Fibroblast growth factor signaling instructs ensheathing glia wrapping of Drosophila olfactory glomeruli

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The formation of complex but highly organized neural circuits requires interactions between neurons and glia. During the assembly of the Drosophila olfactory circuit, 50 olfactory receptor neuron (ORN) classes and 50 projection neuron (PN) classes form synaptic connections in 50 glomerular compartments in the antennal lobe, each of which represents a discrete olfactory information-processing channel. Each compartment is separated from the adjacent compartments by membranous processes from ensheathing glia. Here we show that Thisbe, an FGF released from olfactory neurons, particularly from local interneurons, instructs ensheathing glia to wrap each glomerulus. The Heartless FGF receptor acts cell-autonomously in ensheathing glia to regulate process extension so as to insulate each neuropil compartment. Overexpressing Thisbe in ORNs or PNs causes overwrapping of the glomeruli their axons or dendrites target. Failure to establish the FGF-dependent glia structure disrupts precise ORN axon targeting and discrete glomerular formation.

ensheathing glia | FGF | olfactory glomeruli | neuropil compartimentalization | ORN axon targeting

Glia and neurons interact dynamically to coordinate the development and function of neural circuits. For example, Drosophila midline glia serve as guideposts that provide spatial cues to direct axonal pathfinding in the embryonic ventral nerve cord (1, 2). Neurons in turn provide signals that weave glia into the fabric of the nervous system. For instance, axonal Neuregulin-1 levels dictate the extent of Schwann cell myelination (3). A myriad of other biological processes in the developing and adult brain, including synapse formation (4–7), elimination (8, 9), and regulation of synaptic activity (10–13), require extensive communication and cooperation between neurons and glia.

The Drosophila nervous system contains a diverse array of glial cell types (14), thus offering opportunities to discover new mechanisms for glia–neuron interactions. Ensheathing glia are groups of cells that insulate neighboring neuropil structures by extending their membranous processes along the outer surface of synaptic neuropils or their subcompartments without invading the inner part of the neuropil (15). It remains unknown how ensheathing glia establish such a barrier-like structure, what molecular signals orchestrate this process, and whether the glial barrier is essential for the integrity of the encircled neuropil.

The Drosophila antennal lobe provides an excellent experimental system for tackling these questions with high resolution. The antennal lobe is organized into 50 discrete neuropil compartments, the glomeruli, wherein specific types of olfactory receptor neuron (ORN) axons and projection neuron (PN) dendrites form synapses. Each glomerulus is surrounded by ensheathing glia processes (15–17). In addition, the specific and stereotypic pattern of neuronal projections in the antennal lobe (18, 19) renders the system convenient for exploring the potential neuronal disorganization caused by the malformation of glial structures. Previous studies have used this system to find that in adult Drosophila the ensheathing glia increase their process extension to injured axons (20), help clear degenerating axons (20), and strengthen excitatory interactions between surviving neurons (21). Here we study ensheathing glia in the antennal lobe formation and organization during development.

Results

Pupal Development of Antennal Lobe Ensheathing Glia. To study the development and function of antennal lobe ensheathing glia, we searched for genetic tools that can label these cells specifically during the pupal stage when the olfactory circuit in the antennal lobe is being assembled. We preselected a number of enhancer-GAL4 lines from the FlyLight GAL4 collection based on the published expression pattern in adult animals (22) and from a few MIMIC-GAL4 lines (23). We tested whether these GAL4 lines were active at different pupal stages. We found that at 96 h after puparium formation (hAPF), a late stage in antennal lobe morphology, SPARC-GAL4 (Fig. 1D), GMR56F03-GAL4 (Fig. S1) (24), and GMR10E12-GAL4 (Fig. S1) flies expressed GAL4 predominantly in ensheathing glia. As indicated by the UAS-mCD8GFP reporter that labeled the cell membranes and processes in the presence of GAL4, these GAL4+ cells were located on the periphery of the antennal lobe and extended their processes to wrap around the antennal lobe and around individual glomeruli within it but with minimal invasion into each glomerulus. These morphological features are characteristic of ensheathing glia. We also used UAS-nuclear-LacZ to mark the nuclei of these GAL4+ cells (Fig. 1).

Significance

This research reports that reciprocal interactions between Drosophila olfactory neurons and ensheathing glia mediate the formation of neuronal compartments, groups of synapses that are packed into discrete structures called “glomeruli” that carry specific olfactory information. Ensheathing glia respond to a neuronal cue, the FGF Thise, to pattern the boundaries of the nascent compartments. Neural compartments, in turn, require such glial barriers to separate themselves from neighboring compartments and thus ensure the correct organization of the olfactory circuit. These findings highlight the importance of glia in the assembly and maintenance of neural circuits and the functions of FGF signaling in these processes.

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detected around the antennal lobe, and for Repo, a glia marker in (within 10 μm of the surface of the antennal lobe) was also positive for GAL4+ glia from astrocytes further, we used the GAT antibody (26) to mark astrocytes and found that the majority (∼95%) of the GAL4+ glia are negative for GAT (Fig. S1). In summary, SPARC-GAL4, GRM56F03-GAL4, and GMR10E12-GAL4 mark ensheathing glia around the antennal lobe in the late pupal stage.

SPARC-GAL4 also drove reporter expression at 24 hAPF, enabling us to investigate the development of antennal lobe ensheathing glia. At 24 hAPF, before glomerular formation, ensheathing glia processes were restricted to the periphery of the antennal lobe (Fig. 1A). From 24–48 hAPF, axon terminals of ORNs invade the antennal lobe and target specific subregions to connect with their synaptic partners such as dendrites from PNs (17). We found that around 48 hAPF, when proto-giomeruli first emerged, ensheathing glia processes started to invade the antennal lobe from the lobe surface (Fig. 1B). Even at this initial stage of infiltration, ensheathing glia processes displayed a preference to grow along the borders between adjacent proto-giomeruli instead of growing into them (Fig. 1B2). At 72 hAPF, as glomerular structures became more clearly separable, ensheathing glia processes had encircled most glomeruli (Fig. 1C). These processes remained on glomerular boundaries, with minimal extension into the glomeruli. Ensheathing glia wrapping of olfactory glomeruli was complete by the end of the pupal stage (Fig. 1D).

We also observed an increase in the number of SPARC-GAL4+ ensheathing glia around the antennal lobe from 24–72 hAPF (Fig. 1F). This increase is caused, at least in part, by glial cell proliferation, because we could generate glia clones using mosaic analysis with a repressible cell marker (MARCM) (27) with heat shock-induced mitotic recombination at any point from 24–72 hAPF (see Fig. 3 and Fig. S3 below).

Heartless Knockdown in Ensheathing Glia Reduces Processes and Disrupts the Ensheathment Pattern. To identify the molecular signals that control the extension of ensheathing glia processes, we tested ∼50 candidate adhesion molecules, receptor kinases, and receptor phosphatases by RNAi and screened for potential defects in ensheathing glia development and antennal lobe organization. We found that expression of two independent RNAi targeting nonoverlapping regions of heartless (htl), an FGF receptor (28–30), caused a 70% reduction of ensheathing glia processes within the antennal lobe (Fig. 2A–D). This phenotype is consistent with a previous discovery that FGF signaling is required for astrocytes to infiltrate the larval ventral nerve cord of Drosophila (26). However, the antennal lobe provides a unique opportunity to observe the response of neuronal compartmentalization to glia morphogenesis defects.

We found that many glomeruli became less clearly separable (arrowheads in Fig. 2A2, B2, and C2) when htl was knocked down. To quantify this effect, we used the relative SD (RSD) of the N-cadherin (Ncad) neuropil staining intensity as an index for the degree of compartmentalization, because incomplete antennal lobe compartmentalization would cause obscure glomerular borders and hence smaller variance in the neuropil staining signal. We found a significant reduction of RSD in htl-knockdown compared with wild-type antennal lobes (Fig. 2E). The remaining glomerular borders sometimes also lacked ensheathing glia processes that normally would divide the adjacent glomeruli (Fig. 2A–C). We also observed ensheathing glia processes that extended within glomeruli; such extension into glomeruli is rarely observed in wild-type antennal lobes (Fig. 2B1, arrowhead) and likely results from the poor establishment of glomerular borders. We quantified the localization pattern of the ensheathing glia processes relative to the glomerular compartments by plotting the intensity of the GFP signal derived from glial membranes together with the intensity of Ncad signal marking the neuropil (Fig. 2A–C), and calculated their correlation coefficient (Fig. 2F). In control animals, Ncad and GFP signals exhibited strong anticorrelation (Fig. 2F), because ensheathing glia processes are preferentially located on the

Fig. 1. Ensheathing glia morphogenesis during antennal lobe development. (A–D) Confocal sections of the antennal lobe at 24 (A), 48 (B), 72 (C), and 96 (D) hAPF. Ensheathing glia processes are labeled by SPARC-GAL4-driven (→) UAS-mCD8GFP. Ensheathing glia nuclei are marked by UAS-nuclear-LacZ. Neuronal staining by the Ncad antibody is shown as magenta. Dashed rectangles in center images are enlarged in the right column. D, dorsal; L, lateral. (Scale bars: 10 μm.) (E) Confocal sections of the antennal lobe at 96 hAPF. Glia nuclei stained by the Repo antibody are shown in green and are overlaid on the LacZ channel in E2. (F) Quantification of the number of antennal lobe ensheathing glia cells at different developmental stages and the number of total glia around the antennal lobe at the end of pupae stage. Error bars represent SD. n = 5 for each time point. In this figure, subpanels represent the same sample in different imaging channels, unless otherwise specified.

1E and found that every LacZ+ nucleus around the antennal lobe (within 10 μm of the surface of the antennal lobe) was also positive for Repo, a glia marker in Drosophila (25), confirming that these GAL4+ cells are indeed glia. On average, ∼100 Repo+ cells were detected around the antennal lobe, and ∼56 of these were LacZ+ (Fig. 1F). Repo+/LacZ+ cells are likely astrocytes and cortex glia present in the vicinity of ensheathing glia. To distinguish these
To dissociate the role of htl in controlling glia survival and process extension, we used the MARCM technique (27) to generate single-cell clones of ensheathing glia homozygous for the htl<sup>h422</sup> (30) allele in an otherwise heterozygous background. In this experiment, GMR<sup>R0E12-GAL4</sup> was used to label the homozygous mutant cells. We used UAS-mCD8GFP to visualize glial processes (Fig. 3 E and F) and UAS-nuclear-LacZ (arrows in Fig. 3 E) to visualize cell bodies of ensheathing glia in these MARCM clones. Compared with wild-type cells, the htl<sup>h422</sup> single-cell clones exhibited a 50% reduction in the volume of glial processes (Fig. 3G) and a 50% reduction in total fluorescence intensity (Fig. S3). These reductions were accompanied by a decrease in the number of glomeruli that each ensheathing glia could access, as quantified by the number of glomerular borders to which each glia extended (Fig. 3H). Thus, this mosaic experiment demonstrated that htl is cell-autonomously required for process extension by ensheathing glia.

To determine the subcellular localization of the Htl protein, we used a fosmid transgenic line that produces Htl-GFP from an insertion that contains the extended genomic region covering htl (36) to analyze GFP signal within and around the antennal lobe during pupal development. At 24 hAPF, no Htl-GFP signal was detected inside the antennal lobe (Fig. 3I), consistent with the location of ensheathing glia processes (Fig. 1A). At 48 hAPF, we started to detect Htl-GFP signal between proto-glomeruli (Fig. 3J). By 72 hAPF, Htl-GFP signal was detected at most glomerular borders. [Also, a lower level of signal, which may originate from cell types other than ensheathing glia, such as astrocytes and potentially neurons, was detected within glomeruli (Fig. 3K).] The signal on glomerular borders coincided well with the wrapping pattern of the ensheathing glia over this developmental course (Fig. 1). These data support the hypothesis that Htl mediates FGF signaling to regulate the invasion of the antennal lobe and the wrapping of individual glomeruli by ensheathing glia.

**Thsbe, an FGF Ligand, Is Required for the Wrapping of Glomeruli by Ensheathing Glia.** We next assessed the identity and cellular source of FGF ligands that regulate ensheathing glia development. Htl responds to two FGF ligands, Thsbe (Ths) and Pyramus (Pyr) (37, 38). To test whether ths is required for the glia ensheathing the antennal lobe to form the wrapping pattern, we analyzed ensheathing glia and antennal lobe morphology in animals that carried different ths mutant alleles (39). The phenotypes we observed with loss of htl were all recapitulated in transheterozygous combinations of ths alleles [ths<sup>7507</sup> and Df(2R)ED2258] (40). There was an overall reduction of ensheathing glia processes and cell numbers in the antennal lobe of transheterozygous flies (Fig. 4 A, B, E, and F). Similar to the loss of htl from ensheathing glia, the antennal lobe of ths mutants showed defective compartmentalization as measured by reduced RSD values of Ncad staining (Fig. 4G) and a markedly reduced anti-correlation between the intensity of signal from ensheathing glia processes and the neuropil (Fig. 4H). These findings indicate that ths is required for ensheathing glia to wrap around glomeruli in the antennal lobe and that the failure to form a correct wrapping pattern could disrupt the compartmentalization of the antennal lobe neuropil.

**Ths Is Produced by ORNs, PNs, and Antennal Lobe Local Interneurons.** To identify which cell types express Ths, we took advantage of a MiMIC (Minos mediated integration cassette) insertion (23) located between two coding exons of the ths gene. We converted the MiMIC cassette to an artificial exon that contains the coding sequence for 2A-GAL4 (40). Thus, GAL4 can be produced along with the endogenous N terminus of Ths (encoded by the first two exons) and can drive reporter gene expression in the ths pattern. We then used this Ths-GAL4 to express UAS-mCD8GFP to

![Image](image-url)
visualize the cell bodies and projections of the Ths-producing cells. In the antennal lobe, neuronal processes from PNs, ORNs, and local interneurons (LNs) are all labeled, suggesting that all three major neuronal types produce Ths (Fig. 4I).

To determine the contributions of each of these cell types, we used an intersectional strategy in which Ths-GAL4 was combined with FLP recombinases that are specifically expressed in ORNs (ey-FLP) (41) or PNs (GH146-FLP) (42). With a FLP-out reporter, UAS-FRT-stop-FRT-mCD8GFP (42), we found a number of ORN and PN classes were Ths-GAL4+ based on glomerular labeling (Fig. 4 J and K). At least 45 cell bodies over all sections of the antennal lobe were Ths-GAL4+ without intersection (Fig. 4I); however, only 19 of them (constituting the majority of PNs innervating ~40 glomeruli) were GH146-FLP+ PNs; this finding is consistent with the small number of glomeruli (approximately seven) labeled by the intersection of GH146-FLP and Ths-GAL4 (Fig. 4K). This difference is likely caused by the contribution from LNs, whose cell bodies are also located around the antennal lobe (43, 44), whereas ORN cell bodies are located in the peripheral sensory organs.

We used two approaches to validate the notion that LNs produce Ths. First, we suppressed the expression from most PNs by combining GH146-GAL80 (42) together with Ths-GAL4. After PN-derived signal was largely eliminated, ~27 cell bodies remained around the antennal lobe. The projection pattern of these neurons covered the entire antennal lobe (Fig. 4L), as is characteristic of the majority of LNs (43). Second, we used Ths-GAL4 to label single cells by the MARCM technique and were able to identify MARCM clones for LNs (Fig. 4M). In summary, Ths is produced by a subset of ORNs, PNs, and LNs.
Ths Can Instruct Glomerular Wrapping with a High Spatial Specificity.

We have shown that FGF signaling is necessary for ensheathing glia to extend processes into the antennal lobe and demarcate individual glomeruli. To test whether FGF signaling can instruct ensheathing glia to wrap around selected neuropol compartments, we expressed ths in only one class of ORNs (VA1v) using a GAL4 under the control of the promoter of the odorant receptor specifically expressed in this class (Or7b-GAL4). We observed that overexpression resulted in hyperwrapping of the VA1v glomerulus by ensheathing glia (Fig. 5 B and D). This effect was highly localized, because, in addition to VA1v, only the adjacent VA1d glomerulus was slightly hyperwrapped (Fig. 5D), likely because of the intensified ensheathing glia processes on its border shared with VA1v. Hyperwrapping did not extend to the DA1 glomerulus (Fig. 5D), which is one glomerulus away from the VA1v glomerulus. There also was an excess of glia cells around the hyperwrapped VA1v glomerulus (Fig. 5F).

Similarly, overexpressing Ths in Mz19-GAL4+ PNs, which send dendrites to DA1 and VA1d, caused local hyperwrapping of these glomeruli, as well as a local increase in ensheathing glia cells (Fig. 5 C, E, and G). We have consistently noticed that Mz19-GAL4 activity is stronger in DA1 PNs than in VA1d PNs (46). Accordingly, glial hyperwrapping was more pronounced around DA1 than around VA1d. These results suggest that Ths acts locally as a spatial cue to instruct ensheathing glia to infiltrate the antennal lobe.
Members of the FGF family have diverse functions in a variety of tissues in both vertebrates and invertebrates (49, 50). Vertebrate FGFs regulate not only neural proliferation, differentiation, axon guidance, and synaptogenesis but also gliogenesis, glial migration, and morphogenesis (51–55). Many of these roles are conserved in invertebrates. For example, Ths and Pyr induce glial wrapping of axonal tracts (32, 33), much like the role other FGF members play in regulating myelin sheaths in mammals (55). Ths and Pyr also control Drosophila astrocyte migration and morphogenesis (26); likewise, FGF signaling promotes the morphogenesis of mammalian astrocytes (56). Therefore, studying the signaling pathways in Drosophila will extend our understanding of the principles of neural development.

In ensheathing glia, whose developmental time course and mechanisms have not been well documented before this study, we observed a glial response to FGF signaling reminiscent of the paradigm shown previously (26, 32, 57); however, the exquisite compartmental structure of the Drosophila antennal lobe and genetic access allowed us to scrutinize further the changes of neuropil structure and projection patterns that occurred alongside morphological phenotypes in ensheathing glia. We demonstrated the requirement for Ths in LNs, although it is possible that ORNs and PNs also contribute. We also tested the function of the other ligand, Pyr, in antennal lobe development. We did not detect any change in ensheathing glia morphology with pyr RNAi, and double

Fig. 5. Changes in ensheathing glia processes and numbers in response to local overexpression of ths. (A–C) Confocal sections of adult antennal lobe of the wild type (A) or overexpression of UAS-ths by Or47b-GAL4 (B) or by Mz19-GAL4 (C). SPARC-QF > QUAS-mtdT labels ensheathing glia processes are shown in green; ensheathing glia nuclei marked with QUAS-nuclear-LacZ are shown in magenta. (Scale bar: 10 μm.) (D and E) Quantification of signal intensities of ensheathing glia processes around each glomerulus normalized by the perimeter of the corresponding glomerulus. (f and G) Quantification of ensheathing glia numbers within 5 μm of the surface of each glomerulus. Error bars represent SE. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001; ns (not significant), P > 0.05.

FGF Signaling Ensures Accurate Neuronal Targeting. Last, we examined whether glia-wrapping defects could disrupt neuronal projections to the glomerular compartments. We used membrane proteins under the control of specific odorant receptor promoters (Or88a-mtdT and Or47b-rCD2) to label two ORN classes that project their axons to two adjacent glomeruli, VA1v and VA1d (Fig. 6A). When ths was knocked down with a pan-neuronal driver, C155-GAL4, VA1v and VA1d ORN axons still reached their target area in the ventrolateral antennal lobe. However, they failed to establish exclusive territories for their axonal arborization and instead had partially overlapping axonal terminals (Fig. 6B). Likewise, in ths-mutant animals, VA1v and VA1d ORN axons partially intermingled, rather than remaining confined to their respective glomerular compartments (Fig. 6C; quantified in Fig. 6D). Thus, FGF signaling between neurons and ensheathing glia is essential for antennal lobe compartmentalization and for the targeting accuracy of ORN axons.

Discussion

The use of discrete neuropil compartments for organizing and signaling information is widespread in invertebrate and vertebrate nervous systems. In both the fly antennal lobe and vertebrate olfactory bulb, axons from different ORN classes are segregated into distinct glomeruli (47). The rodent barrel cortex also uses discrete compartments, the barrels, to represent individual whiskers (48). In this study, we showed that FGF signaling between neurons and glia mediates neuronal compartment formation in the Drosophila antennal lobe.

Fig. 6. Loss of ths results in defects in olfactory receptor neuron targeting. (A–D) Confocal sections of the adult antennal lobe, with Or88a-mtdT and Or47b-rCD2 labeling ORN axons that target VA1d (magenta) and VA1v (green) glomeruli, respectively. Images within the dashed rectangles in the far left column are enlarged in columns 2–4. (A) Wild type. (B) C155-GAL4-driven ths RNAi. (C and D) ths mutant flies showed severe intermingling of VA1d and VA1v ORN terminals (C) and a mild spillover of VA1d ORN axons (D, arrow). thsRNAi denotes the DH2;ED2238 allele. (Scale bar: 10 μm.) (E) Quantification of the accuracy of ORN axon targeting: 1, normal; 2, mild spillover; 3, severe intermingling. Each symbol represents one fly with two antennal lobes scored separately and averaged. Error bars represent SD. * P < 0.05; ** P < 0.01. In this figure, subpanels represent the same sample in different imaging channels, unless otherwise specified.
RNAi against ths and pyr did not enhance the phenotype compared with ths knockdown alone.

FGF signaling in glomerular wrapping appears to be highly local. In our overexpression experiments, the hyperwarping effect was restricted to the glomerulus where the ligand is excessively produced and did not spread to nearby nonadjacent glomeruli. These experiments suggest that Ths communicates locally to instruct glial ensheathment of the glomeruli rather than diffusing across several microns to affect nearby glomeruli. Because heparan sulfate proteoglycans are known to act as FGF coreceptors by modulating the activity and spatial distribution of the ligands (50, 58, 59), we speculate that Ths in the antennal lobe may be subject to such regulation to limit its diffusion and long-range effect.

Our data showed that deficient ensheathment of antennal lobe glomeruli is accompanied by imprecise ORN axon targeting. However, we cannot determine whether these targeting defects reflect initial axon-targeting errors or a failure to stabilize or maintain the discrete targeting pattern. Previous models for the establishment of antennal lobe wiring specificity suggested that the glomerular map is discernable by the time glia processes start to infiltrate the antennal lobe (17). Because of a lack of class-specific ORN markers for early developmental stages, the relative timing between when neighboring ORN classes refine their axonal targeting to discrete compartments and when ensheathing glial barriers are set up still remains unclear. Nevertheless, our discovery that FGF signaling functions in the formation of discrete neuronal compartments in the antennal lobe highlights an essential role for glia in the precise assembly of neural circuits.

Methods

Immunostaining. Tissue dissection and immunostaining were performed according to previously described methods (60). Primary antibodies used in this study include rat anti-DNcad [DN-Ex #8; 1:40; Developmental Studies Hybridoma Bank (DSHB)], chicken anti-gFP (1:1,000; Aves Labs), rabbit anti-DsRed (1:500; Clontech), mouse anti-rCD2 (OX-34; 1:200; AbD Serotec), rat anti-TH (1:1 µg/mL; Roche), mouse nc82 (1:35; DSHB), mouse anti-Repo (1:50; DSHB), rabbit anti-γ-Galactosidase (1:1,25; MP Biomedicalals), rabbit anti-GAT (1:3,000; a gift from Marc Freeman, Vollum Institute, Oregon Health and Science University, Portland, OR), and mouse anti-γ-Galactosidase (1:1,000; Promega). Secondary antibodies were raised in goat or donkey against rabbit, mouse, rat, and chicken antiserum (Jackson Immunoresearch) conjugated to Alexa 405, FITC, biotin, or 647. Confocal images were collected with a Zeiss LSM 780 laser scanning microscope and were processed with Zen (Zeiss), ImageJ (NIH), and Imaris (Bitplane) software.

Mosaic Analysis. The hsFlp MARCM analyses were performed as previously described (27, 61) with slight modifications. GMIR0162-GAL4 was used for labeling ensheathing glia in adult-stage Drosophila. Flies were kept at 18 °C and were heat shocked for 30 min at 37 °C between 0 and 24 hAPF.

Data Analysis. Confocal sections of antennal lobes (Figs. 2 and 4) were analyzed by ImageJ software to measure integrated intensity value, area size, mean, and SD for the region of interest (ROI) manually selected based on Ncad counterstaining. Process intensity was calculated using the integrated intensity value of the antennal lobe section crossing the DA1 and DM6 glomeruli normalized by the area size of the antennal lobe of that section. The Plot Profile function in ImageJ was used to measure signal intensities for ensheathing glia processes and Ncad along the selected lines. Glia cell number was counted manually based on LacZ staining within 10 μm of the surface of the antennal lobe determined by Ncad counterstaining. To quantify the MARCM clones (Fig. 3), confocal sections of marked ensheathing glia were processed by Imaris software by manually thresholding the images by a set of consistent parameters, followed by automatic measurement of the volume and total intensity of glia processes. In the overexpression experiment (Fig. 5), the ROI was selected manually by including the inner region and the boundaries of the glomerulus of interest based on Ncad counterstaining. Process intensity was defined as the integrated intensity value divided by the perimeter of the ROI selection. The glia number was counted manually based on LacZ signal within 5 μm of the surface of each glomerulus. ORN targeting defects (Fig. 6) were scored by an experimenter blind to the genotype. Graphs were generated using the GraphPad Prism software; mean ± SD were shown on the graphs. Statistical significance was calculated with GraphPad Prism using a two-tailed Student’s t test (Figs. 2–5) or a Mann–Whitney U test for the nonparametric data in Fig. 6.

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Supporting Information

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Fig. S1. Two FlyLight-GAL4 lines and SPARC-GAL4 label antennal lobe ensheathing glia. Confocal sections of adult antennal lobe are shown. (A1, B1, C1, and D1) Ensheathing glia processes are labeled by GMR56F03-GAL4– (at 25 °C) (A1 and C1), GMR10E12-GAL4– (at 18 °C) (B1), or SPARC-GAL4– (D1) driven (> UAS-mCD8GFP). Ensheathing glia nuclei are marked by UAS-nuclear-LacZ (A2 and B2). Glia nuclei stained by the Repo antibody are shown in green. (A3 and B3) The channel for Repo staining is overlaid on the nuclear-LacZ channel. (C and D) Colabeling of ensheathing glia (mCD8GFP and nuclear-lacZ) and the astrocyte marker GAT, visualized by antibody staining (white). GAT staining is overlaid on the nuclear-LacZ channel (C2 and D2) to show that, with a few exceptions (arrows), GAT does not label GAL4+ cells. (E) Colabeling of ensheathing glia [SPARC-QF > QUAS-mtdT and QUAS-nuclear-lacZ (green and magenta, respectively)] and astrocytes (Alrm-GAL4 > mCD8GFP, white). The GFP channel is overlaid on the nuclear-LacZ channel in E2 to show that the glia marked by SPARC-QF are not astrocytes. (Scale bar: 10 μm.) In this figure, subpanels represent the same sample in different imaging channels, unless otherwise specified.
**Fig. S2.** P35 suppresses the reduction in ensheathing glia numbers caused by htl knockdown. (A and B) Projections of confocal sections along the z axis for the wild type (A) and UAS-htl RNAi (VDRC 27180) together with UAS-P35 driven by SPARC-GAL4 (B). Ensheathing glia cell bodies marked by SPARC-GAL4 > UAS-nuclear-LacZ are shown in red; Ncad antibody staining for antennal lobe neuropil is shown in blue. (C) Quantification of the number of ensheathing glia cells in each antennal lobe. (D and E) Confocal sections of the antennal lobe at 96 hAPF of wild type (D) and UAS-htl RNAi (VDRC 27180) together with UAS-P35 driven by SPARC-GAL4 (E). Ensheathing glia processes are labeled by SPARC-GAL4 > UAS-mCD8GFP. Neuropil compartments are stained with Ncad antibody. (F–H) Quantification of the intensity of ensheathing process within the antennal lobe (F), relative SD of Ncad staining (G), and the correlation coefficient for the intensities of ensheathing glia processes and neuropil staining (H). (Scale bars: 10 μm.) Error bars represent SD. **P < 0.01; ****P < 0.0001; ns (not significant), P > 0.05. In this figure, subpanels represent the same sample in different imaging channels, unless otherwise specified.

**Fig. S3.** Additional examples of MARCM clones and analysis. (A–C) Wild-type ensheathing glia processes labeled by GMR10E12-GAL4 > UAS-mCD8GFP are shown in green. Ensheathing glia cell bodies marked by UAS-nuclear-LacZ are shown in blue. Neuropil staining by the Ncad antibody is shown in magenta. (Scale bar: 10 μm.) D, dorsal; L, lateral. An ensheathing glia located on the dorsal surface of the antennal lobe (A3) extends processes ventrally and half-encircles the DA3 glomerulus (A1). An ensheathing glia located on the dorsal surface of the antennal lobe (B2) extends processes laterally to access glomeruli in the center of the antennal lobe (B1 and B3). An ensheathing glia located on the most anterior surface of the antennal lobe (C3) extends processes into posterior antennal lobe (C4). (D) Quantification of the total intensity of processes from each ensheathing glia (wild-type and Htl<sup>Ab42/Ab42</sup>) labeled by MARCM. Error bars represent SD. **P < 0.01. In this figure, subpanels represent the same sample in different imaging channels, unless otherwise specified.
Fig. 54.  *Orb*^{0449-GAL4} labels LNs in the antennal lobe. (A) *Orb*^{0449-GAL4} > UAS-mCD8GFP are shown in green. (A1) A projection of confocal sections along the z axis at 24 hAPF. Approximately 23 LNs are labeled. (A2) A confocal section of antennal lobe at 96 hAPF. A total of ~55 LNs are labeled over all confocal sections of the antennal lobe. (B) ey-FLP intersects with *orb*^{0449-GAL4} together with UAS-FRT-stop-FRT-mCD8GFP to show that *orb*^{0449-GAL4} is inactive in the majority of ORNs, except for two classes that project to the medial side of the antennal lobe (arrow). (C) The GH146-FLP intersection shows a rare case in which an interneuron is labeled (one cell out of 10 antennal lobes); no cells are labeled around the other antennal lobes. Neuropil staining by the Ncad antibody is shown in magenta. (Scale bar: 10 μm.)

Fig. 55. Loss of Ths from ORNs and PNs does not cause defects in ensheathing glia or glomeruli. (A) GH146-GAL4 drives RNAi against ths. Ensheathing glia processes are labeled by SPARC-QF > QUAS-mtdT. (B) ey-FLP MARCM combined with a cell-lethal strategy creates a ths mutant in nearly all ORNs. Ensheathing glia processes are labeled by GMR56F03-GAL4 > UAS-mCD8GFP. Neuropil staining by the Ncad antibody is shown in magenta. (Scale bar: 10 μm.) (C–F) Quantification of ensheathing glia process intensities (C and E) and relative SD of Ncad staining intensities (D and F). Error bars represent SD. ns (not significant), *P* > 0.05. In this figure, subpanels represent the same sample in different imaging channels, unless otherwise specified.