REGULAR ARTICLE

Morphological characterization of single fan-shaped body neurons in *Drosophila melanogaster*

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Abstract The fan-shaped body is the largest substructure of the central complex in *Drosophila melanogaster*. Two groups of large-field neurons that innervate the fan-shaped body, viz., F1 and F5 neurons, have recently been found to be involved in visual pattern memory for "contour orientation" and "elevation" in a *rut*-dependent manner. The F5 neurons have been found to be responsible for the parameter "elevation" in a *for*-dependent manner. We have shown here that the F1 neuron also affects visual memory for "contour orientation" in a *for*-dependent way. With the help of Gal4/UAS and FLP-out techniques, we have characterized the morphological features of these two groups of neurons at single neuron resolution. We have observed that F1 or F5 neurons are groups of isomorphic individual neurons. Single F1 neurons have three main arborization regions: one in the first layer of the fan-shaped body, one in the ventral body, and another in the inferior medial protocerebrum. Single F5 neurons have two arborization regions: one in the fifth layer of the fan-shaped body and the other in the superior medial protocerebrum. The polarity of the F1 and F5 neurons has been studied with the Syt-GFP marker. Our results indicate the existence of presynaptic sites of both F1 and F5 neurons located in the fan-shaped body and postsynaptic sites outside of the fan-shaped body.

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Introduction

In the midbrain of insects, the central complex with its highly regular interconnections is a putative center related to functions ranging from motor control to visual information processing (Strausfeld 1976, 1999; Strauss 2002; Liu et al. 2006; Heinze and Homberg 2007). In the cockroach *Blaberus discoidalis*, lesions in the central complex affect normal turning (Ridgel et al. 2007) and the central complex responds to visual signals and mechanical stimulation of the antennae (Ritzmann et al. 2008). In the honeybee *Apis mellifera*, intrinsic neurons of the protocerebral bridge of the central complex process visual stimuli (Homberg 1985). In the locust *Schistocerca gregaria*, electrophysiological experiments have shown flight-correlated activity changes in neurons of the lateral accessory lobe, which is an accessory area of the central complex (Homberg 1994). Recently, intracellular recordings from particular interneurons of the central complex have revealed their sensitivity to polarized light and a topographic representation of E-vector orientations in the columnar organization of the protocerebral bridge (Vitzthum et al. 2002; Heinze and Homberg 2007).

In *Drosophila*, the central complex consists of the protocerebral bridge, the ellipsoid body, the fan-shaped body, the superior arch, the paired noduli, and two closely associated accessory structures: the ventral bodies and the
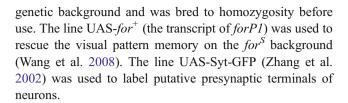
lateral triangles (Hanesch et al. 1989; Renn et al. 1999). Evidence from behavioral experiments in *Drosophila* central complex mutants implies that the central complex play a role in motor control (Strauss and Heisenberg 1993; Ilius et al. 1994; Martin et al. 1999). The H³-2-deoxyglucose incorporation pattern in the central body varies in response to diverse visual stimulation patterns (Bausenwein et al. 1994). The fan-shaped body, as the largest substructure of the central complex, is a unique three-dimensional matrix mirror, symmetrically organized across the midline of the proto-cerebrum (Hanesch et al. 1989; Rein et al. 2002). It is composed of six roughly horizontal layers or eight vertical segments (Hanesch et al. 1989). Each layer of the fan-shaped body is innervated by a group of large-field neurons, the so-called F neurons, that arborize both in the fan-shaped body and in regions outside the central complex (Hanesch et al. 1989). They consequently make interconnections between the central complex and other neuropils in the protocerebrum (Hanesch et al. 1989). Among these large-field F neurons, the F1 neurons that innervate the first layer of the fan-shaped body participate in the *rutabaga* (*rut*)-dependent memory for the visual pattern "contour orientation", whereas the F5 neurons that innervate the fifth layer of the fan-shaped body participate in the visual pattern "elevation" in both a *rut*- and *foraging* (*for*)-dependent manner (Liu et al. 2006; Wang et al. 2008).

Here, we demonstrate that specific expression of the *for*[+] gene in F1 neurons can rescue the memory defect for pattern parameter "orientation", but not for "elevation" on a *for*[S] background. To understand the relationship between function and structure, we have used several Gal4 lines with expression in the fan-shaped body and the FLP-out technique to label single neurons with an mCD8-GFP reporter (Golic and Lindquist 1989; Struhl and Basler 1993; Wong et al. 2002). The morphological features of individual F1 and F5 neurons in the fan-shaped body are described, and the polarity of the F1 and F5 neurons has been investigated with a Syt-green fluorescent protein (Syt-GFP) marker.

Materials and methods

Fly strains

All of the flies were raised on standard food at room temperature with a 12-h light/12-h dark cycle at 60% humidity. The strains Gal4-NP6561, Gal4-NP6510 (Liu et al. 2006), and Gal4-c205 were used to label the F1 and F5 neurons of the fan-shaped body. The lines yw;UAS-mCD8-GFP (Lee and Luo 1999) and hsFLP;CyO/Sp;UAS>rCD2, y[+] >mCD8-GFP (Wong et al. 2002) were used as reporters. The *for* allelic variant line *for*[S] (Osborne et al. 1997) was outcrossed for eight generations to obtain a wild-type *CS*

genetic background and was bred to homozygosity before use. The line UAS-*for*[+] (the transcript of *forP1*) was used to rescue the visual pattern memory on the *for*[S] background (Wang et al. 2008). The line UAS-Syt-GFP (Zhang et al. 2002) was used to label putative presynaptic terminals of neurons.

Clone induction

For FLP-out clonal analysis, the above-mentioned Gal4 lines were crossed with hsFLP;CyO/Sp;UAS>rCD2, y[+] >mCD8-GFP to obtain Gal4 flies carrying hsFLP and UAS> rCD2, y[+] >mCD8-GFP. On the day before eclosion, pupae on the wall of the vial were given a mild heat shock by placing the vial directly into a 37°C water bath to remove the FLP-out cassette rCD2, y[+] in a subset of the neurons, thereby allowing the expression of mCD8-GFP in these neurons and CD2 expression in the remaining neurons. The duration of the heat shock was 40–45 min for Gal4-c205, Gal4-NP6561, and Gal4-NP6510. The eclosed flies were then transferred into a fresh vial and 2– to 5-day-old flies were used for dissection.

Immunohistochemistry

Dissection of adult brains was performed in cold PBS (phosphate-buffered saline) to remove the cuticle and connective tissues. After a brief wash in PBS, samples were fixed in freshly prepared 4% paraformaldehyde in PBS for 1.5 h at room temperature, rinsed (3×15 min) in PBT (PBS with 0.5% Triton X-100) followed by blocking with PNT (10% normal goat serum in PBT) for 2 h at room temperature. Subsequently, samples were incubated with primary antibodies diluted in PNT (mouse nc82, dilution 1:20, kindly provided by E. Buchner, University of Würzburg, Germany; rabbit anti-GFP, dilution 1:1000, Invitrogen, Ore.) overnight at 4–8°C. After being rinsed (3×15 min) in PBT at room temperature, samples were incubated with secondary antibodies diluted in PNT (goat anti-mouse Alexa-488-conjugated antibody, dilution 1:100, Invitrogen; goat anti-rabbit Alexa-555-conjugated antibody, dilution 1:1000, Invitrogen). After further rinses (3×15 min) in PBT at room temperature, brains were mounted in Vectashield (Vector Laboratories, Burlingame, Calif.). To protect brains from pressure by the coverslip, spacer rings of approximately 180 μm in height were used.

Imaging and three-dimensional reconstruction

Mounted whole brains of adult flies were scanned by Olympus FV500 or Leica TCS SP5 confocal microscopes. Stacks of optical sections at 0.2 μm spacing were collected by an Olympus 60× or Leica 63× objective lens with 1024×1024 pixel resolution at middle scanning

speed. The raw confocal images were imported into AutoDeblur (Media Cybernetics, Md.) to perform three-dimensional deconvolution and subjected to three-dimensional reconstruction by Leica Deblur. The images were then processed by ImageJ (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, Md., USA, http://rsb.info.nih.gov/ij/, 1997–2008). To obtain clear images of three-dimensional reconstructions, the GFP signal in a few image stacks that had high noise was cropped out before making the three-dimensional recon-structions. Because of the limited working distance of the objective lens, several samples were scanned along the Z axis in both directions to make composite images. Figures were prepared by Adobe Photoshop (Adobe System, Calif.). The terminology and axis of *Drosophila* brains were in accord with those of Strausfeld and co-workers (Strausfeld 1976; Strausfeld and Seyan 1985).

Visual pattern memory assay

The procedure of the visual pattern memory assay in the fly simulator was the same as previously described (Wang et al. 2008).

Results

F1 and F5 neurons are sufficient to rescue visual pattern memory for two parameters, respectively

Our previous results showed that rut^+ rescue of the two kinds of large-field neurons, viz., F1 and F5 neurons, could rescue the memory defect for the distinct visual pattern parameters, "contour orientation" and "elevation", on the *rut*-mutant background, respectively (Liu et al. 2006). Recently, we found that specific expression of the for^+ gene in the F5 neurons could rescue the memory defect for parameter "elevation" on the for^S background (Wang et al. 2008). We then tested whether F1 neurons were also involved in *for*-dependent visual pattern memory for "contour orientation". As NP6561 and NP6510 are the only known GAL4 lines that label F1 neurons specifically, and as their overall expression patterns are extremely similar because the distance between the two P{GAL4} insertion loci is only 3 bp (Flybase Consortium at http://flybase.org/static_pages/docs/consortium.html), the GAL4 line NP6561 was then assigned to drive the local expression of for^+ on a homozygous for^S background (UAS-for^+/+; for^S/for^S;NP6561/+). As shown in Fig. 1, the control flies (UAS-for^+/+;for^S/for^S and for^S/for^S;NP6561/+) showed no memory for "contour orientation" (Performance Index$_8$ [PI$_8$]=0.04±0.06, t-test, t=0.73, P=0.47; PI$_8$=−0.04±0.07, t=−0.58, P=0.57), whereas specific expression of the for^+
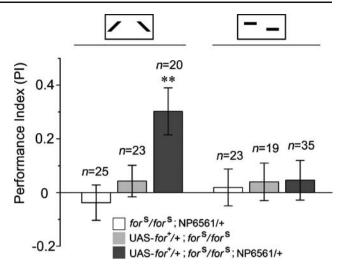

Fig. 1 Rescue of the memory for "contour orientation" in for^S flies by expression of the for^+ transcript in F1 neurons. In the presence of the pattern parameter "contour orientation" (*left*), the memory scores of the for^S flies with only the GAL4 driver (for^S/for^S;NP6561/+) or with only UAS-for^+ (UAS-for^+/+; for^S/for^S) are not significantly higher than zero. The flies of genotype UAS-for^+/+; for^S/for^S;NP6561/+ show memory scores significantly higher than zero. In the case of the parameter "elevation" (*right*), memory scores of all the three fly lines are not significantly different from zero (*error bars* SEMs, *n* numbers of flies tested). **$P<0.01$
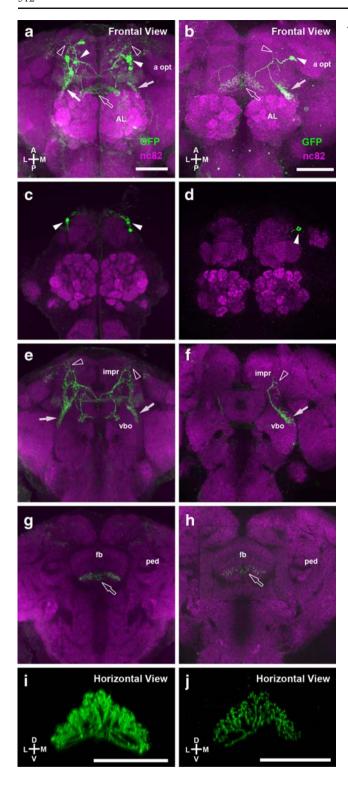
gene in F1 neurons by NP6561 was able to rescue the defect effectively in the memory for "contour orientation" in for^S flies (PI$_8$=0.30±0.09, t=3.41, P=0.003). On the other hand, the expression of the for^+ gene in F1 neurons by NP6561 was unable to rescue the defect in the memory for "elevation" in for^S flies (UAS-for^+/+;for^S/for^S : PI$_8$=0.04±0.071, t=0.55, P=0.59; for^S/for^S;NP6561/+: PI$_8$=0.019±0.069, t=0.27, P=0.79; UAS-for^+/+;for^S/for^S; NP6561/+: PI$_8$=0.045±0.074, t=0.61, P=0.55). Thus, F1 neurons are specific for the *for*-dependent visual pattern memory for "contour orientation".

These results, together with our previously reported results, show that F1 and F5 neurons are specifically involved in visual pattern memory for "contour orientation" and "elevation", respectively.

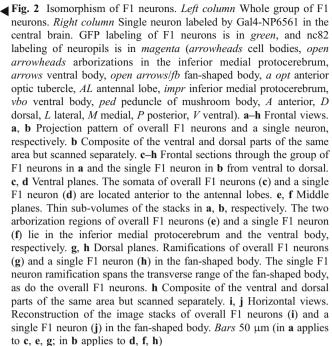### F1 neurons are a group of isomorphic neurons with three main arborization domains

Since F1 neurons are responsible for storing visual pattern memory for "contour orientation", we wanted to know the morphological features of these F1 neurons, which could be labeled by Gal4-NP6561 or GAL4-NP6510 combined with UAS-mCD8-GFP (Figs. 2, 3). The somata of about seven F1 neurons were located anterior to the antennal lobe in each hemisphere of the adult fly brain (Fig. 2c). The primary neurites from each soma converged into a bundle that ran dorsally and then posteriorly into the fan-shaped

neurons. A representative single F1 neuron in the protocerebrum is shown in Fig. 2b. The soma was located anterior to the antennal lobe, near the anterior optic tubercle (Fig. 2d), and a primary neurite extended dorsally into the inferior medial protocerebrum where it branched into mainly three fibers to form arborizations in the inferior medial protocerebrum (Fig. 2f), the ventral body (Fig. 2f), and the fan-shaped body (Fig. 2h, j). When compared from different viewing angles, the single F1 neurons labeled by both Gal4-NP6561 and Gal4-NP6510 showed some similar features (Fig. 3). Both of them had their cell bodies located anterior to the antennal lobe and generate mainly three arborization regions.

Figure 3d shows the ramification of F1 neurons in the first layer of the fan-shaped body: a main fiber invaded the most ventral margin of the fan-shaped body (Fig. 3c) and generated dorsally running processes with varicosities in a thin coronal layer (Fig. 3d). The ramification pattern in the fan-shaped body was grossly symmetrical across the midline of the central brain (Figs. 2b, 3d, h). Individual F1 neurons showed similar arborization patterns in the fan-shaped body (Fig. 3d, h). Further understanding of the organization of F1 neurons was provided by double neuron visualization (Fig. 3i–l). In the majority of cases, two neurons from each side of the protocerebrum projected their long fibers medially and posteriorly to the contralateral sides and finally arborized in the fan-shaped body where their ramification regions overlapped (Fig. 3l). In a few cases, the branches in the fan-shaped body were organized differently, such that the fiber innervating the fan-shaped body bifurcated into two

body. Three arborization fields were generated by the primary neurites in the inferior medial protocerebrum (Fig. 2e), the ventral body (Fig. 2e), and the fan-shaped body of the central complex (Fig. 2g, i).

We analyzed the structure of the F1 neurons in detail with a FLP-out technique that enabled us to label single
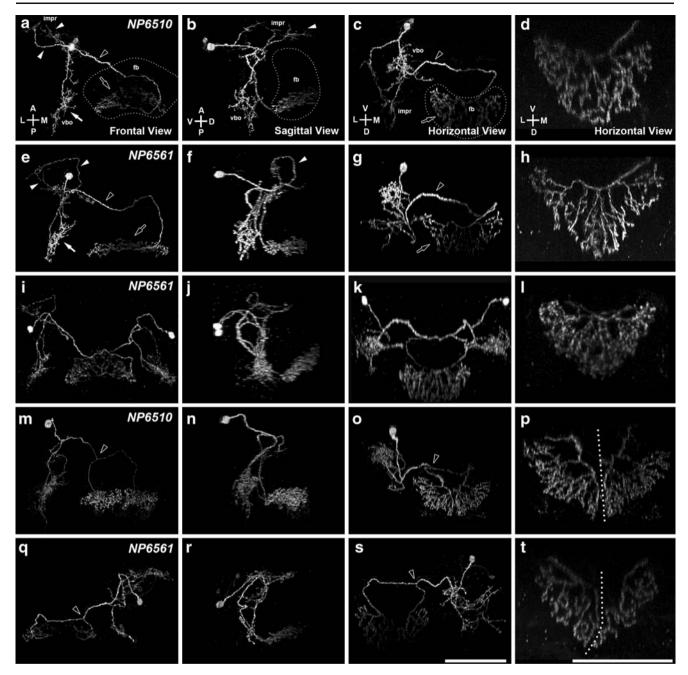
Fig. 3 Morphological features of F1 neurons. *Each row* Same sample viewed from different angles. The arborization regions in the inferior medial protocerebrum (*impr*) and ventral body (*vo*) are indicated by *arrowheads* (**a**, **b**, **e**, **f**) and *arrows* (**a**, **e**), respectively. The arborizations in the fan-shaped body (*open arrows* in **a**, **c**, **e**, **g**) are generated by the main fibers (*open arrowheads*). **a–h** Three arborization regions of single F1 neurons labeled by Gal4-NP6510 (**a–d**) and Gal4-NP6561 (**e–h**). *Dashed lines* in **a–c** indicate fan-shaped body (*fb*). **i–l** Projection pattern of double F1 neurons. Two F1 neurons with their cell bodies located contralaterally have trajectories symmetrically arranged across the middle line of the central brain (**i**, **k**). Their ramifications in the fan-shaped body seem to overlap (**l**). **m–t** A rare type of ramification pattern in the fan-shaped body labeled by Gal4-NP6510 (**m–p**) and Gal4-NP6561 (**q–t**). Their main fibers bifurcate before innervating the first layer of the fan-shaped body. The points of bifurcation are indicated by *open arrowheads* (**m**, **o**, **q**, **s**). The two branching regions are located in the same plane but do not overlap (*dashed lines* in **p**, **t**). *Bars* 50 μm (in **s** applies to *first three columns*, in **t** applies to *last column*)

secondary fibers (Fig. 3m, q, o, s): one crossed the midline to the contralateral side, whereas the other did not. Both fibers then projected posteriorly and ipsilaterally to form two adjacently aligned arborization regions that together covered the whole first layer of the fan-shaped body (Fig. 3p, t).

The branching of F1 neurons in the inferior medial protocerebrum showed different morphological features. In

some cases, we observed abundant arbors with spiny terminals (Fig. 4a–f) and, in others, short fibers with few branches (Fig. 4g–l).

With regard to the arborizations in the ventral body, fibers projected by primary neurites extended into the ventral body (Fig. 4). In most cases, the arborizations were generated by two fibers in a thin anterio-posteriorly

extending layer (Fig. 4d, e, g, h, j, k). Interestingly, additional fibers were occasionally found to innervate the ventral body (Fig. 4k).

In brief, F1 neurons are a group of isomorphic neurons with three main branches: one in the first layer of the fan-shaped body, one in the ventral body, and the other in the inferior medial protocerebrum.
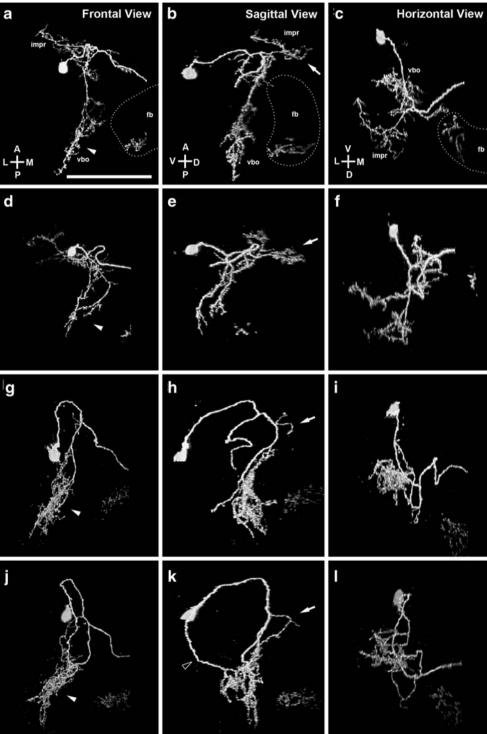

Fig. 4 Arborization patterns of F1 neurons in the inferior medial protocerebrum (*impr*) and ventral body (*vbo*). Gal4-NP6561 was used to label F1 neurons. *Each row* Same sample from different angles. *Dashed lines* in **a–c** indicate fan-shaped body (*fb*). **a–c**, **d–f** Two samples of arborization patterns with dense branches in the inferior medial protocerebrum. **b**, **e** Sagittal view (*arrows* arborization regions). **g–i**, **j–l** Two samples of arborization patterns with sparse branches in the inferior medial protocerebrum. **h**, **k** Sagittal view (*arrows* arborization regions). **a**, **d**, **g**, **j** Arborization regions in the ventral body (*arrowheads*). Additional fibers are occasionally found to innervate the ventral body (*open arrowhead* in **k**). *Bar* 50 μm
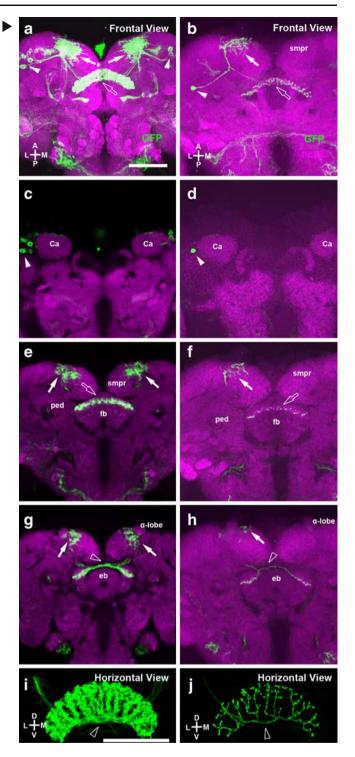
Fig. 5 Isomorphism of F5 neurons. *Left column* Whole group of F5 neurons. *Right column* Single neuron labeled by Gal4-c205 in the protocerebrum. GFP labeling of F5 neurons is in *green*, and nc82 labeling of neuropils is in *magenta* (*arrowheads* cell bodies, *arrows* arborizations in the superior medial protocerebrum, *open arrows/fb* fan-shaped body, *smpr* superior medial protocerebrum, *Ca* calyx of mushroom body, *α-lobe* α-lobe of mushroom body, *ped* peduncle of mushroom body, *eb* ellipsoid body). **a**, **b** Projection pattern of overall F5 neurons and a single neuron, respectively. **c–h** Frontal sections through the group of F5 neurons in **a** and the single F5 neuron in **b** from dorsal to ventral. **c**, **d** Dorsal planes. The cell bodies of F5 neurons are located laterally to the calyxes of the mushroom bodies. **e**, **f** Middle planes. **g**, **h** Ventral planes (*open arrowheads* shafts at the ventral edge of the fan-shaped body). **i**, **j** Horizontal view of the ramifications of overall F5 neurons and a single F5 neuron, respectively, in the fan-shaped body (*open arrowheads* shafts). *Bars* 50 μm (in **a** applies to **a-h**; in **i** also applies to **j**)

F5 neurons belong to a group of isomorphic neurons with two arborization domains

A group of F5 neurons marked by Gal4-c205 was composed of about eight to ten neurons with somata that were loosely arranged near the calyx of the mushroom body at each side of the protocerebrum (Fig. 5a, c). The primary neurites that projected from each soma converged into a tightly packed bundle near the lateral edge of the calyx and ran ventrally and anteriorly into the superior medial protocerebrum. There, the bundle bifurcated: one gave rise to dense arborizations, the majority of which were dorsal to the α/α'-lobes of the mushroom body (Fig. 5a, e, g), whereas the other projected medio-posteriorly into the fifth layer of the fan-shaped body, where it branched dorsally into horizontal processes that formed an anteriorly convex stratum (Fig. 5a, e, g, i).

Using the FLP-out system to dissect the expression patterns at single neuron resolution, we found that individual F5 neurons projected in the same direction as the whole group (Fig. 5b). Primary neurites extended into the superior medial protocerebrum where they gave rise to two fibers: the shorter extended anteriorly and formed arborizations with spiny terminals in the superior medial protocerebrum (Fig. 5f), whereas the longer fiber innervated the most ventral margin of the fan-shaped body where it bifurcated into a shaft (Fig. 5h, j) and formed ramifications with bleb-like terminals in the fifth layer of the fan-shaped body (Fig. 5f, j). As shown in Fig. 5i and j, the single F5 neuron shared a similar arborization pattern in the fan-shaped body as did the whole group. Moreover, its arborization domain took up the full extent of the fifth stratum of the fan-shaped body, like that of the whole group.
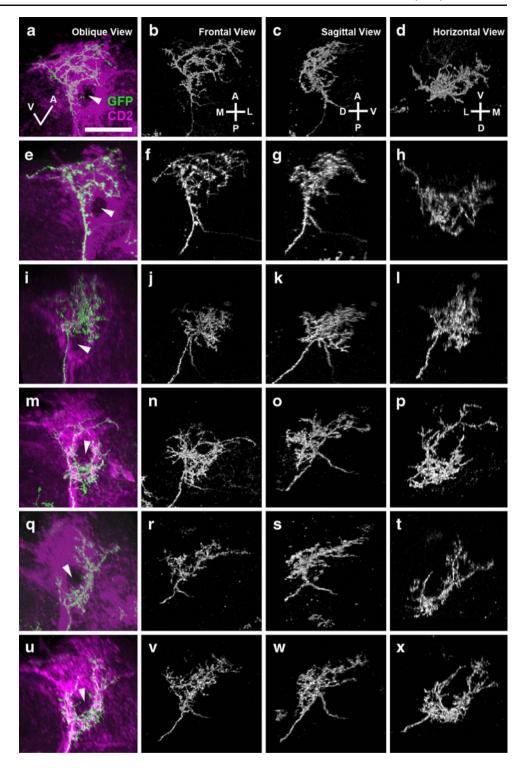
Furthermore, we found that the dendritic arborizations of F5 neurons in the superior medial protocerebrum could be divided into two categories according to location of their arborizations. Individual F5 neurons in the superior medial

protocerebrum had relatively sparse arbors (Fig. 6). Three-dimensional reconstruction provided us with different viewing angles for observing the morphology of these neurons. From an oblique view, we found a small canal that was not innervated by the dendrites of F5 neurons in the tufted arborization labeled by CD2 (Fig. 6a, e, i, m, q, u). According to the relative positions of dendrites to the canal, arborization patterns of individual neurons in the superior

Fig. 6 Two kinds of single
F5 neuron arborizations in the
superior medial protocerebrum.
Gal4-c205 was used to label F5
neurons. *Each row* Same sample
from different angles. *First col-
umn* Branches of a single neuron
labeled by GFP (*green*) super-
imposed over branches of over-
all F5 neurons labeled by CD2
(*magenta*). A canal in the
branching region of overall F5
neurons is visible from an
oblique view (*arrowheads* in **a**,
e, **i**, **m**, **q**, **u**). **a–l** Three samples
of single neurons with arboriza-
tion regions located anterior to
the canal. **m–x** Three samples of
single neurons with arborization
regions located posterior to the
canal. *Bar* 20 μm

medial protocerebrum could be divided into two main
categories. In the first category, an anteriorly running shaft
gave rise to small branches that were located anterior to the
canal and formed the anterior part of the overall arboriza-
tion (Fig. 6a–l). This arrangement was encountered in more
than half of the single neuron samples. In the second
category, the branches of the F5 neurons in the superior

medial protocerebrum occupied the posterior part of the
whole arborization volume (Fig. 6m–x). Compared with the
arborization patterns in the superior medial protocerebrum,
the ramifications of individual F5 neurons in the fan-shaped
body showed more common characteristics (Fig. 5j).

In brief, individual F5 neurons belong to a group of
isomorphic neurons with two arborization domains: one in

the superior medial protocerebrum and the other in the fifth layer of the fan-shaped body.

Presynaptic sites of both F1 and F5 neurons are mainly located in the fan-shaped body
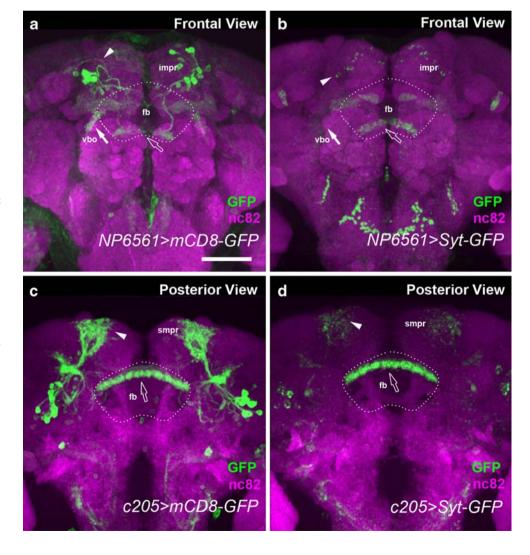
To identify the possible presynaptic sites in F1 and F5 neurons, a reporter line, UAS-Syt-GFP, was used. In this line, GFP is fused to a pre-synaptic vesicle membrane protein, synaptotagmin (Syt), in order to visualize presynapses (Zhang et al. 2002). In Gal4-NP6561 flies carrying UAS-Syt-GFP, stronger GFP labeling could only be observed in the first layer of the fan-shaped body, whereas weaker GFP signal was found in neuronal cell bodies of the inferior medial protocerebrum and the ventral body (Fig. 7b). From the observations that F1 neurons had bleb-like terminals in the fan-shaped body and spine-like ends in the ventral body and the inferior medium protocerebrum (Hanesch et al. 1989), we suggested that the branches in the inferior medial protocerebrum and the

ventral body should contain postsynaptic sites, and that branches in the fan-shaped body should contain presynaptic sites.

Similar to the F1 neurons, stronger GFP labeling was mainly present in the fifth layer of the fan-shaped body in Gal4-c205 flies carrying UAS-Syt-GFP (Fig. 7d). As shown in Fig. 7d, GFP labeling was found to be weaker in the cell bodies and the anterior arborization region in the superior medial protocerebrum. These results were consistent with the observation that F5 neurons showed spine-like terminals in the superior medial protocerebrum and bleb-like terminals in the fifth layer of the fan-shaped body (Hanesch et al. 1989). We suggest that the aborizations in the superior medial protocerebrum should contain postsynaptic sites, and that the branches in the fan-shaped body should contain presynaptic sites.

Taken together, our observations indicate the existence of presynaptic sites of both F1 and F5 neurons located in the fan-shaped body and postsynaptic sites outside of the fan-shaped body.

Fig. 7 Possible pre- and postsynaptic sites of F1 and F5 neurons. GFP labeling of neurons is in *green*, and nc82 labeling of neuropils in the protocerebrum is in *magenta*. GFP signals in the inferior/superior medial protocerebrum (*impr/smpr, arrowheads*), ventral body (*vbo, arrows*), and fan-shaped body (*fb, open arrows*). **a, b** Expression pattern of Gal4-NP6561, as visualized with mCD8-GFP or Syt-GFP, respectively. Syt-GFP is mainly present in the fan-shaped body domain of F1 neurons. Compared with the mCD8-GFP signal in the inferior medial protocerebrum and the ventral body, the Syt-GFP signal is weak. **b** is a composite image of the ventral and dorsal parts of the same sample scanned from both sides. **c, d** Expression pattern of Gal4-c205 visualized with mCD8-GFP or Syt-GFP, respectively. The fan-shaped body domain of F5 neurons is strongly labeled by Syt-GFP but is weak in cell bodies and the superior medial protocerebrum when compared with the corresponding labeling by mCD8-GFP. *Bar* 50 μm

Discussion

Here, our results have shown that ectopic expression of *for*⁺ in the F1 neurons rescues visual memory for pattern parameter "countour orientation", but not that for "elevation". We have analyzed the morphological features of large-field F1 and F5 neurons at single neuron resolution in the protocerebrum of the fruitfly. Projection patterns of individual F1 or F5 neuron are similar to those of the whole group. Outside of the fan-shaped body, F1 neurons have two arborization domains in the ventral body and the inferior medial protocerebrum, whereas F5 neurons have one arborization domain in the superior medial protocerebrum. Both F1 and F5 neurons innervate the fan-shaped body with bleb-like terminals that are strongly labeled by presynaptic-specific Syt-GFP.

Our behavioral results have revealed that, with regard to the *for*-dependent visual memory, F1 neurons are also specific for visual pattern "countour orientation". This observation, together with our former report on F5 neuron function in *for*-dependent visual memory, parallels our previous results obtained on a *rut* genetic background and further confirms the conclusion that F1 and F5 neurons are crucial for visual memory for the patterns "elevation" and "contour orientation" (Liu et al. 2006; Wang et al. 2008), as summarized in Table 1.

Our results concerning the morphology of F1 and F5 neurons parallel the Golgi staining results of the fan-shaped body neurons by Hanesch et al. (1989). Both F1 and F5 neurons have their ramifications filled in the fan-shaped body in the transverse and longitudinal range. Moreover, both F1 and F5 neurons innervate the fan-shaped body laterally; they might be the F*l*-neurons, since their fibers reach the fan-shaped body laterally and extend along the ventral surface of the fan-shaped body neuropil, as F*l*-neurons do (Hanesch et al. 1989). According to Hanesch et al. (1989), the group of F*l*-neurons is heterogeneous. The F*l*-neurons, which only have small branching regions in the ventral body, seem to be analogous to our F1 neurons with their few branches (Fig. 3i, l) in the inferior medial protocerebrum. The F5 neurons may belong to the F*l*-neurons with arborizations in the medio-dorsal protocerebrum and sparse branches in

the fan-shaped body. Interestingly, we have also found F1 neurons with extra branches (Figs. 2c, g, 3c, f) in the inferior medial protocerebrum; these probably belong to the F*l*-neurons.

We have investigated the polarity of the F1 and F5 neurons by comparing overexpressed Syt-GFP, a presynaptic marker, and the mCD8-GFP signal by using the same Gal4 lines. In F1 neurons, a lower level of Syt-GFP signal has been observed outside the fan-shaped body. Large parts of F1 neurons in the inferior medial protocerebrum and ventral body are devoid of Syt-GFP, whereas the Syt-GFP signal in the fan-shaped body is much stronger. In F5 neurons, the Syt-GFP signal in the superior medial protocerebrum is greatly decreased, whereas the Syt-GFP signal in the fan-shaped body is strong as compared with the mCD8-GFP signal. Because the Syt-GFP and mCD8-GFP signals have been indirectly compared based on different samples, we can only conclude here that the regions devoid of Syt-GFP signal contain postsynaptic sites, whereas regions with strong Syt-GFP signal contain presynaptic sites. Thus, at least some F1 and F5 neurons have presynaptic sites in the fan-shaped body and postsynaptic sites in the branching domains outside the fan-shaped body. Meanwhile, we cannot exclude the possibility that presynaptic sites occur in branching domains outside the fan-shaped body and postsynaptic sites are present within the fan-shaped body. This technical limitation might be resolved by a strategy suggested by Ito and co-workers in which Syt-GFP and DsRed S197Y are co-expressed so that the overall neuronal morphology can be visualized with DsRed S197 and the presynaptic sites can be marked by Syt-GFP in the same neurons (Verkhusha et al. 2001; Otsuna and Ito 2006).

Interestingly, we have found a few F1 neurons that have their arborization domain in the fan-shaped body, but that are composed of two bordering but separate sub-domains. Their main fibers bifurcate near the ellipsoid body; the two secondary fibers extend contralaterally along the outer edge of the ellipsoid body and form two arborization domains that jointly cover the whole first layer of the fan-shaped body (Fig. 3p, t). A comparable phenomenon has been observed in *Drosophila* visual projection neurons, viz., LT32 neurons, that show "misrouting projections" but can finally find their correct projection target (Otsuna and Ito 2006). Similarly,

Table 1 Gal4 driver lines able to rescue the memory for two visual pattern parameters in the flight simulator on various genetic background are listed. Visual memory is defective in *forS* and *rut* mutant flies but can be respectively restored by ectopic expression of the wild-type form of *for* and *rut* in fan-shaped body neurons. In both cases, F1 neurons are always related to the memory for pattern "contour orientation", whereas F5 neurons are always related to the memory for "elevation"

Visual pattern parameters	Fan-shaped body neuron types and corresponding Gal4 lines		Genes	References
Contour orientation	F1	NP6561, NP6510	*rutabaga*	Liu et al. 2006
Elevation	F5	C205, C5, C271, 104y, 121y, 154y, 210y		
Contour orientation	F1	NP6561	*foraging*	This work
Elevation	F5	C205		Wang et al. 2008

these F1 neurons might have their main fibers "mis-bifurcated". The two secondary fibers could recognize each other and cooperate to form a normal tiling arborization pattern without overlapping.

This morphological study of the fan-shaped body should help us to understand the function and to disclose more properties of this important central brain structure.

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