in the PL. Most PL neurons seem to send axons ipsilaterally to posterior lateral regions (arrows), to exit the brain through the regions marked by asterisks. **(B)** A plane ventral to the clusters of PI somata. A pair of somata (long arrowheads, ventro-medial neurons) posteriorly sends axons that bifurcate twice (white arrows) to bear three processes in the ipsilateral brain hemisphere. One fiber marked by arrowheads appears to project posteriorly toward the thoracic ganglia (TG). For the projection pattern of the processes, see Fig. 5A2. **(C)** Dorsal region of the SEG neuropil. Two lines of fibers running posteriorly were labeled. **(D)** Somata of SEG neurons in a plane ventral to C. Two pairs are located in the anterior region (large arrows) and one pair in the posterior (small arrows). **(E, F)** Sagittal views of a left brain hemisphere with anterior to the left. **(E)** PI and SEG neurons are in focus. Single, double, and triple white arrowheads show axon bundles of PL, PI, and SEG neurons, respectively. The three different groups of neurons exit posteriorly from the brain through the region marked with an asterisk and project to the ring gland. A long arrowhead indicates the soma of the left ventro-medial neuron marked with a long arrowhead in B. Large arrows indicate somata of the anterior SEG neurons, and the small arrow the soma of a posterior SEG neuron. **(F)** Somata of PL neurons are in focus. Ef, esophageal foramen. Scale bars, 100 μm in A, E (also applies to F); 50 μm in B (also applies to C, D).

S.D.) (n=20 somata, from two larvae). Processes from PI neurons extended ventrally and appeared to decussate in the midline to exit posteriorly from the brain through the contralateral NCC (Fig. 3A, E). The PI neurons also projected sparse fibers in the superior protocerebral neuropil (small arrows in Fig. 5A1).

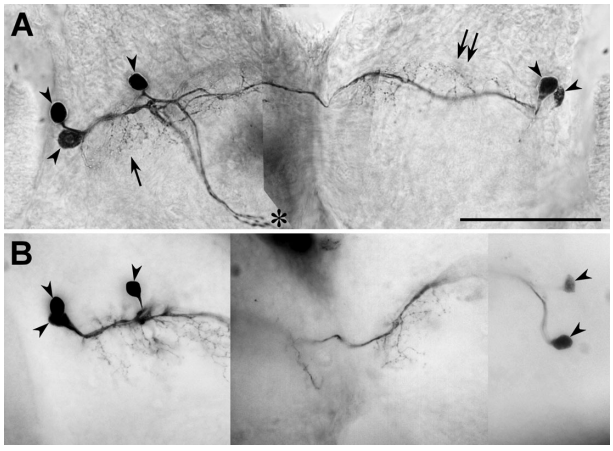

Fig. 4. Neurons in the brain backfilled with $NiCl_2$ unilaterally through a distal part of the ring gland in a whole-mount preparation of the larval blow fly, *P. terraenovae*. **(A)** Dorsal view with anterior to the top. **(B)** Frontal view with dorsal to the top, turned over horizontally. **(A, B)** Three pairs of PL-i neurons were labeled in the left brain hemisphere and two pairs of PL-c neurons in the right. Arrowheads indicate somata of PL-i and PL-c neurons. PL-i neurons extend fine fibers in the superior protocerebral neuropil (arrow). PL-c neurons extend axons contralaterally and bear fine branches along the neurites (double arrow). PL-i and PL-c neurons run in the posterior lateral region and exit the brain through the region indicated by an asterisk. Scale bar, 100 μm in A (also applies to B).

Another pair of neurons, from which axons ran posteriorly in the ipsilateral brain hemisphere and bifurcated twice to bear three processes, was labeled in a ventro-medial region of the brain (Fig. 3B, E). Here we call this type of neuron a ventro-medial neuron. The first branch extended medially, the second joined a dense plexus around the esophageal foramen, probably to exit the brain through the ipsilateral NCC, and the third branch appeared to project posteriorly toward the thoracic ganglia (Figs. 3B, 5A2).

In the PL of each hemisphere, 11 to 14 somata were stained (PL neurons, Figs. 2B, 3A). Their diameters were 17.2±3.2 μm (mean±S.D.) (n=40 somata, from three larvae). The PL neurons projected many fine branches in the superior protocerebral neuropil (Figs. 2B, 3A, 5A1, B1). An axon bundle from the PL neurons ran in a posterior lateral region of the brain neuropil (Fig. 3A). This is considered to correspond to the posterior lateral tract identified in the adult brain (Shiga et al., 2000). In the SEG, three pairs of somata were labeled in the ventral cortex (SEG neurons, Figs. 2C, D, 3D). Their diameters were 17.9±2.6 μm (mean±S.D.) (n=10 somata, from two larvae). Two pairs had somata in the anterior region. The two pairs projected fibers dorsally and appeared to exit the brain through the contralateral NCC (Fig. 2C). The axons bore many fine branches below the esophagus (Figs. 2C, 3C). The last one, a posterior pair, also appeared to send axons to the contralateral side, but did not bear branches beneath the esophagus (Fig. 2D).

Dense fiber projections were observed in the anterior region of the tritocerebral neuropil and in the dorso-anterior region of the SEG neuropil (Figs. 2B, 5B1). At least some fibers in this region are considered to originate in a subset of PL neurons, because corazonin immunocytochemistry

has identified three pairs of PL neurons with fibers in the SEG neuropil in the larval brain of *P. terraenovae* (Cantera et al., 1994).

Neurons projecting to a distal part of the ring gland

Either HRP or $NiCl_2$ was unilaterally introduced to fill neurons sending fibers to a distal part of the ring gland, a region containing the CA and part of the PG. Such backfills succeeded in six larvae and revealed two types of PL neurons, PL neurons with an ipsilateral projection (PL-i neurons) and PL neurons with a contralateral projection (PL-c neurons) to the distal part of the ring gland. There were, at most, three PL-i neurons and two PL-c neurons in each brain hemisphere labeled by such unilateral backfills (Figs. 4, 5D). The PL-i neurons sent fibers to the superior protocerebral neuropil (arrow in Figs. 4A, 5D). The PL-c neurons bore many fine processes along their neurites (double arrow in Figs. 4A, 5D). The two types of PL neurons projected fibers to neither the tritocerebral nor SEG neuropils (Figs. 4, 5D). In five of the six larvae examined, no somata were labeled in either the PI or SEG whereas in the other one, a SEG neuron was weakly labeled; this weak labeling was probably caused by dye leakage.

Peptide immunocytochemistry

Among eight antibodies tested in the current study, four — those against bombyxin, Bom-PTTH, *Manduca sexta*-allatotropin, and ovary ecdysteroidogenic hormone I — labeled neither cells nor fibers in the larval cephalic neuroendocrine system of *P. terraenovae*. The other four antibodies, those against β-pigment-dispersing hormone (β-PDH), sulfated CCK-8, FMRFamide, and *Gryllus*-PDF, labeled several groups of cells in the larval cephalic ganglia as well as the fibers in the aorta and ring gland. Here we report the immunoreactive cells and fibers in the larval cephalic neuroendocrine system.

CCK-8-immunoreactive cells

The anti-CCK-8 antiserum labeled cells in the PI and PL of the protocerebrum, and also in the SEG. A pair of cells was labeled in the PI and SEG, and three pairs in the PL (Figs. 6A, 9A). Axons from the PI cells crossed over the midline to project ventrally. Their terminal region was not resolved in the current study because of weak immunolabeling. By confocal microscopy, axons of the PL cells appeared to run in the posterior

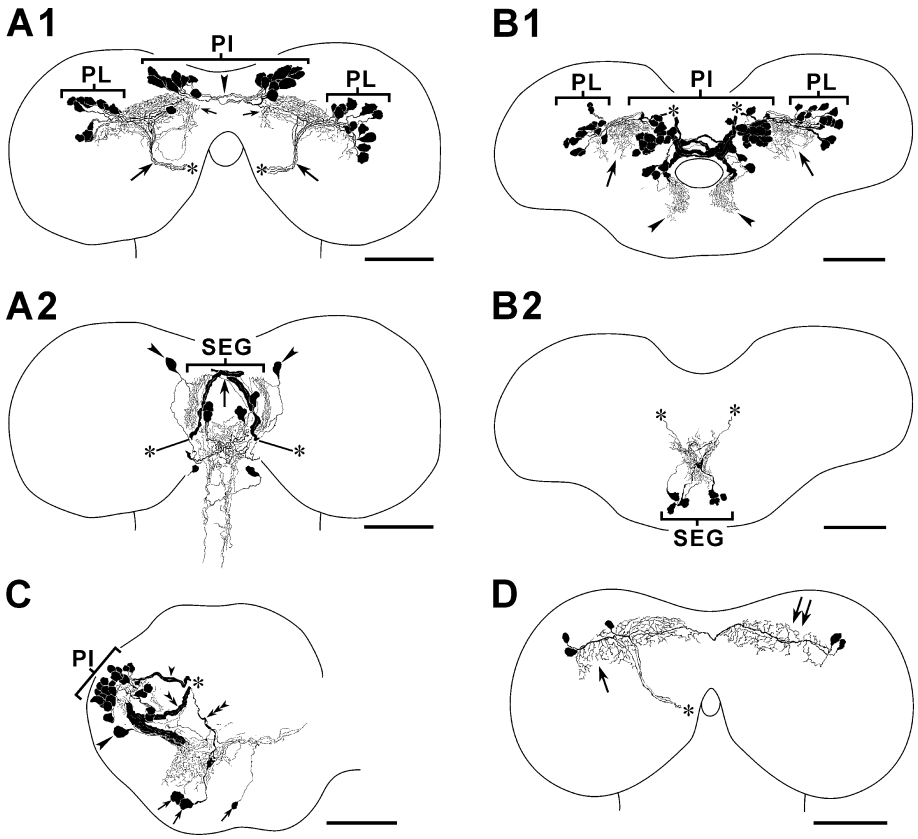

Fig. 5. Drawings of neurons in the brain and subesophageal ganglion (SEG) backfilled through the ring gland, either bilaterally through the proximal part **(A–C)** or unilaterally through the distal part **(D)** in the larval blow fly, *P. terraenovae*. **(A)** Dorsal views with anterior to the top (A1 is dorsal to A2). **(A1)** Somata and fibers labeled in the dorsal region of the brain. Somata of PI and PL neurons, and a pair of distinct fiber tracts (large arrows) originating from PL neurons, are visible. Sparse fibers originating in PI neurons were labeled in the superior protocerebral neuropil (small arrows). The axons of PL neurons exit the brain through the regions marked by asterisks. Some fibers project to the contralateral brain hemisphere (arrowhead). **(A2)** Somata and fibers labeled in the ventral part of the brain, and the SEG. Three pairs of SEG neurons and a pair of somata in the ventro-medial region of the brain (long arrowheads, ventro-medial neurons) are visible. Axons from somata in the ventro-medial region produce three processes. One extends medially, and another joins the dense fiber plexus surrounding the esophagus. The latter probably exits the brain ipsilaterally through the nervus corporis cardiaci (NCC). The third process projects posteriorly toward the thoracic ganglia. Fiber bundles originating from somata in the PI (arrow) appear to decussate in the midline and exit the brain through the contralateral NCC (asterisks). **(B)** Frontal views with dorsal to the top (B1 shows PI and PL neurons, and B2 shows SEG neurons). **(B1)** Many fine fibers derived from the axons of PL neurons are visible in the superior protocerebal neuropil (arrows). Dense, fine branches are labeled around the esophageal foramen (long arrowheads). The axons of PI and PL neurons exit the brain through the regions marked by asterisks. **(B2)** SEG neurons dorsally extend the axons, which appear to decussate under the esophagus and exit the brain contralaterally from the regions marked with asterisks. **(C)** Sagittal view of the left brain hemisphere with anterior to the left. Three distinctive fiber tracts from PL neurons (single arrowhead), PI neurons (double arrowhead), and SEG neurons (triple arrowhead) are visible. These axons exit from the brain through the region marked with an asterisk. A long arrowhead indicates the soma of a ventro-medial neuron, and arrows indicate somata of SEG neurons. **(D)** Three PL-i neurons and two PL-c neurons (dorsal view with anterior to the top). PL-c neurons bear fine branches along the neurites (double arrow). PL-i neurons extend fibers in the superior protocerebral neuropil (arrow). Their axons exit the brain from the region marked by an asterisk. Scale bars, 100 μm.

lateral region of the brain neuropil and project anteriorly toward the ring gland (Fig. 6A). Cells in the other two brain regions also showed CCK-8 immunoreactivity. Three or four pairs of cells were labeled in the posterior region of the lateral protocerebrum, and one or two pairs in the dorso-posterior region of the brain, close to the midline (Figs. 6A, 9A). The former bore short processes close to the cells (Fig. 9A). The signal intensity in the two groups of cells was weak and variable among preparations. Besides these, a pair of ascending fibers was labeled in the brain neuropil, probably originating in cells located in the thoracico-abdominal ganglion (arrowheads in Fig. 9A). In the ring gland, about six pairs of cells were labeled in the lateral and proximal regions, but none in the distal (Fig. 6B, C). These cells extended fibers to the proximal region of the ring gland. Immunoreactive fibers were found in the aorta. These fibers were possibly derived from somata in the brain and ring gland (Fig. 6C). Also in *Calliphora vicina*, cells in the PI and a proximal part of the ring gland have been reported to be immunoreactive with an anti-gastrin/CCK antiserum (Cantera, 1988).

FMRFamide-immunoreactive cells

The anti-FMRFamide antiserum labeled a large number of cells in the brain and SEG. The distribution of FMRFamide-immunoreactive cells in *P. terraenovae* is comparable to that of FMRFamide-immunoreactive or expressing cells in larvae of *D. melanogaster* (White et al., 1986; Schneider et al., 1991, 1993). About four or five pairs of cells were labeled in the PI, and six or seven pairs in the PL (Figs. 7A, B, 9B). In the dorso-medial region of the brain, a pair of cells was labeled (arrowheads in Fig. 7B). In the dorso-posterior region of the brain, three or four pairs of cells were labeled (arrows in Fig. 7B). Besides these, a large number of addi-

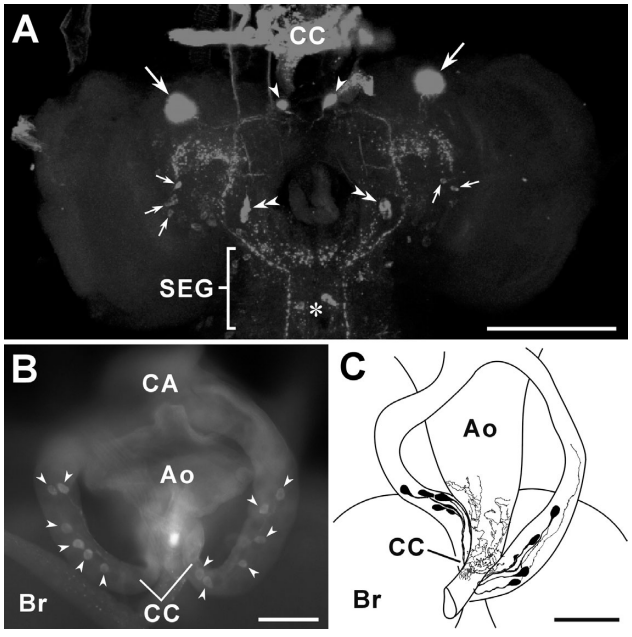

Fig. 6. CCK-8 immunoreactivity in the larval cephalic ganglia and ring gland of the blow fly, *P. terraenovae*. (A, B) Dorsal views with anterior to the top. **(A)** Confocal stacks. Large arrows indicate a clump of three cells in the PL. Because of strong immunoreactivity in the somata, three cells are indistinguishable in the confocal stacks. A pair of cells was labeled in the PI (arrowheads) and subesophageal ganglion (SEG, asterisk), and two pairs in the dorso-posterior region of the brain close to the midline (double arrowheads). Two or three pairs of cells were labeled in the posterior region of the lateral protocerebrum (small arrows). **(B)** Six pairs of cells were labeled in a lateral and proximal part of the ring gland (arrowheads). **(C)** A drawing of CCK-8-immunoreactive cells in the ring gland. Fibers projecting to the aorta (Ao) are visible. Br, brain; CA, corpus allatum; CC, corpus cardiacum. Scale bars, 100 μm.

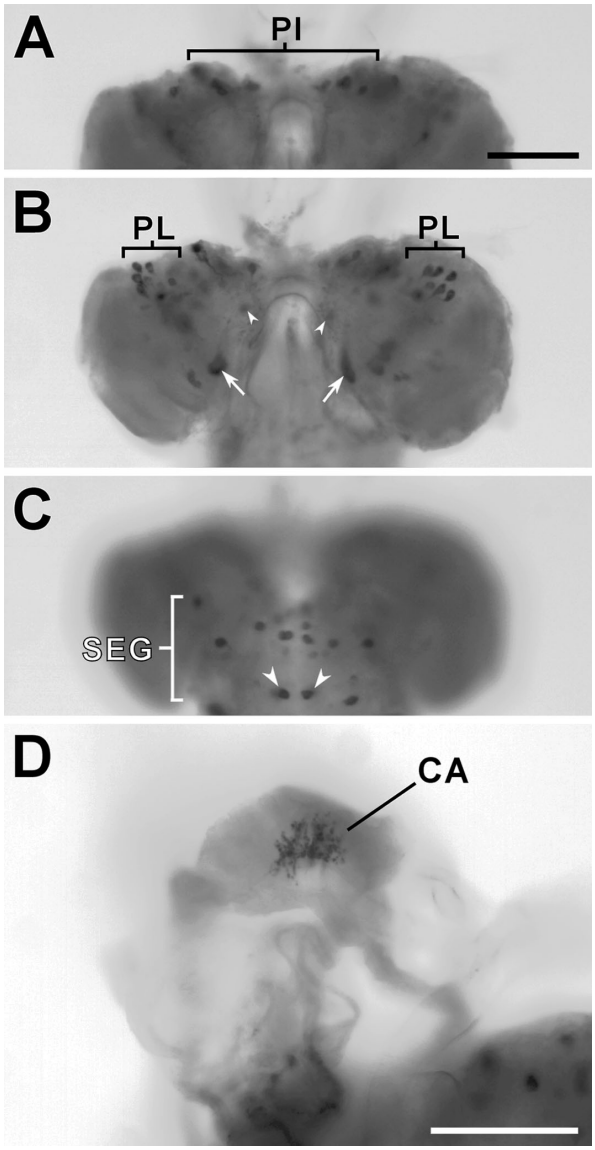

Fig. 7. FMRFamide immunoreactivity in the larval cephalic ganglia and ring gland of the blow fly, *P. terraenovae*. **(A–D)** Dorsal views with anterior to the top. (A, B) Cells in the **(A)** PI and **(B)** PL immunolabeled with the anti-FMRFamide antiserum. In addition, a pair of cells was labeled in the dorso-medial region (arrowheads, out of focus) and three pairs in the dorso-posterior region (arrows). Additional cells were also labeled in the brain. **(C)** Immunolabeled cells in the anterior region of the subesophageal ganglion (SEG) and those in the posterior (arrowheads) are visible. **(D)** Dense, varicose FMRFamide-immunoreactive fibers in the corpus allatum (CA). Scale bars, 100 μm in A (also applies to B, C), D.

tional cells showed FMRFamide immunoreactivity in the brain (Fig. 9B). In the ventral cortex of the SEG, two groups of cells were labeled. The first, of six or seven pairs of immunoreactive cells, was located in the anterior region, and the second, of two pairs, located medially in the posterior region (Figs. 7C, 9B). In our preparations, we could not resolve the axon pathways of cells in the PI, PL and SEG because of the complexity of the network of immunoreactive fibers. In the distal part of the ring gland or the CA, dense immunoreactive fibers were observed (Fig. 7D).

***Gryllus*-PDF-immunoreactive cells**

We compared neurons labeled by the anti-β-PDH antiserum with those immunopositive to the anti-*Gryllus*-PDF antiserum in the brain and SEG of *P. terraenovae*, and found that they were identical in cell location and fiber projection patterns. In this report, we refer only to cells immunoreactive with the anti-*Gryllus*-PDF antiserum. Three different types of cells were labeled in the brain. The first group, consisting of a pair of cells, was located in the PI. The axons crossed the midline, projected ventrally along the foramen of the esophagus (Figs. 8A, 9C), and appeared to terminate in the dorsal region of the SEG neuropil beneath the esophagus. The second, consisting of three cells, was located in the PL (Figs. 8B, 9C). The third group consisting of four cells was located in the vicinity of the optic neuropil (Figs. 8B, 9C). These cells bore small branches close to them (inset of Fig. 8B) and their axons projected dorsally to bear many varicose fibers in the dorso-lateral protocerebral neuropil (Figs. 8B, 9C). Besides these, a pair of ascending fibers, which probably originating in cells in the thoracico-abdominal ganglion, was labeled in the brain neuropil (arrowheads in Fig. 9C). The terminal region was close to that of the PDF-immunoreactive cells in the vicinity of the optic neuropil. PDF-immunoreactive varicose fibers were detected in the wall of the aorta, but not in the ring gland (Fig. 8C).

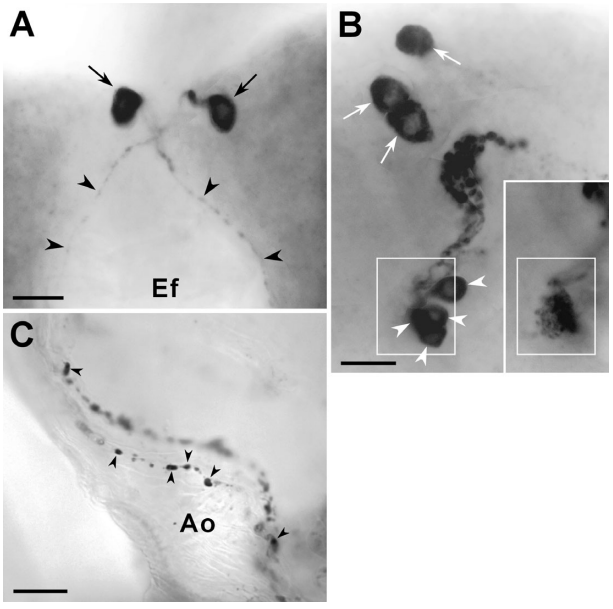

Fig. 8. *Gryllus*-PDF immunoreactivity in the larval cephalic ganglia and aorta of the blow fly, *P. terraenovae*. **(A, B)** Dorsal views with anterior to the top. **(A)** A pair of cells labeled in the PI (arrows). The axons decussate in the midline to run ventrally and posteriorly along the esophageal foramen (Ef, arrowheads). **(B)** Three cells in the PL labeled by the anti-PDF antiserum (arrows). Four PDF-immunoreactive cells close to the optic neuropil in a left brain hemisphere were also visible (arrowheads). The cells extend small fine branches close by (inset) and also project varicose fibers to the dorso-lateral protocerebral neuropil. Inset: A region slightly dorsal to the main image (the rectangles show the same area). **(C)** PDF-immunoreactive varicose fibers on the wall of the aorta (Ao, arrowheads). Scale bars, 20 μm in A, B (also applies to inset of B), C.

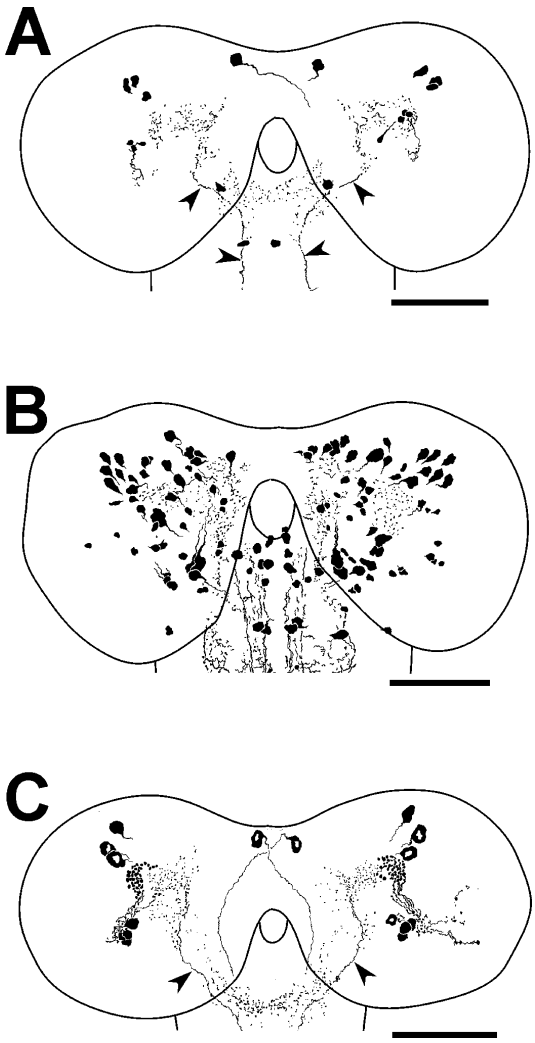

Fig. 9. Drawings of cells and fibers labeled with the antisera against **(A)** CCK-8, **(B)** FMRFamide, and **(C)** *Gryllus*-PDF in the larval cephalic ganglia of the blow fly, *P. terraenovae*. Dorsal views with anterior to the top. **(A)** A pair of cells was labeled in each of the PI and SEG, and three pairs in the PL, with two other types of cells labeled in the brain. Besides these, a pair of ascending fibers is seen in the brain neuropil (arrowheads). **(B)** Cells in the PI, PL, and SEG labeled by the anti-FMRFamide antiserum are visible, and additional cells also in the brain. **(C)** A pair of cells is located in the PI and three pairs in the PL. Four additional pairs of cells, located close to the optic neuropil, project varicose fibers to the dorso-lateral protocerebral neuropil. Besides these, a pair of ascending fibers was labeled in the brain neuropil (arrowheads). Scale bars, 100 μm.

DISCUSSION

The current study revealed four kinds of neurons, PI, ventro-medial, PL, and SEG neurons, projecting to the ring gland in *P. terraenovae*.

PI neurons

Brains with a small number of cerebral neurons backfilled revealed that a subset of PI neurons projected the axons to the contralateral brain hemisphere to join the CC, and sent fibers to the SEG and/or tritocerebral neuropil as well as the protocerebral neuropil. PI neurons with contralateral projection have also been identified by intracellular dye injection in the larval brains of *B. mori* (Ichikawa, 1991) and *M. sexta* (Copenhaver and Truman, 1986), and by the GAL4-UAS system of targeted gene expression in *D. melanogaster* larvae (Siegmund and Korge, 2001). Therefore, this seems to be a common feature of PI neurons, at least in the holometabolous insect larval brain.

A pair of neurons in the ventro-medial region

In the ventro-medial region of the brain, a pair of cells (ventro-medial neurons) was labeled by filling from the proximal ring gland. Although fiber projections from these cells were not completely resolved, the cell location and axon trajectory were distinct from those of PI neurons and reminiscent of ventromedial neurons (VM neurons) identified by the GAL4-UAS system in the larval brain of *D. melanogaster* (Siegmund and Korge, 2001). The *Drosophila* VM neurons are considered to be eclosion hormone-producing cells because of their immunoreactivity to this hormone, and they extend a fiber anteriorly to terminate in the CC through the ipsilateral NCC (Siegmund and Korge, 2001). According to their morphological properties, the ventro-medial neurons of *P. terraenovae* seem to be counterparts of the *Drosophila* VM neurons and might be involved in ecdysis. Since the ventro-medial neurons were not labeled by backfilling from a distal part of the ring gland, these neurons seem to have terminals in the CC or in the aorta, as do the *Drosophila* VM neurons.

PL neurons

Two types of PL neurons projecting to a distal part of the ring gland, those with ipsilateral projections (PL-i neurons) and those with contralateral projections (PL-c neurons), were identified in the current study. There are three PL-i and two PL-c neurons per hemisphere. In the larval brains of *D. melanogaster* and *Sarcophaga crassipalpis* also, the same numbers of PL-i and PL-c neurons project fibers to the distal part of the ring gland (Giebultowicz and Denlinger, 1985; Siegmund and Korge, 2001). In *D. melanogaster*, three PL-i neurons (CA-LP) innervate the CA and two PL-c neurons (PG-LP) innervate the PG (Siegmund and Korge, 2001).

The larval PL-c neurons of *P. terraenovae* share some morphological properties, i.e., cell number, cell location, dense collateral fibers along the neurite, and remarkable contralateral projection, with the PTTH-producing PL neurons of *B. mori* and *D. melanogaster* larvae (Mizoguchi et al., 1990; McBrayer et al., 2007) and *M. sexta* pupae (O'Brien et al., 1988). These morphological properties suggest that the PL-c neurons of *P. terraenovae* produce PTTH. Lepidopteran PTTH stimulates ecdysteroid biosynthesis in the PG, whereas the *Drosophila* homolog of lepidopteran PTTH (*Drosophila* PTTH) regulates developmental timing and body size, but not ecdysteroid production (McBrayer et al., 2007). According to the morphological similarity, it is possible that the PL-c neurons of *P. terraenovae* also control developmental timing and body size, as do the *Drosophila* PTTH-producing neurons. Because of less similarity between dipteran PTTH and lepidopteran PTTH (McBrayer et al., 2007), the antiserum against Bom-PTTH might not label any somata in *P. terraenovae*.

In *M. sexta* larvae, eight to ten 1b cells in the PL are considered to regulate the CA, because these cells and fibers in the CA show immunoreactivity to *M. sexta*-allatostatin (Žitňan et al., 1995). In terms of cell location and projection pattern, the 1b cells in *M. sexta* are similar to the PL-i neurons innervating the distal part of the ring gland in *P. terraenovae*. In *D. melanogaster* also, CA-LP1 and CA-LP2 neurons have somata in the PL and ipsilaterally project fibers to the CA (Siegmund and Korge, 2001). It is an intriguing question whether the PL-i neurons are involved in the regulation of JH biosynthesis in flies.

Backfills from a proximal part of the ring gland will label neurons terminating in the CC, and those sending axons passing through the CC to the aorta, PG, and CA, and also possibly to the frontal ganglion and stomatogastric system. Backfills from the proximal part revealed 11–14 cells, whereas those from the distal part composed of the PG and CA revealed five cells (three PL-i and two PL-c somata per hemisphere), indicating that 6–9 neurons terminate in the CC or pass through it to innervate the aorta, frontal ganglion, or stomatogastric system. Among them, at least three PL neurons, which are immunoreactive with anti-corazonin antiserum, pass through the CC without branching there to innervate the ventral wall of the aorta, with fiber arborization in the protocerebral and SEG neuropils (Cantera et al., 1994).

Fiber arborization of the PI and PL neurons was observed in a restricted region of the superior protocerebral neuropil, mainly between the soma cluster in the PI and that in the PL in each hemisphere. It seemed that each PI or PL neuron bore fibers close to the soma and along the axon. Observation from the frontal, dorsal, and sagittal directions showed that the projection area was small, but there dense arborization was found. This region must contain dendritic fields of PI and PL neurons. Their putative dendritic fibers in the protocerebral neuropil probably serve as an input region for interneurons of the third or fourth order representing sensory information. However, it seems that neurons giving them sensory inputs have not been identified in any species. PL neurons also seem to receive internal information, such as the circadian rhythm. In the larval brains of *D. melanogaster* and *P. terraenovae*, PDF-positive neurons, putative larval circadian clock neurons, with somata close to the optic neuropil, project varicose fibers toward fibers derived from the axons of PL neurons at the superior-lateral protocerebral neuropil (Siegmund and Korge, 2001; Hamanaka et al., 2005). These observations suggest that larval PL neurons receive circadian rhythm information, which possibly serves to control the production and/or release of neurosecretory materials.

SEG neurons

SEG neurons appear to send axons to the contralateral NCC, and produce an arborization in the anterior region of

the SEG neuropil. In the larval brain of *D. melanogaster*, the GAL4-UAS system reports two types of SEG neurons (CC-MS1 and CC-MS2), both of which innervate the CC, and there are two pairs of CC-MS1 in the anterior region of the SEG and a pair of CC-MS2 in the posterior region (Siegmund and Korge, 2001). Two types of SEG neurons identified in the current study might be counterparts of CC-MS1 and 2 in *D. melanogaster*, according to their cell number and location. In *S. bullata* larvae, about eight pairs of somata in the SEG express Neb-pyrokinin-2, which is presumably released into the hemolymph through the aorta and ring gland to accelerate puparium formation (Verleyen et al., 2004). In *D. melanogaster* larvae, 20 cells in the SEG, all of which express *hugin* and a subset of which projects to the CC, have been demonstrated to modulate feeding behavior (Melcher and Pankratz, 2005; Bader et al., 2007). Some of SEG neurons identified in the current study might control feeding behavior and also puparium formation.

Peptide immunocytochemistry

CCK-8, FMRFamide, and PDF immunoreactivities were detected in cells in the PI and PL of the brain, and in the SEG as well as in fibers in the aorta and ring gland. These results suggest that some of the cephalic neurons projecting to the aorta and ring gland signal using CCK-8, FMRFamide, and PDF-like peptides, although double labeling by backfills and immunocytochemistry will be required for ultimate proof.

Gastrin/CCK peptides are members of the gastrointestinal peptide family (Strand, 1999). Peptides homologous to gastrin/CCK, referred to as sulfakinins, have been isolated in several insect species (reviewed by Nässel, 2002). It is likely that the anti-CCK-8 antiserum recognizes insect sulfakinins, because CCK-8 and the C-terminal amino acid sequence of insect sulfakinins share five amino acids. A sulfakinin gene has been cloned in *D. melanogaster* and *Calliphora vomitoria* (Nichols et al., 1988; Duve et al., 1995). In *D. melanogaster*, the sulfakinin gene encodes three kinds of peptides, drosulfakinins 0, I, and II. Immunocytochemistry using antisera against a unique N-terminal extension of the respective peptides has revealed several types of drosulfakinin-immunoreactive cells in the larval brain and SEG of *D. melanogaster* (Nichols and Lim, 1996). Among them, the distribution of drosulfakinin 0-immunoreactive cells is quite similar to that of CCK-8-immunoreactive cells in *P. terraenovae*. However, it is unlikely that the anti-CCK-8 antiserum recognizes drosulfakinin 0 or its homolog, because drosulfakinin 0 has no sequence similarity with insect sulfakinins (Nichols et al., 1988). The spatial expression pattern of sulfakinins in *P. terraenovae* might therefore be different from that in *D. melanogaster*.

Sulfakinins have been identified as a feeding satiety factor in the German cockroach, *Blattella germanica* (Maestro et al., 2001), the desert locust, *Schistocerca gregaria* (Wei et al., 2000), and the blow fly, *Phormia regina* (Downer et al., 2007). CCK-8-like or sulfakinin peptides might therefore be involved in feeding regulation also in *P. terraenovae* larvae.

FMRFamide and FMRFamide-related peptides (FaRPs), which possess -RFamide residues in the C terminus, are a diverse and well-studied family of neuropeptides, identified mainly as a myomodulator in invertebrates (reviewed by Orchard et al., 2001; Nässel, 2002). In *D. melanogaster*, there are five types of FaRPs: dFMRFamides (2, 3, 4, 5, 6 and 8), dromyosuppressin, drosulfakinins (1 and 2), neuropeptide F, and short neuropeptides Fs (1 and 2) (reviewed by Nässel, 2002). Because the antiserum against FMRFamide recognizes -RFamide residues in the C terminus (Weber et al., 1981), a variety of peptides including FaRPs could be detected by this antiserum. In the *D. melanogaster* larval brain, a subset of PI neurons produces myosuppressin (Wegener et al., 2006). Some PI cells immunoreactive to FMRFamide in the current study are likely to express myosuppressin. Furthermore, in *D. melanogaster* a subset of PL neurons produces corazonin and short neuropeptide F (Wegener et al., 2006), and it has also been found histologically that three pairs of PL neurons with projection to the ring gland co-express corazonin and short neuropeptide F (Nässel et al., 2008). *Protophormia terraenovae* corazonin-immunoreactive PL neurons identified by Cantera et al. (1994) are therefore likely to express *Drosophila* short neuropeptide F homologs as well, which could have been detected by the anti-FMRFamide antiserum in the current study.

Interestingly, the CA of *P. terraenovae* is innervated by FMRFamide-immunoreactive fibers. These fibers are most likely to originate in FMRFamide-immunoreactive cells in the PL, since backfills from the distal part of the ring gland or the CA stained only PL-i and PL-c neurons. In *D. punctata*, an anti-RFamide antiserum labels fibers in the CA as well as cells in the PI and PL, and endogenous FIRFamide promotes JH biosynthesis in the CA by attenuating allatostatin activity (Stay et al., 2003). Also in *P. terraenovae*, a subset of FMRFamide-immunoreactive cells in the PL with projection to the CA may modulate JH biosynthesis by the CA.

Pigment-dispersing factor (PDF) is a neuropeptide well known as an output neuromodulator from circadian clock neurons in the optic lobe of the adult brain of *D. melanogaster* (Renn et al., 1999). The clock neurons already appear in the larval stage (Helfrich-Förster, 1997). Homologous cells have also been identified in the lateral part of the larval brain in the flies *Musca domestica* and *P. terraenovae* (Pyza et al., 2003; the present study). Additionally, PI and PL cells as well as fibers in the aorta in *P. terraenovae* were immunoreactive with the anti-PDF antiserum. Since a pair of PI cells appeared to have terminals in the dorsal region of the SEG neuropil, PDF-immunoreactive fibers in the aorta are likely derived from PL cells. In the adult brain of *P. terraenovae*, PDH-immunoreactive PL neurons with fibers in the aorta and CC have been identified (Hamanaka et al., 2004; 2007). A subset of larval PL cells might release PDF-like peptides from the aorta into the hemolymph. In *D. melanogaster* larvae, however, in-situ hybridization has not confirmed expression of the *pdf* transcript anywhere else except for four pairs of lateral neurons close to the optic neuropil (Park et al., 2000). PDF immunoreactivity in the PL cells and aorta might be due to PDF-like peptides, but not authentic PDF. It has been suggested that in *P. terraenovae* and *Locusta migratoria*, PDF-like peptides function as neurohormones released from the abdominal ganglia (Nässel et al., 1993; Persson et al., 2001). The present results imply that brain neurons may also employ PDF-like peptides as neurohormones.

In the cephalic ganglia, similar types of cells were labeled by different antisera. For example, the anti-CCK-8 and anti-*Gryllus*-PDF antisera similarly labeled two types of cells, i.e., a pair of cells in the PI and three pairs of cells in

the PL, and the anti-FMRFamide antiserum also labeled cells in the PI and PL. CCK-8 and *Gryllus*-PDF share Asp in the second position from the C terminus and an amide residue at the C terminus. CCK-8 and FMRFamide share Phe in the first position and Met in the third position from the C-terminus, and an amide reside in the C terminus as well. The sequence similarity is quite restricted, and furthermore it has been demonstrated that cross reactivity between the anti-CCK-8 and anti-FMRFamide antisera hardly occurs in the adult brain of *P. terraenovae* (Hamanaka et al., 2007). Therefore it seems that the similar distribution pattern implies co-localization of their immunoreactivities, and thus of different peptides rather than cross reactivity. In the adult brain of *P. terraenovae*, co-localization of β-PDH, CCK-8, corazonin, and FMRFamide immunoreactivities has been demonstrated (Hamanaka et al., 2007).

In *Drosophila* larvae, Wegener et al. (2006) made a comprehensive schematic representing peptide phenotypes of cephalic neurosecretory cells by comparison of a mass spectrometric peptide profiling of the ring gland with previously reported histological results. Also in *P. terraenovae*, future studies combining biochemistry and molecular biology will be necessary to fully understand the true peptide products in these neurons.

The present study revealed the number, cell location and fiber distribution of neurons projecting to the ring gland in *P. terraenovae*. In comparison with *D. melanogaster* and *S. crassipalpis,* cyclorrhaphan larvae seem to share comfeatures in the morphology of the cephalic neuroendocrine system. Due to its large body size, *P. terraenovae* is amenable to physiological studies including pharmacological and electrophysiological experiments. We await subsequent biochemical or physiological analyses of the identified cephalic neurons in order to understand the neuroendocrine mechanisms underlying important developmental and physiological phenomena.

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