Published in "The Journal of Comparative Neurology 502(5): 848 - 856, 2007"
which should be cited to refer to this work.

Genetic Dissection of Neural Circuit Anatomy Underlying Feeding Behavior in *Drosophila*: Distinct Classes of *hugin*-Expressing Neurons

RÜDIGER BADER,[1*] JULIEN COLOMB,[2*] BETTINA PANKRATZ,[1] ANNE SCHRÖCK,[1] REINHARD F. STOCKER,[2] AND MICHAEL J. PANKRATZ[1*]
[1]Institut für Genetik, Forschungszentrum Karlsruhe, 76021 Karlsruhe, Germany
[2]Department of Biology, University of Fribourg, CH-1700 Fribourg, Switzerland

ABSTRACT

The *hugin* gene of *Drosophila* encodes a neuropeptide with homology to mammalian neuromedin U. The *hugin*-expressing neurons are localized exclusively to the subesophageal ganglion of the central nervous system and modulate feeding behavior in response to nutrient signals. These neurons send neurites to the protocerebrum, the ventral nerve cord, the ring gland, and the pharynx and may interact with the gustatory sense organs. In this study, we have investigated the morphology of the *hugin* neurons at a single-cell level by using clonal analysis. We show that single cells project to only one of the four major targets. In addition, the neurites of the different *hugin* cells overlap in a specific brain region lateral to the foramen of the esophagus, which could be a new site of neuropeptide release for feeding regulation. Our study reveals novel complexity in the morphology of individual *hugin* neurons, which has functional implication for how they coordinate feeding behavior and growth.

Feeding is one of the most conserved activities of animals. Although animals have evolved a wide spectrum of feeding behaviors in terms of food preferences and foraging strategies, there is a fundamental need to regulate food intake relative to growth, reproductive, and metabolic needs. Since the groundbreaking work on the cloning of the gene encoding the hormone leptin (Zhang et al., 1994), much progress has been made in mammals in analyzing the roles of various neuropeptides in food intake and energy balance regulation. These have come mostly from knockout experiments and subsequent physiological analysis of feeding behavior and metabolic consequences. However, the neural circuits in the brain that mediate the activities of these genes, and how these circuits function under various nutrient conditions and experiences, remain largely unknown (Broberger, 2005).

Drosophila provides a genetically accessible system for studying the neural circuits that control innate behaviors such as feeding and mating. We recently identified a group of neurons in the *Drosophila* central nervous system (CNS), named *hugin* neurons, that modulates feeding behavior in response to nutrient signals (Melcher and Pankratz, 2005). We also provided evidence that *hugin* is a *Drosophila* homolog of the mammalian gene encoding the neuropeptide neuromedin U (Melcher et al., 2006), which has been shown to regulate food intake and body weight in rodents (Howard et al., 2000; Hanada et al., 2004; Brighton et al., 2004). These observations suggested that *hugin* and neuromedin U may be part of a conserved neural pathway for regulating feeding behavior and metabolism.

In the *Drosophila* larva, *hugin* is expressed in 20 cells of the subesophageal ganglion (SOG; Meng et al., 2002; Melcher and Pankratz, 2005). The *hugin* neurons send

The first two authors contributed equally to this work.

Grant sponsor: Deutsche Forschungsgemeinschaft (to M.J.P.); Grant sponsor: Swiss National Funds; Grant number: 3100AO-105517 (to R.F.S.).

*Correspondence to: Michael J. Pankratz, Institut für Genetik, Forschungszentrum Karlsruhe, 76021 Karlsruhe, Germany.
E-mail: michael.pankratz@itg.fzk.de

neurites to the protocerebrum, the ventral nerve cord, the central neuroendocrine organ (known as the ring gland), and the pharynx. Furthermore, arborizations of the *hugin* neurons in the SOG lie in close proximity to axon terminals of specific gustatory sensory neurons (Stocker, 1994; Melcher and Pankratz, 2005; Scott, 2005; Amrein and Thorne, 2005), leading to the proposal that *hugin* neurons may represent second-order interneurons that mediate taste information (Melcher and Pankratz, 2005). The connectivity pattern of the *hugin* neurons also raised the issue of the target specificity of individual *hugin* neurons. There was already some evidence that certain groups of *hugin* neurons projected to specific targets, as deduced from double staining with different markers (Melcher and Pankratz, 2005). However, the limited availability of markers allowed identification of only some of the groups, namely, those that projected to the ring gland and the pharynx; it could not be determined whether *hugin* cells that projected to the protocerebrum or the ventral cord also showed unique target specificity. Moreover, no information could be attained at single-cell resolution, because the available markers labelled groups of cells and not individual cells.

To overcome these shortcomings, we have used in this study genetically produced clones to analyze, at the single-cell level, the morphology of individual neurons of the *hugin* neural cluster in the *Drosophila* larva. Our results indicate that single neurons project to single targets. Furthermore, they revealed complexities in the morphology of individual *hugin* neurons that were not apparent from studying the entire neuronal cluster. This has led to the identification of a novel region bordering the esophageal foramen and the SOG that could be involved in feeding regulation.

MATERIALS AND METHODS

Clonal analysis

Flies harboring hugS3-Gal4 were crossed with those carrying the flp out constructs [y w hsFLP; Sp/CyO;UAS > CD2y+ > CD8-GFP; a gift of Barry Dickson (IMP, Vienna, Austria)]. A 24-hour egg collection was heat shocked for 2 hours at 37°C. The larval brains were prepared at late third instar. This procedure resulted in a wide range of flp out events in terms of numbers of clones per brain. For single-cell clones, the following numbers of independent samples were scanned: protocerebrum, 14; ring gland, 10; pharynx, 7; ventral nerve cord, 4.

Histochemistry and fluorescence microscopy

Immunofluorescent stainings were carried out essentially as described by Melcher and Pankratz (2005). Images were taken with a Zeiss (Oberkochen, Germany) LSM 510 Meta in transmission mode or a Leica TCS SP2 (Wetzlar, Germany). Primary antibodies were applied overnight at 4°C. Secondary fluorescent antibodies were applied for 1 hour at room temperature. Samples were mounted in Mowiol and analyzed with a Zeiss LSM 510 Meta in confocal multitracking mode, generating optical 1–1.5-μm sections (using a Zeiss ×40/1.2-W C-Apochromat lens) or 2.5-μm sections (using a Zeiss ×25/0.8Imm Plan-Neofluar lens). For direct detection of YFP fluorescence, larval brains of appropriate genotype were dissected in chilled *Drosophila* Ringer's solution on ice, and mounted without fixation in PBS. Primary antibodies used for immunofluorescence were anti-GFP (dilution 1:1,000; Abcam, Cambridge, United Kingdom; catalogue No. ab6556) as well as the neuronal markers 22C10 (dilution 1:100), anti-elav (Elav-9F8A9; dilution 1:300; both Developmental Studies Hybridoma Bank, Iowa City, IA), and anti-nc82 (dilution 1:50; gift of Erich Buchner, Wuerzburg, Germany). See below for detailed information on the primary antibodies used. The secondary antibodies used were Alexa 488-coupled anti-mouse antibody (dilution 1:200) from goat, Cy3-coupled anti-mouse antibody (dilution 1:200) from goat (Molecular Probes, Eugene, OR), and Cy2- or Cy3-coupled anti-rabbit antibody (dilution 1:200) from goat (Jackson Immunoresearch, West Grove, PA). Nuclear counterstaining was performed with Draq5 (dilution 1:1,000; Biostatus Ltd., Leicestershire, United Kingdom), together with secondary antibodies.

For stainings with a CD2 background, the procedure was adapted from the protocol of Ramaekers et al. (2005). Briefly, young third-instar larvae (72–96 hours AEL) were predissected in phosphate buffer (PB; 0.1 M, pH 7.2). The brains attached to the body wall were fixed for 20 minutes in PB containing 3.7% formaldehyde and subsequently rinsed in PBT (0.3% Triton X-100 in PB). They were further dissected and placed for 2 hours in PBT in 5% normal goat serum (NGS) at room temperature for blocking. Subsequently, they were incubated with a cocktail of primary antibodies overnight at 4°C. Primary antibodies included two neuronal markers: the mouse monoclonal anti-ChATB1 (dilution 1:500; Developmental Studies Hybridoma Bank) and nc82 (dilution 1:20) from A. Hofbauer (University of Regensburg). In addition, we used rabbit polyclonal anti-GFP (dilution 1:1,000; Molecular Probes; A6455) and monoclonal anti-rat-CD2 (dilution 1:100; Serotec GmbH, Düsseldorf, Germany; MCA154R). After several rinses in PBT, samples were incubated overnight in PBT-NGS with the secondary antibodies anti-rabbit Alexa 488-conjugated antibody from goat (dilution 1:200) and anti-mouse Cy3-conjugated antibody from goat (dilution 1:200; Molecular Probes). After several rinses, brains were mounted in Vectashield (Vector, Burlingame, CA), with nail polish used as spacer. The CNS was mounted with the ventral nerve cord on top. Images of the periphery were taken by using a fluorescence microscope (Leica DM R) equipped with a CCD camera. Stacks of confocal images at 0.93-μm focal plane spacing were collected with a Bio-Rad MRC 1024 confocal microscope and Laser Sharp image-collection software. Images were then processed in Image J freeware (http://rsb.info.nih.gov/ij/index.html), and curves (input to output options) were readjusted for each color independently but always on the whole picture. The intensity of nonspecific background staining was lowered by using the "dust and scratches" filter in Adobe Photoshop 7.0 for Macintosh.

Information on primary antibodies

Rabbit anti-GFP antibody (Abcam) was raised against a highly purified recombinant GFP made in *Escherichia coli* and is reactive against all versions of *Aequora victoria* GFP, such as S65T-GFP, RS-GFP, YFP, and EGFP; rabbit anti-GFP (Molecular Probes) was raised against GFP isolated directly from *Aequora victoria* and recognizes the ectopically expressed GFP; anti-22C10 is a monoclonal mouse antibody that recognizes a protein band of 500 kDa present in *Drosophila* wild-type extracts, which was iden-

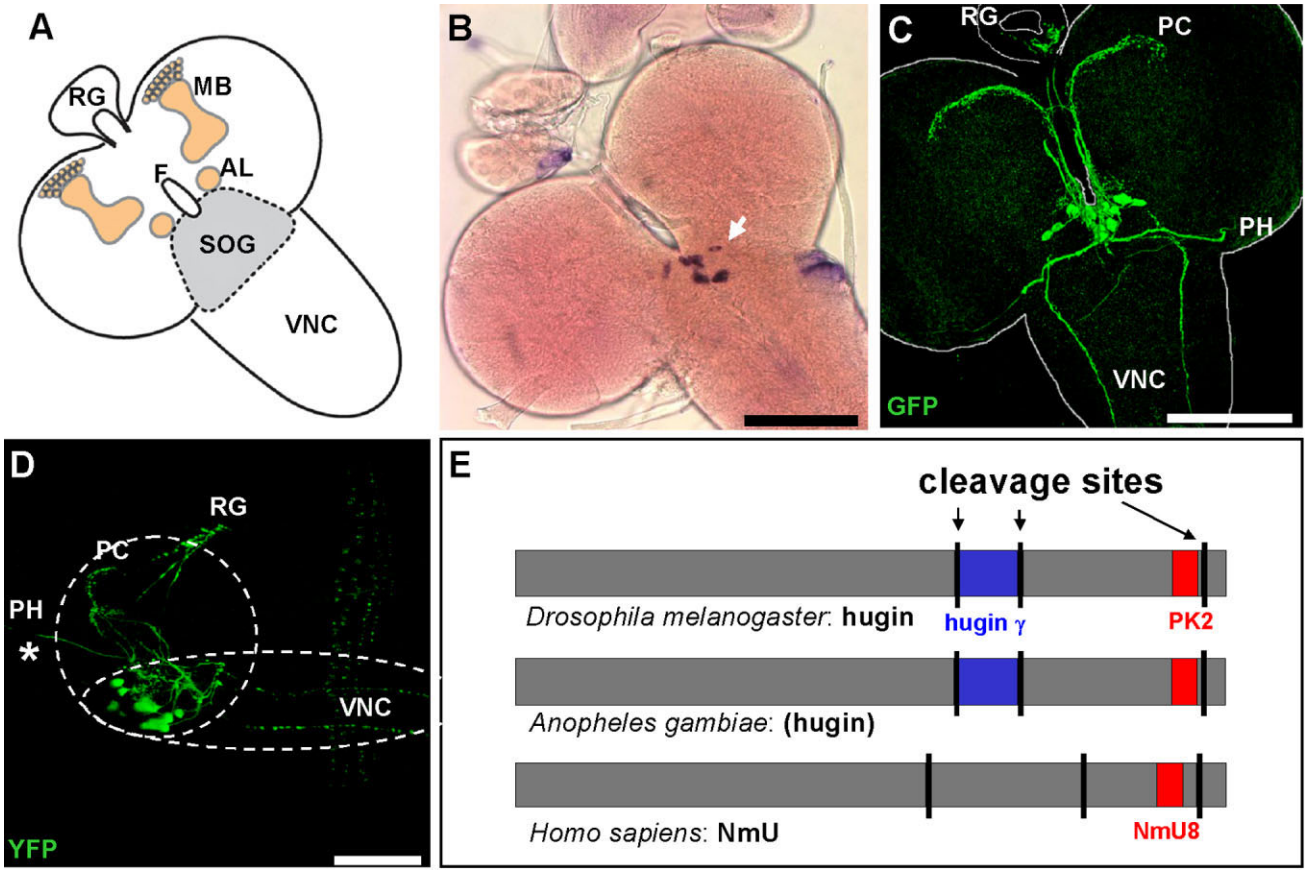

Fig. 1. The *hugin* neural cluster and gene structure. **A:** Schematic drawing of the CNS of a third-instar *Drosophila* larva. Antennal lobe (AL), foramen of the esophagus (F), mushroom body (MB), ring gland (RG), subesophageal ganglion (SOG), ventral nerve cord (VNC). **B:** In situ hybridization showing *hugin* gene expression. The *hugin*-positive cells are located in the SOG (arrow). Note that not all *hugin* cells are visible in this focal plane. **C:** Immunohistochemical staining against GFP expressed under the control of a *hugin* promoter. The four major targets are shown: protocerebrum (PC), VNC, RG, and pharynx (PH). **D:** Lateral view of the CNS (marked by dashed lines) of a living larva expressing YFP under the control of a *hugin* promoter. Neurites leaving the CNS toward the PH are marked by a star. **E:** Homology of the *hugin* prepropeptide to *Anopheles hugin* homolog and human NmU prepropeptide based on the cleavage pattern. Scale bars = 50 μm.

tified as the protein Futsch and which is homologous to vertebrate microtubule-associated protein 1B (MAP1B; Hummel et al., 2000). It localizes to the microtubule compartment of the cell, associates with microtubules in vitro, and is used as a neuropile marker; anti-elav is a monoclonal antibody raised in mouse against a hybrid protein P10.ATG produced in *E. coli*, which consists of the first 260 amino acids of phage T7 gene fused to the entire 483 amino acid *Drosophila* Elav, an mRNA binding protein that regulates neuroglian alternative splicing in neurons (Lisbin et al., 2001). The antibody was shown to be specific by the absence of labelling in *Drosophila* Elav-null embryos (Koushika et al., 1996); anti-nc82 is a monoclonal antibody raised in mouse by injection of *Drosophila* head homogenate and identifies a protein of 190 kDa in Western blots of homogenized *Drosophila* heads that was identified as Bruchpilot, a protein present in chemical synapses (Wagh et al., 2006); the anti-ChATB1 antibody was raised by injection of extracts of bacterially expressed *Drosophila* dChAT protein. DChAT (*Drosophila* choline acetyltransferase) is the synthetic enzyme for acetylcholine and thus a marker for neurons with a cholinergic phenotype. The antibody was shown to react with a single band at a position of approximately 80 kDa in crude fly head samples (Takagawa and Salvaterra, 1996); anti-CD2 was raised in mouse against activated T helper cells from rat and was shown to recognize the rat CD2 cell surface antigen, a 50–54-kDa glycoprotein expressed by thymocytes and mature T cells (Whiteland et al., 1995). This antibody recognizes the ectopically expressed CD2 protein as demonstrated by the absence of labelling in wild-type brains (data not shown).

RESULTS

The *hugin* gene is expressed exclusively in 20 neurons in the SOG of the *Drosophila* larva, where they project to four distinct targets: the protocerebrum, the ventral nerve cord, the ring gland, and the pharynx (Fig. 1A–D; Melcher and Pankratz, 2005). The *hugin* gene encodes a prepropeptide that can give rise to several potential neuropeptides (Meng et al., 2002). The cleavage pattern is conserved in insects and mammals (Fig. 1E), and one of the peptides, PK2, is homologous to the mammalian

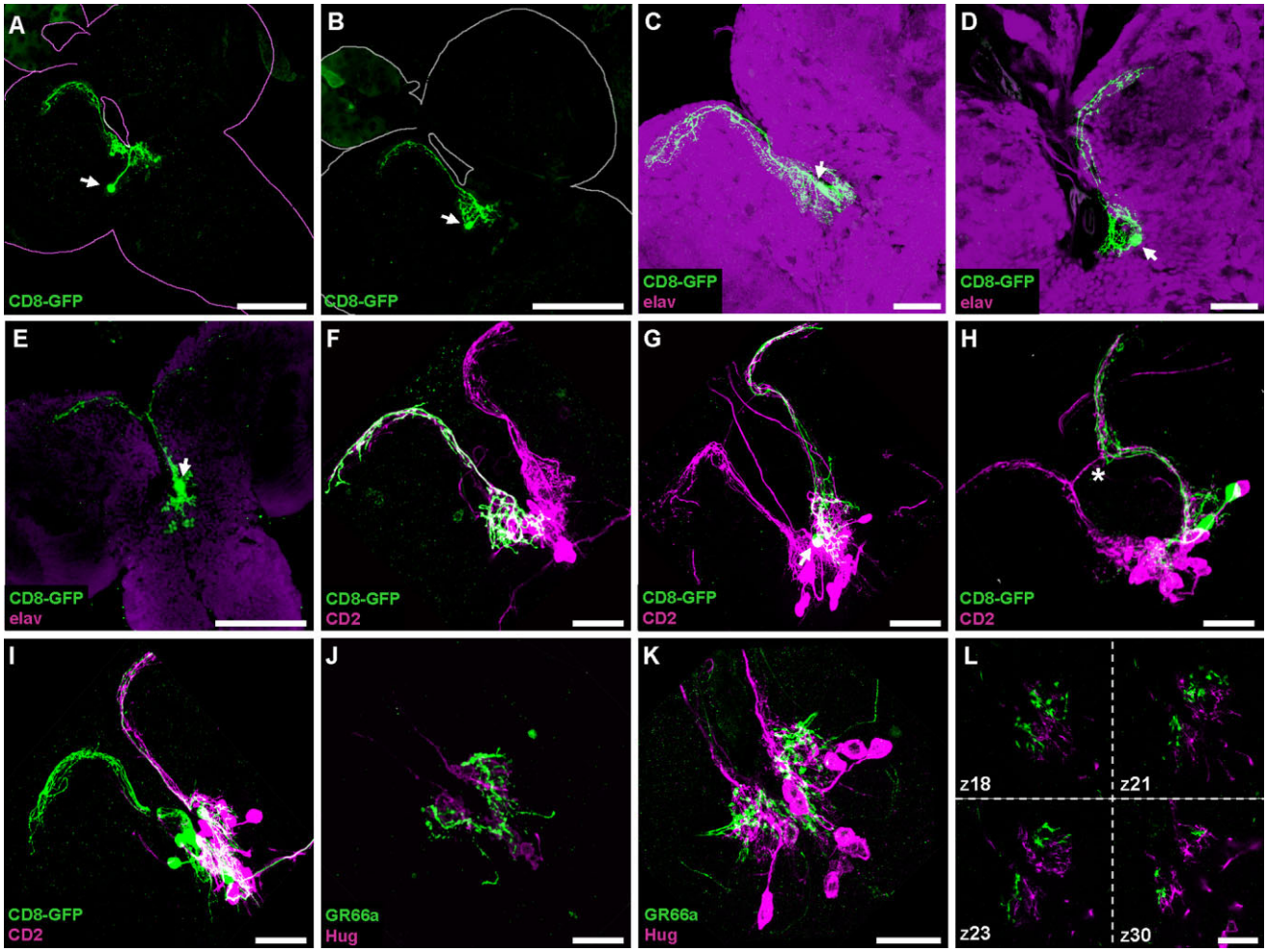

Fig. 2. Neurites to the protocerebrum. Flp out clones of *hugin* cells stained against GFP (green), without additional staining (A,B), with elav background staining (C–E), or on top of non-flp out *hugin* cells stained against CD2 (magenta; F–I). Overlap of neurites from GR66a-expressing gustatory receptor neurons and *hugin* neurons (J–L). **A–D:** Single cells projecting to the ipsilateral side of the protocerebrum (arrow: cell bodies). **E:** Single cell projecting to both sides of the protocerebrum. **F,G:** Single cells projecting to the ipsilateral hemisphere. **H:** Two cells projecting to the right hemisphere; note the thin connection between the hemispheres (asterisk). **I:** Four cells projecting to the left side of the protocerebrum, one cell projecting to the right side, one cell projecting to the pharynx. **J,K:** GR66a neuron terminals (green) overlap the arborizations of *hugin* cells (magenta) lateral to the esophageal foramen. **L:** Single confocal stacks (four representative numbered stacks) from Z-projections in K. Scale bars = 20 μm in C,D,F–L; 50 μm in A,B,E.

NmU8 (Melcher et al., 2006). To determine the morphology of individual *hugin* neurons, we used the flp out technique (Wong et al., 2002) to generate single cells marked with a fluorescent genetic marker. We describe below the details of each neurite target. Because we do not know at this point whether the different neurites are axons or dendrites, we have avoided using these terms in the descriptions.

Neurites to the protocerebrum

Single clones of *hugin* cells were obtained that show neurites to the ipsilateral protocerebrum (Fig. 2A–I). Different representative clones are shown to illustrate the variations in the morphology of single cells (see also Supplementary Fig. 1). These cells also show arborization ventrolateral to the foramen of the esophagus in a region that is innervated by gustatory receptor neurons expressing GR66a (Fig. 2K,L). We have previously observed connections between the left and the right protocerebrum (Melcher and Pankratz, 2005; Fig. 2H), and we have obtained clones in which neurites branch onto both hemispheres (Fig. 2E). Although we have not observed any cells that go only contralaterally, this is not proof of the nonexistence of such cells. We also obtained clones in which four cells on one side of the CNS all project to the ipsilateral protocerebrum (Fig. 2I). If one assumes the same for the other brain hemisphere, this implies that the protocerebrum is innervated by at least eight cells.

Neurites to the ventral nerve cord

Single *hugin* cells also project down the ventral nerve cord (Fig. 3A–D). The morphology of these neurons is striking. In addition to a long process travelling down contralaterally along the lateral neuropile border of the

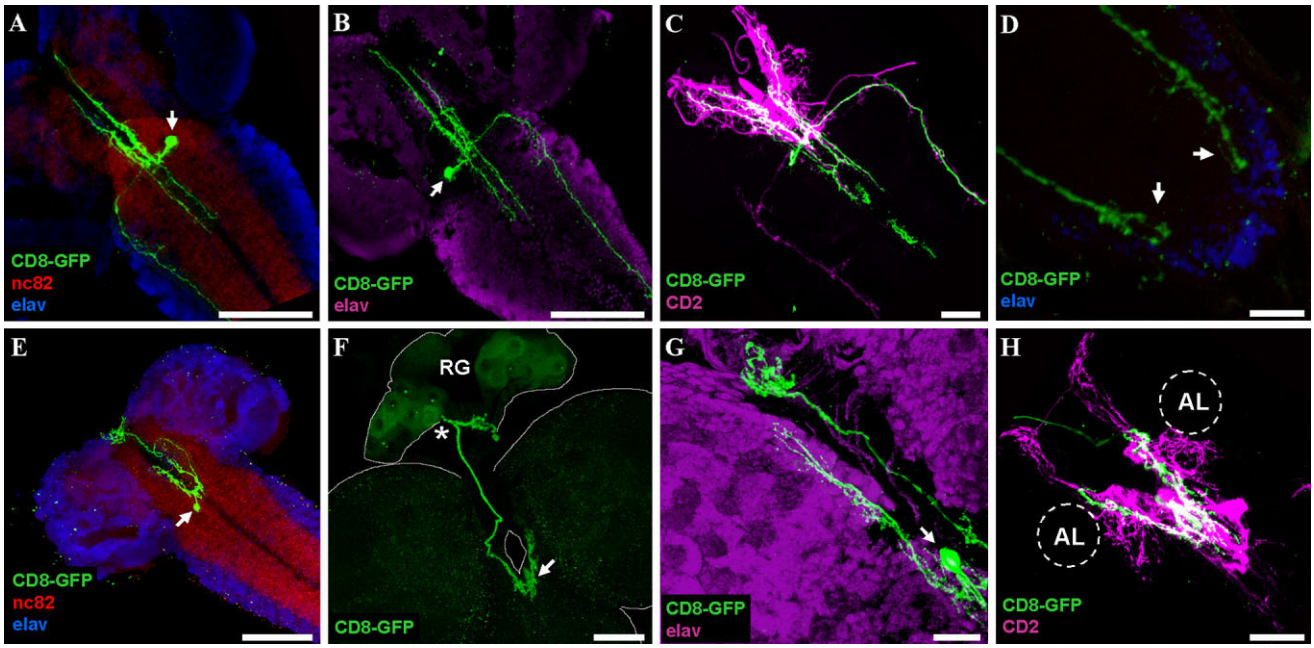

Fig. 3. Neurites to the ventral nerve cord and to the ring gland. **A,B:** Single *hugin* cells (green) projecting to the ventral nerve cord (VNC; arrows: cell bodies). **C:** Single cell projecting to the VNC (green); non-flp out *hugin* cells are shown in magenta. **D:** Magnification of neurite terminals of *hugin* cells at the the posterior end of the VNC. **E–G:** Single cells projecting to the ring gland (RG; arrows: cell bodies). The asterisk in F marks the point of entry into the RG. **H:** Single cell projecting to the RG (green) in the context of the other *hugin* cells (magenta). The position of the antennal lobes (AL) is outlined for orientation. Scale bars = 20 μm in C,D,F–H; 50 μm in A,B,E.

ventral cord, there are four shorter fibers projecting up and down just left and right of the midline (Fig. 3A–C). The long neurites that extend down the lateral side of the ventral nerve cord branch out at the tip (Fig. 3D); the precise targets are not known. The two fibers that project in the anterior direction pass along each side of the foramen and end at the medial part of the protocerebrum. In Figure 3C, focusing on the ipsilateral processes in the ventral nerve cord, we can see that at least one CD2-labeled neurite follows the green-labeled one. We thus conclude that at least two neurons per hemisphere innervate the ventral cord. We have previously shown that there are 20 *hugin* neurons, of which there are four pharyngeal and four ring gland neurons (Melcher and Pankratz, 2005). It is likely, then, that the number of ventral cord neurons is four and that the number of protocerebral neurons is eight.

Neurites to the ring gland

Single *hugin* cells projecting to the contralateral side of the ring gland were also observed (Fig. 3E–H). In addition, these cells are characterized by an ipsilateral process that stops lateral to the esophageal foramen. The neurite length observed can vary (e.g., compare Figure 3F with G). We consistently observe fibers projecting to the border of the antennal lobe and the SOG (Fig. 3H); however, the processes sometimes extend farther dorsally (Fig. 3G). This may be due to thinning of fibers in the more dorsal regions. In the ring gland, the *hugin* cells establish dense arborizations on the side ipsilateral to the entering fiber and weaker arborizations after crossing to the other side (Fig. 3F). Possible target cells in the ring gland are located in the corpora cardiaca (Siegmund and Korge, 2001).

Neurites to the pharynx

The fourth class of *hugin* neurons projects to the anterior pharynx, close to the cephalopharyngeal skeleton (Fig. 4A–C). The neurites leave the SOG, make a U-turn, and end at the anterior part of the dorsal pharyngeal muscles. Whether the pharyngeal neurons in fact innervate the muscles is not known. Neurites can be seen that cross the midline and those that do not, but, because the pharyngeal neurons are located close to the midline, this distinction is sometimes difficult (Fig. 4D,E). In addition, these neurons have short neurites along each side of the foramen (Fig. 4E–G).

The *hugin* gustatory circuit and the olfactory system

The close intermingling of axon endings of gustatory receptor neurons and arborizations of *hugin* neurons in the SOG suggested that *hugin* neurons could act as gustatory interneurons (Pankratz and Melcher, 2005). One of the questions raised by the earlier study was to which class of *hugin* neurons these arborizations belonged. The current analysis suggests that all *hugin* neurons may have taste inputs (see Fig. 2J–L); however, neurons that project to the protocerebrum are the only ones showing arborizations in the lateral part of the SOG (see Fig. 2F,G), whereas other classes of neurons have distinct but overlapping neurites in the medial part of the SOG (compare Figs. 3C,H and 4F). Because the SOG lies in close proximity with the antennal lobes, we investigated the morphological relationship between the protocerebral *hugin* neurons and the antennal lobes, the first relay center for olfactory signaling. First, we asked whether the arborizations of the protocerebral neurons overlapped with

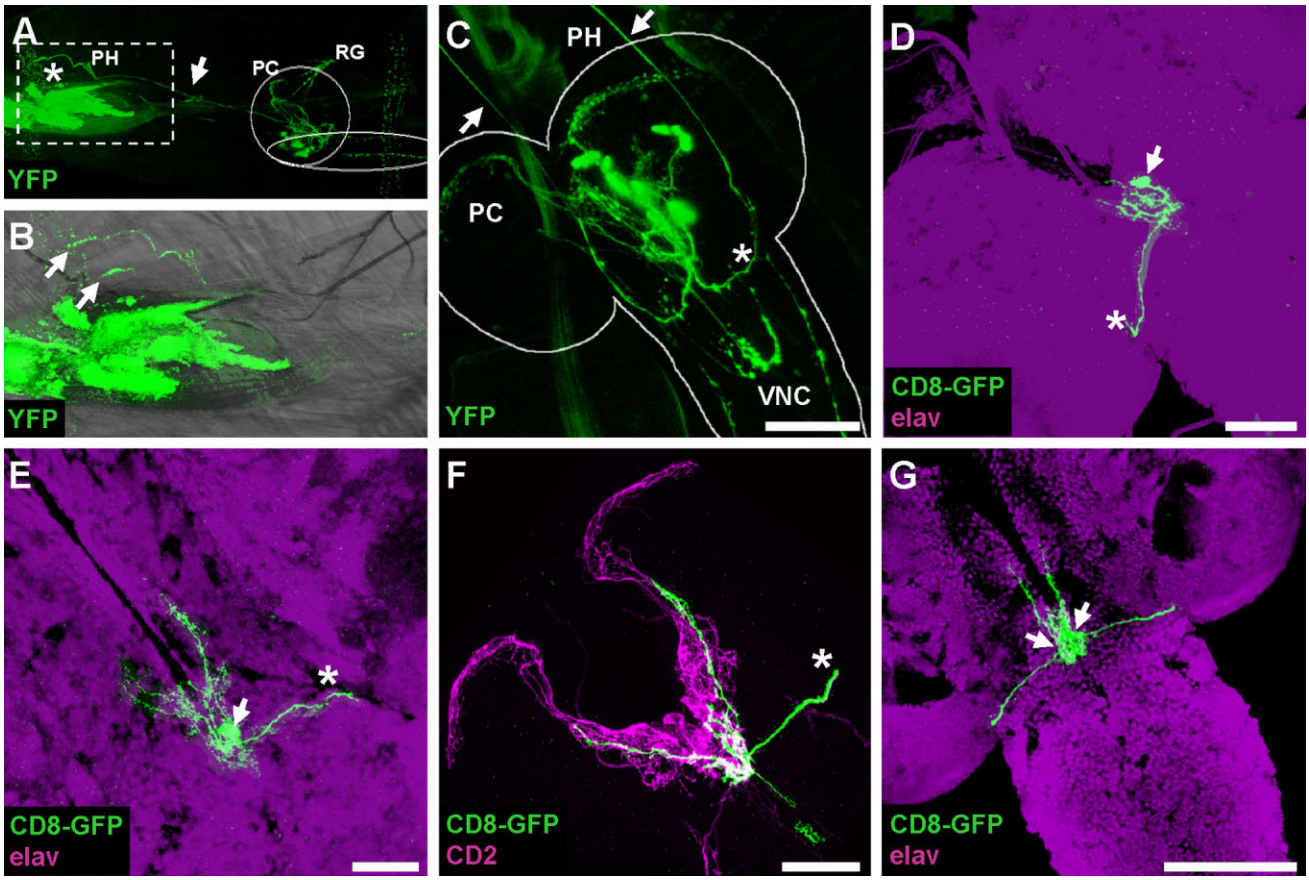

Fig. 4. Neurites to the pharynx. **A:** Two photon microscope image of the head region of a living third-instar larva expressing YFP under the control of a *hugin* promoter. The head is oriented to the left. The fiber extending to the PH is marked by an arrow. Asterisk marks the ending of the neurites. The strong autofluorescence below the star is from the cephalopharyngeal skeleton. The boxed area is magnified in B. **B:** Pharynx region of living larva; arrows point to the neurite terminals. **C:** Two photon microscope image of a living larva. The picture is taken from a ventral view. The CNS is marked by white line; neurites to the pharynx (PH) are marked by arrows. The U-turn made by the neurites is marked by a star. Neurites to the protocerebrum (PC) and the ventral nerve cord (VNC) are also visible. **D,E:** Single *hugin* cells (arrows) with neurites (asterisk) leaving the CNS toward the PH. **F:** Cells projecting to the PH (asterisk) and VNC, both shown in green, on top of non-flp out *hugin* cells (magenta). **G:** Two cells (arrows) with neurites leaving the CNS. Scale bars = 20 μm in D–F; 50 μm in C,G.

the antennal lobe. Protocerebrum-specific *hugin* clones in nc82 neuropile background staining indicated that the arborizations lie just at the border of the antennal lobe but do not intermingle with it (Fig. 5A). This is supported by clones in the adult, where the antennal lobes are significantly larger relative to the SOG (Fig. 5B). We next analyzed the neurites to the larval protocerebum with respect to the mushroom body calyx, a secondary olfactory relay center. Consistent with our earlier results, the *hugin* neurites lie dorsal to the mushroom body calyx (Fig. 5C–F). Thus, at the morphological level, we do not see an overlap of *hugin* neurons with the central olfactory pathway. However, insofar as *hugin* encodes a secreted peptide, an influence on the olfactory system cannot be excluded.

The *hugin* neuronal architecture at the foramen-SOG boundary

The arborizations of the protocerebral *hugin* neurons lie in a region lateral to the foramen that border the SOG. Other classes of *hugin* neurons do not show such arborization, but they all show neurites into a region directly juxtaposed to the foramen and the SOG. The architecture of the *hugin* neurons within this region indicates that the neurites lie in close proximity to each other (Fig. 5G–J). The ventral nerve cord neurons (Fig. 5K–M), the ring gland neurons (Fig. 5N,O), and the pharyngeal neurons (Fig. 5P–R) all have neurites that extend just lateral to the foramen. For the ventral cord neuron, there is also a small arborization at the bottom end of the foramen, i.e., at the border to the SOG (Fig. 5L,M). For the pharyngeal neuron, two additional spiked neurites can be seen extending dorsally and ventrally in a similar region (Fig. 5Q,R). Thus, in addition to having specific targets outside the SOG, the different *hugin* neurons have overlapping neurites near the SOG. These observations suggest that the region bordering the lateral foramen and the SOG might have a special role in mediating *hugin* neuronal function.

DISCUSSION

Single *hugin* neurons project to only one of the four major targets

Understanding how the brain controls behavior requires a thorough knowledge of the underlying neural

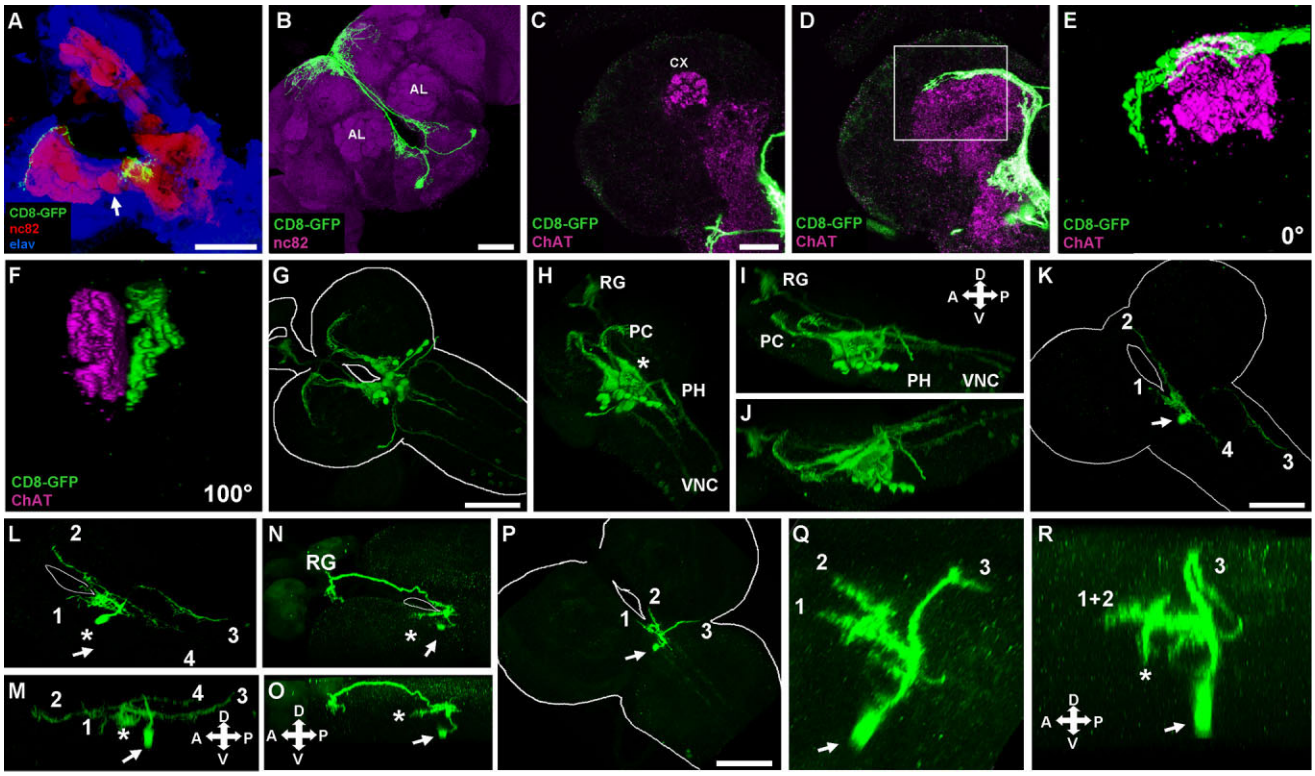

Fig. 5. Position of *hugin* neurites relative to the olfactory system and to the foramen. **A:** Single *hugin* cell (green) in the larval CNS projecting to the protocerebrum. The arrow marks the larval antennal lobe (AL), which is stained by the synaptic marker nc82 (red). **B:** Adult brain with three *hugin* cells projecting to the protocerebrum; antennal lobes (AL) are marked by nc82 (magenta). **C,D:** Adjacent stacks of left hemisphere of a larval brain; *hugin* cells are stained in green. The neuropile including the mushroom body calyx (CX) is marked by ChAT (magenta), which labels cholinergic neurons. **E,F:** The region in D marked by a rectangle, showing the terminals of the protocerebral *hugin* neurons (green) relative to the calyx (magenta). F is rotated by 100°. **G–J:** 3-D reconstruction of 14 *hugin* cells, rotated at different angles. The cell bodies are located on the ventral side of the SOG. All neurites initially proceed dorsally toward the foramen before extending to their final targets. The asterisk in H denotes the arborization located lateral to the foramen. Directional arrows are included in figures showing a lateral view of the *hugin* neurons: anterior (A), posterior (P), dorsal (D), ventral (V). **K–M:** 3-D reconstruction of a single cell projecting to the vental nerve cord. The numbers are for orientation during image turning. The small arborization at the bottom end of the foramen is marked by a star. Arrows mark the cell bodies. **N,O:** 3-D reconstruction of a single cell projecting to the ring gland (see Fig. 3F). Asterisks mark the short processes that project laterally to the foramen. **P–R:** 3-D reconstruction of a single cell projecting to the pharynx. The numbers are for orientation during image turning. In addition to the two short neurites running on each side of the foramen (1 and 2), there are processes (asterisk) extending dorsally and ventrally. Q and R are magnifications of P. Scale bars = 20 μm in C; 50 μm in A,D,G,K,P.

circuitry. Although many behaviors have been studied, and numerous genes required for specific behaviors have been identified, the cellular details of the underlying neural circuits are far less well understood. The *hugin* neuronal circuit provides an opportunity to dissect, at single-cell resolution, the connectivity patterns of a cluster of neurons modulating feeding behavior.

Our analysis revealed that the 20 cells of the *hugin* cluster consists of four classes, whose cell bodies are arranged fairly symmetrically straddling the midline: eight projecting to the protocerebrum and four each to the ventral nerve cord, to the ring gland, and to the anterior pharynx (Fig. 6). Within a given neuronal class, it is further possible that individual neurons have distinct ipsi- or contralateral neurites.

Different *hugin* neurons share overlapping neurites at the foramen-SOG border

In addition to having unique neurite targets, the *hugin* neurons also possess additional, partially overlapping patterns of innervation in a region ventrolateral to the foramen. These findings further demonstrate the necessity for a single-cell analysis, in that it has revealed insights into the pattern that would have gone undetected because of overlapping innervations from other neurons. The class of *hugin* neurons projecting to the protocerebrum has extensive arborizations in the lateral SOG, where external chemosensory organ neurons project (see accompanying article by Colomb et al., 2007). These arborizations intermingle with the terminals of the gustatory receptor neurons. Whether they make synaptic contacts is unknown. These arborizations could also represent sites of neuropeptide release; i.e., *hugin* neuropeptides in this region may influence the transmission of incoming gustatory signals. In either case, the fact that the arborizations lateral to the SOG belong to the *hugin* neurons projecting to the protocerebrum is consistent with the view that *hugin* may act in higher brain centers to mediate gustatory information (Melcher and Pankratz, 2005).

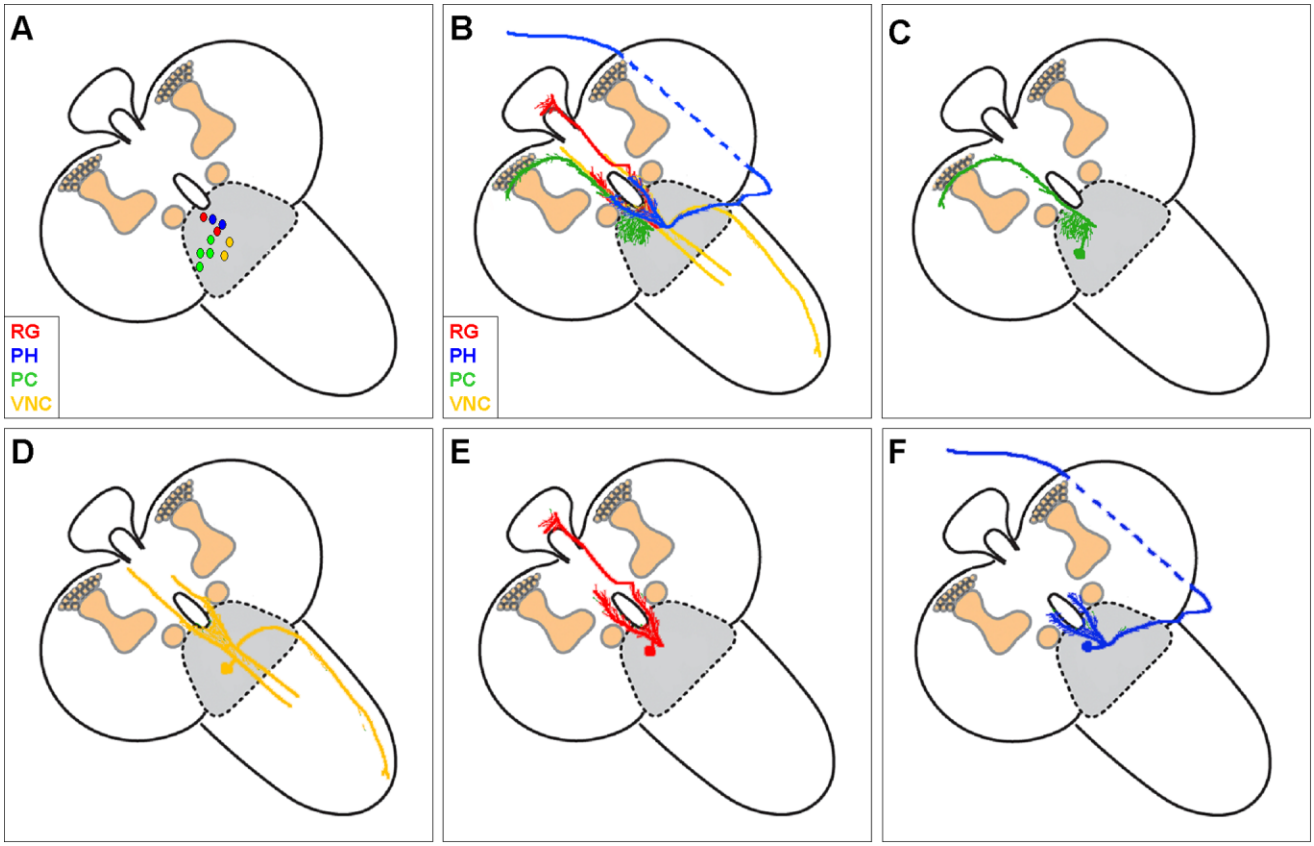

Fig. 6. Summary of different classes of *hugin* neurons. **A:** Schematic drawing of the locations of the *hugin* cell bodies; only those in the left hemisphere are shown. **B:** Summary of the four classes of *hugin* neuronal targets. **C:** Schematic drawing of a single cell projecting to the PC. **D:** Schematic drawing of a single cell projecting to the VNC. **E:** Schematic drawing of a single cell projecting to the RG. **F:** Schematic drawing of a single cell projecting to the PH. The gray area represents the SOG. For all classes, note the additional neurites in a region ventrolateral to the foramen near the SOG.

This does not mean, however, that only the protocerebral neurons mediate taste information. Other classes of *hugin* neurons show extensive innervations within the medial part of the SOG, and the accompanying analysis of GR neuronal projection in the larvae (Colomb et al., 2007) demonstrates that the taste projection targets cover different areas of the SOG. Therefore, *hugin* neurons may mediate taste information at different regions of the CNS, including the protocerebrum, the SOG, and the ventral nerve cord.

The classes of *hugin* neurons that project to the ventral nerve cord, ring gland, and pharyngeal muscles also have additional neurites that run lateral to the foramen. The ventral nerve cord neurons project along both sides of the foramen, ending near the top of the protocerebrum. The ring gland neurons have short neurites on the ipsilateral side (i.e., opposite from the neurites that go to the ring gland), whereas the pharyngeal neurons send out short neurites on both sides of the foramen. The ventral nerve cord and the pharyngeal neurons also have short neurites that project dorsally and ventrally near the foramen. Thus, all four cell types, in addition to their innervations outside the SOG, have neurites that terminate at or near the border between the foramen and SOG. This region may thus have a special function in integrating the activities of the different *hugin* neurons. Because the region is very close to the foramen, i.e., the canal in the CNS through which the esophagus passes, it is possible that *hugin* neuropeptides are secreted directly onto the esophagus at this site. In this regard, we point out that a specific set of *hugin* neurons innervates the ring gland, the major neuroendocrine organ of *Drosophila* larva, which is also innervated by the insulin-producing cells (Brogliolo et al., 2001; Rulifson et al., 2002). We also pointed out earlier that the lateral arborizations intermingling with incoming taste neuronal terminals might also be a site of neuropeptide release. It is thus possible that the *hugin* neuropeptides are released at multiple sites: the ring gland is used for global control of growth and metabolism; the lateral arborizations are used for regional modulation of gustatory signaling through the protocerebrum; the newly defined region targetted by the different classes of *hugin* neurons at the foramen-SOG border is utilized for local control of feeding. This region could represent the tritocerebrum. This brain structure has not been precisely defined anatomically in the *Drosophila* larva, but based on embryonic studies (Hirth et al., 2001) the larval tritocerebrum could be located in a region ventrolateral to the foramen. In either case, it would be interesting to see

whether other neuropeptide-producing neurons in the brain also project to this region.

Neural circuit for integration of gustatory and metabolic signals

In sum, we have analyzed a neuronal cluster whose members all express a common gene, *hugin*, which can be divided into four different classes, each having a specific morphology and neurite pattern. Two of these classes are confined to the CNS, whereas the other two project to peripheral targets as well. The protocerebral neurons may mediate gustatory information, and the targets of ring gland and pharyngeal neurons are clearly relevant for feeding behavior, growth, and metabolism. These structural considerations suggest that the *hugin* neural circuit might function in integrating external sensory and internal metabolic information to regulate feeding and growth.

ACKNOWLEDGMENTS

We thank Matthias Bauer, Margret Bülow, Barry Dickson, and Christoph Melcher for their contributions.

LITERATURE CITED

Amrein H, Thorne N. 2005. Gustatory perception and behavior in *Drosophila melanogaster*. Curr Biol 15:R673–R684.

Brighton PJ, Szekeres PG, Willars GB. 2004. Neuromedin U and its receptors: Structure, function, and physiological roles. Pharmacol Rev 56:231–248.

Broberger C. 2005. Brain regulation of food intake and appetite: molecules and networks. J Intern Med 258:301–327.

Brogiolo W, Stocker H, Ikeya T, Rintelen F, Fernandez R, Hafen E. 2001. An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. Curr Biol 11:213–221.

Colomb J, Grillenzoni N, Ramaekers A, Stocker RF. 2007. Architecture of the primary taste center of *Drosophila melanogaster* larvae. J Comp Neurol 502:834–847.

Hanada R, Teranishi H, Pearson JT, Kurokawa M, Hosoda H, Fukushima N, Rukue Y, Serino R, Fujihara H, Ueta Y, Ikawa M, Okabe M, Murakami N, Shirai M, Yoshimatsu H, Kangawa K, Kojima M. 2004. Neuromedin U has a novel anorexigenic effect independent of the leptin signaling pathway. Nat Med 10:1067–1073.

Hirth F, Loop T, Egger B, Miller DF, Kaufman TC, Reichert H. 2001. Functional equivalence of Hox gene products in the specification of the tritocerebrum during embryonic brain development of *Drosophila*. Development 23:4781–4788.

Howard AD, Wang R, Pong SS, Mellin TN, Strack A, Guan XM, Zeng Z, Willisams DL, Feighner SD, Nunes CN, Murphy B, Stair JN, Yu H, Jiang Q, Clements MK, Tan CP, McKee KK, Hrenluk DL, McDonald TP, Lynch KR, Evans JF, Austin CP, Caskey CT, Van der Ploeg LH, Liu Q. 2000. Identification of receptors for neuromedin U and its role in feeding. Nature 406:70–74.

Hummel T, Krukkert K, Roos J, Davis G, Klambt C. 2000. *Drosophila* Futsch/22C10 is a MAP1B-like protein required for dendritic and axonal development. Neuron 26:357–370.

Koushika SP, Lisbin MJ, White K. 1996. ELAV, a *Drosophila* neuron-specific protein, mediates the generation of an alternatively spliced neural protein isoform. Curr Biol 6:1634–1641.

Lisbin MJ, Qiu J, White K. 2001. The neuron-specific RNA-binding protein ELAV regulates neuroglian alternative splicing in neurons and binds directly to its pre-mRNA. Genes Dev 15:2546–2561.

Melcher C, Pankratz MJ. 2005. Candidate gustatory interneurons modulating feeding behavior in the *Drosophila* brain. PLoS Biol 3:e305.

Melcher C, Bader R, Walther S, Simakov O, Pankratz MJ. 2006. Neuromedin U and its putative *Drosophila* homolog *hugin*. PLoS Biol 4:e68.

Meng X, Wahlström G, Immonen T, Komer M, Tirronen M, Predel R, Kalkkinen N, Heino T, Sariola H, Roos C. 2002. The *Drosophila hugin* gene codes for myostimulatory and ecdysis-modifying neuropeptides. Mech Dev 117:5–13.

Ramaekers A, Magnenat E, Marin EC, Gendre N, Jefferies GS, Luo, L, Stocker RF. 2005. Glomerular maps without cellular redundancy at successive levels of the *Drosophila* larval olfactory circuit. Curr Biol 15:982–992.

Rulifson EJ, Kim SK, Nusse R. 2002. Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. Science 296:1118–1120.

Scott K. 2005. Taste recognition: food for thought. Neuron 48:455–464.

Siegmund T, Korge G. 2001. Innervation of the ring gland of *Drosophila*. J Comp Neurol 431:481–491.

Stocker RF. 1994. The organization of the chemosensory system in *Drosophila melanogaster*: a review. Cell Tissue Res 275:3–26.

Takagawa K, Salvaterra P. 1996. Analysis of choline acetyltransferase protein in temperature sensitive mutant flies using newly generated monoclonal antibody. Neurosci Res 24:237–243.

Wagh DA, Rasse TM, Asan E, Hofbauer A, Schwenkert I, Durrbeck H, Buchner S, Dabauvalle MC, Schmidt M, Qin G, Wichmann C, Kittel R, Sigrist SJ, Buchner E. 2006. Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in *Drosophila*. Neuron 49:833–844.

Whiteland JL, Nicholls SM, Shimeld C, Easty DL, Williams NA, Hill TJ. 1995. Immunohistochemical detection of T-cell subsets and other leukocytes in paraffin-embedded rat and mouse tissues with monoclonal antibodies. J Histochem Cytochem 43:313–320.

Wong AM, Wang JW, Axel R. 2002. Spatial representation of the glomerular map in the *Drosophila* protocerebrum. Cell 109:229–241.

Zhang Y, Proenca R, Maffei M, Barone M, Friedman JM. 1994. Positional cloning of the mouse obese gene and its human homologue. Nature 372:425–432.