Netrin Signaling Defines the Regional Border in the Drosophila Visual Center

Takumi Suzuki, Chuyan Liu, Satoru Kato, ..., Satoko Hakeda-Suzuki, Takashi Suzuki, Makoto Sato

makotos@staff.kanazawa-u.ac.jp

HIGHLIGHTS
Netrin regulates boundary formation in combination with Slit in the fly brain
Dual Netrin functions as attractant and repellent explain boundary formation

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Netrin Signaling Defines the Regional Border in the Drosophila Visual Center

Takumi Suzuki,1,5,6 Chuyan Liu,2,5 Satoru Kato,3,5 Kohei Nishimura,3,5 Hiroki Takechi,4,5 Tetsuo Yasugi,1 Rie Takayama,1 Satoko Hakeda-Suzuki,4 Takashi Suzuki,4 and Makoto Sato1,2,3,7,*

SUMMARY
The brain consists of distinct domains defined by sharp borders. So far, the mechanisms of compartmentalization of developing tissues include cell adhesion, cell repulsion, and cortical tension. These mechanisms are tightly related to molecular machineries at the cell membrane. However, we and others demonstrated that Slit, a chemorepellent, is required to establish the borders in the fly brain. Here, we demonstrate that Netrin, a classic guidance molecule, is also involved in the compartmental subdivision in the fly brain. In Netrin mutants, many cells are intermingled with cells from the adjacent ganglia penetrating the ganglion borders, resulting in disorganized compartmental subdivisions. How do these guidance molecules regulate the compartmentalization? Our mathematical model demonstrates that a simple combination of known guidance properties of Slit and Netrin is sufficient to explain their roles in boundary formation. Our results suggest that Netrin indeed regulates boundary formation in combination with Slit in vivo.

INTRODUCTION
Compartmental subdivision of the brain into each unique region is essential for the development and function of the brain. The established compartmental borders play crucial roles in controlling the behavior of signaling molecules that regulate cell fate as well as in isolating cells in each individual region (Kiecker and Lumsden, 2005; Batlle and Wilkinson, 2012). Although these borders inhibit cell migration across them during development, the inhibition of cell mixing is also crucial for the stabilization of tissue homeostasis in developed organisms because its failure contributes to the accelerated invasion of tumor cells (Abercrombie, 1979; Cortina et al., 2007; Cayuso et al., 2015)

Border formation along each compartment is known to be regulated by three mechanisms: differential affinity in cellular adhesion, interfacial tension between different cell populations, and cell repulsion by intercellular signaling (Batlle and Wilkinson, 2012). Importantly, all of these mechanisms involve molecular machineries located at the cell membrane. In contrast, the mechanism of border formation by diffusible guidance molecules is only poorly understood.

The fly visual center is composed of four ganglia: the lamina, medulla, lobula, and lobula plate. Neurons in these ganglia are mainly derived from two distinct progenitor pools, the outer proliferation center (OPC) and the inner proliferation center (IPC). The neurons in each ganglion are located in specific regions to form sharp compartment boundaries and never intermingle with each other at the interfaces between ganglia. However, the mechanisms that inhibit cell mixing at the borders between ganglia have remained unclear. Previously, we and another group showed that cell-cell interaction through Slit-Robo signaling, a repulsive axon guidance signaling pathway, is involved in the inhibition of cell mixing between the lamina and the IPC (Tayler et al., 2004) and also between the OPC and IPC during larval development (Suzuki et al., 2016). However, because the cell mixing occurs only partially even by severe disruption of Slit-Robo signaling, additional signaling pathways likely also participate in the formation of these borders.

Here, we show that Netrin signaling, another axon guidance signaling pathway, regulates the formation of the border between the OPC and IPC. The ligands Netrin A (NetA) and Netrin B (NetB) are expressed in the IPC, whereas their receptors Frazzled (Fra, Drosophila homologue of Deleted in colorectal cancer [DCC]) (Kolodziej et al., 1996) and Unc5 are expressed in lamina glial cells located at the border between the OPC.
Netrin and Its Receptors Are Expressed in Each Domain of the Optic Lobe during Larval Development

We have previously reported that Slit-Robo signaling is important for the proper arrangement of medulla neurons by establishing the border between the OPC and IPC (Figure 1A) (Suzuki et al., 2016). This border was not completely disrupted in slit, robo3, or robo2 mutants, suggesting that other signaling pathways are also involved in the border formation. To identify other regulatory signaling pathways, we conducted expression screening for typical axon guidance molecules and found that Netrin and its receptors are expressed in the medulla primordium. First, we examined the localization patterns of the ligands NetA and NetB. NetA localization was exclusively found in a subset of the lateral IPC cells (Figures 1D and 1G, white arrows), whereas NetB-myc was localized in the Bsh+ OPC-derived neurons (Figure 1C1, yellow arrows) and in a subset of the lateral IPC cells (Fas3+ located next to the lamina (Figure 1C2, white arrows) (Brankatschk and Dickson, 2006). Next, we examined expression patterns of Netrin receptors using fra-LacZ, an enhancer trap line for fra, in situ hybridization for unc5 mRNA, and antibodies against Fra and Unc5. fra-LacZ was expressed in the glia precursor cell (GPC)-derived neurons (Eya+) that are located in the innermost area of the OPC (Figure 1E1, white arrows) and also in the lamina glial cells (Repo+; Figure 1E2, white arrows). The fra-LacZ-positive cells near the lamina glial cells are most likely neuroepithelial cells in the IPC (Figure 1E2, asterisks). We also observed Fra protein localization in the lamina glial cells (data not shown). The localization pattern of Unc5 protein was quite similar to that of Fra, with localization in the lamina glial cells (data not shown). As observed in Figure 1F2, the marker for unc5 mRNA was also expressed in the GPC-derived neurons visualized by omb-Gal4 UAS-nlsGFP (Figure 1F1; white arrows; a subset of GFP-positive cells). Taken together, Netrin ligands are expressed in neurons derived from IPC and OPC, whereas both of their receptors are expressed in the GPC-derived neurons and in lamina glial cells (Figure 1G). The lamina glia cells project their processes inside the medulla neuropil as discussed later (Figures 3F and 3G).

Netrin Signaling Regulates the Formation of the Border between OPC and IPC

To reveal the roles of Netrin signaling, we first examined the effects of NetA and NetB mutations on the arrangement of OPC and IPC cells (Suzuki et al., 2016). Throughout this study, Bsh, Fas3, and Ncad antibodies were used to visualize OPC-derived neurons, IPC cells, and the neuropil structure, respectively. IPC cells penetrated the OPC region and suppressed the formation of neuropil structures in NetABD brains (Figures 2A, 2B, and 2R). At the same time, distribution of OPC-derived neurons was disrupted. However, we have never observed penetration of OPC cells into the IPC region. Ectopic IPC cells were only rarely observed in NetA4 and NetB4 larvae, suggesting that NetA and NetB act redundantly (data not shown). These results suggest that Netrin plays important roles in the establishment of the boundary between OPC and IPC that restricts IPC cells within IPC during larval development. Interestingly, the cells that usually express Netrins were misplaced in NetAB mutants brains, suggesting that the cells that express Netrin receptors play important roles in boundary formation.

Therefore, we examined contributions of Fra and Unc5 receptors by analyzing loss-of-function mutants. In fra3/fra4 larvae, the IPC cells invaded the OPC (Figures 2C and 2R) and the distributions of OPC-derived
neurons were dramatically disrupted, as observed in NetAB$^D$ larvae. Similar defects were observed in unc5 null mutant brains (Figures 2D and 2R). Again, invasion of the OPC cells into the IPC region was never observed. Thus, these results suggest that Netrin signaling plays important roles in establishing the boundary between the OPC and IPC to prevent IPC cells from penetrating the OPC. Note that Fas3$^+$ ectopic IPC cells contain neuroblasts (NBs), which extensively proliferate (Figures 2F–2H). These ectopic NBs may derive from IPC, because there are many IPC NBs near the boundary between the IPC and OPC in control brains (Figure 2E). Consistent with the observation that the OPC cells do not penetrate the IPC, there is no OPC NB near the IPC-OPC boundary. The Fas3$^+$ IPC NBs may penetrate the OPC destroying the boundaries in the mutant brains. We did not find any positional bias in the above-mentioned defects. The invasion of IPC cells equally occurred in the ventral, dorsal, and central part of the brain.
The area of the ectopic IPC cells found in the OPC region was extremely variable, which might be related to the timing of IPC NB penetration during development. Therefore, we simply compared the numbers of brain samples showing the boundary defect (Figure 2R).

We also found that ectopic IPC cells were frequently surrounded by glial cells visualized with repo-Gal4 UAS-CD8GFP in NetABD and fra3/fra4 mutants (Figures 2I–2K; n = 29/29 and 22/25, respectively), suggesting that the arrangement of glial nuclei in the mutant medulla and found that glial cells (Repo+) appeared within and around Ncad-negative regions in NetABD and fra3/fra4 mutants (Figures 2L–2N; n = 26/49 and 9/25, respectively). We examined the distribution of glial cells in the lamina as well as in the medulla primordium in these mutants because lamina glial cells are thought to be important for the formation of the border by acting as a source of Slit (Tayler et al., 2004). Glial cells were always observed within the Ncad+ lamina plexus region in the controls (Figure 2O). However, a subset of lamina glial cells was ectopically found outside the lamina plexus in NetABD and fra3/fra4 mutants (Figures 2P and 2Q; n = 18/45 and 16/28, respectively). Thus, these results suggest that Netrin signaling controls the distribution of glial cells.
Glia-Specific Inhibition of Netrin Signaling Disrupts the Border between the OPC and IPC

As observed in Figure 1, Netrin receptors are expressed in the lamina glial cells in addition to the GPC-derived neurons. Moreover, Netrin signaling dysfunction resulted in ectopic appearance of Fas3+ IPC cells surrounded by glial cells in the OPC (Figures 2I–2K). These observations raise a possibility that Netrin signaling is activated in lamina glial cells to regulate the border formation. To examine this possibility, we conducted glial-cell-specific suppression of Netrin signaling using RNAi lines against fra and unc5. We induced fra RNAi and unc5RNAi under the control of repo-Gal4 and observed ectopic appearance of Fas3+ IPC cells in the medulla primordium (Figures 3A–3C and 3M). A similar result was obtained by slit RNAi (Figures 3D and 3M; slit\textsuperscript{Df1228}, n = 11/31; slit\textsuperscript{P10229} n = 12/20; slit\textsuperscript{CD5627}, n = 7/10), indicating that Netrin signaling as well as slit expression in glial cells is essential for the formation of the border between the OPC and the IPC. Ectopic NetB expression in glial cells also caused a similar boundary defect (Figures 3E and 3M), suggesting that Netrin ligand expression needs to be restricted to the OPC-derived neurons and IPC cells. The numbers of brain samples showing the boundary defect were compared (Figure 3M).

There are three layers of lamina glial cells, namely, epithelial, marginal, and medulla glia (Poect et al., 2001). To examine which types of lamina glial cells regulate the border formation, we used two Gal4 drivers expressed in different subsets of lamina glial cells, R25A01-Gal4 (Edwards et al., 2012) and dll-Gal4. R25A01-Gal4 is exclusively expressed in the medulla glia, the third glial sheath located between the lamina and the medulla (Figure 3F, arrows), whereas dll-Gal4 is expressed in both epithelial and marginal glia, the first and second glial sheaths, respectively (Figure 3G, arrows). These cells project long glial processes inside the medulla neuropil (yellow arrows in Figures 3F and 3G) (Poect et al., 2001). First, we examined the distribution of lamina glial cells in the NetAB\textsuperscript{+} mutant and found that R25A01-Gal4\textsuperscript{+} and dll-Gal4\textsuperscript{+} glial cells visualized with GFP ectopically appeared in the OPC (Figures 3H and 3I, arrows; n = 13/72 and 12/58, respectively). Next, we knocked down Netrin signaling in lamina glial cells. Induction of unc5 RNAi under the control of R25A01-Gal4 resulted in ectopic Fas3+ IPC cells (Figures 3J and 3K, arrows; n = 26/37 and unc5\textsuperscript{CD56310}, n = 15/25), and R25A01-Gal4\textsuperscript{+} glial cells appeared ectopically in the OPC (Figure 3L; unc5\textsuperscript{CD5627}, n = 5/12 and unc5\textsuperscript{CD56310}, n = 9/25). Although unc5 RNAi under the control of dll-Gal4 caused boundary defects (data not shown), dll-Gal4 is weakly expressed throughout the optic lobe (Figure 3G). We need to use Gal4 drivers that are specifically expressed in the epithelial and/or marginal glia to clarify their roles. However, the aforementioned results suggest that Netrin signaling in lamina glial cells regulate the border formation.

Mathematical Modeling of Boundary Formation by Slit and Netrin

We and others have previously reported that Slit-Robo signaling is required for the boundary formation in the fly optic lobe (Taylor et al., 2004; Suzuki et al., 2016). To investigate how Slit/Robo and Netrin, two major axon guidance pathways, regulate the boundary formation, we propose a simple mathematical model that describes their mutual interaction via Slit and Netrin signaling pathways by simply focusing on neuron and glia. In general, Slit always causes repulsion upon binding to Robo receptors, which means glia repel neurons (Figure 4A). In contrast, Netrin signaling regulates either attraction or repulsion. Fra and Unc5 are known as an attractive and repulsive receptor, respectively (Keleman and Dickson, 2001; Timofeyev et al., 2012). However, the lamina glial cells express both Fra and Unc5, and therefore it is not clear if Netrin acts as an attractant or a repellent in the developing fly optic lobe. In this situation, Netrin function could be switched depending on its concentration. According to the results of in vitro culture experiments, Netrin may act as an attractant when its concentration is low, whereas it may act as a repellent when its concentration is high (Taylor et al., 2015).

Based on this idea, we formulated a mathematical model of boundary formation by Slit and Netrin (Figure 4B). We assume that the interaction between IPC cells and lamina glia is more important compared with that between OPC cells and lamina glia, because IPC cells including IPC NBs always invaded the OPC by penetrating through the lamina glial cells in various mutant backgrounds (Figure 2). In addition, lamina glia-specific knockdown of unc5 caused the similar boundary defects (Figures 3J–3L). For simplicity, we focused only on the relationship between IPC neuronal cells (including NBs) and lamina glial cells, and ignored OPC- and GPC-derived neurons. We assume that slit, fra, and unc5 are expressed in glial cells and that netrin and robo are expressed in neurons (Figure 4A).

Here, G, N, A, and R represent the density of glia, neuron, Netrin, and Slit, respectively (Figure 4B). The changes in the distributions of neuron and glia are calculated with the initial condition in which the two
Figure 3. Suppression of Netrin Signaling in Glial Cells Disrupts the Border Formation

(A–E, F2, G2, and H–L) Lateral views of the developing medulla at the late third larval instar stage (medial sections). (A–E) Ectopic IPC cells (Fas3+, magenta, arrows), neuropil structure (Ncad+, blue), and glial cell membrane (UAS-CD8GFP+); green in (A–C) are compared. (A) Control. (B–D) unc5, fra, and slit knock down under the control of repo-Gal4, respectively. (E) Ectopic NetB expression under the control of repo-Gal4. (F–L) Lamina glial cells are visualized by (F, H, and J–L) R25A01-Gal4 UAS-CD8GFP and (G and I) dll-Gal4 UAS-CD8GFP (arrows). Frontal (F1 and G1; anterior view of Figure 1A3 focusing on glial process) and lateral views (F2, G2; see Figure 1A1), in which the processes of lamina glial cells are found within the medulla neuropil (yellow arrows) (F1 and G1) lateral to the top. (H and I) In NetAB mutant, ectopic lamina glial cells co-expressing Repo (magenta) are observed (arrows). (J–L) unc5 RNAi under the control of R25A01-Gal4 UAS-CD8GFP (green) induces ectopic IPC cells (K; Fas3+; magenta; arrow) and ectopic glial cells (L; Repo+; magenta; arrows). (J) Control.

(M) Frequency of samples showing the boundary defect is compared in control, fra RNAi, unc5 RNAi, slit RNAi, and NetB ectopic expression under the control of repo-Gal4. Examined sample numbers are shown at the bottom. Statistically tested by Fisher’s exact test (p < 0.03).
**A**

Glia: $\frac{\partial G}{\partial t} = \frac{(r_g + a_g)A}{A_{\text{MAX}} - a_g} \nabla \cdot (G \nabla A) + p_G(C_{\text{MAX}} - G - N)G$

Neuron: $\frac{\partial N}{\partial t} = r_n \nabla \cdot (N \nabla R) + p_n(C_{\text{MAX}} - G - N)N$

Netrin: $\frac{\partial A}{\partial t} = d_n \Delta A - k_n A + n_n N + g_n G$

**B**

**C**

$\alpha_r$

**D**

Initial condition (t=1) $A(\text{Neuron})/R(\text{Glia})$

**E**

Control (t=100K) $A(\text{Neuron})/R(\text{Glia})$

**F**

$C_{\text{max}}=A_{\text{max}}=R_{\text{max}}=1$

**G**

$C_{\text{max}}=A_{\text{max}}=R_{\text{max}}=1$

**H**

Degree of overlap $\text{Glia}$ Diffusion ($C_{\text{max}}=a_n=1$) Migration vs. $\text{Neuron}$ vs. $\text{Glia}$

**I**

Degree of overlap $\text{Glia}$ Diffusion ($C_{\text{max}}=a_n=1$) Migration vs. $\text{Neuron}$ vs. $\text{Glia}$

**J**

Fra mutant ($a_n=0$) $A(\text{Neuron})/R(\text{Glia})$

**K**

unc5 mutant ($a_n=0$) $A(\text{Neuron})/R(\text{Glia})$

**L**

slit mutant ($p_n=0$) $A(\text{Neuron})/R(\text{Glia})$

**M**

Netrin mutant ($n_n=0$) $A(\text{Neuron})/R(\text{Glia})$

**N**

Ectopic Netrin in glia ($p_n=0.1$) $A(\text{Neuron})/R(\text{Glia})$
cell types form partially overlapping but separated clusters (Figure 4D). Since NetB-myc and slit-LacZ signals were undetectable in neurons and glial cells, respectively, in the early third instar larval stage (data not shown), A and R are set to 0 as an initial condition. Note that our mathematical model is dimensionless. The distance, time, and density do not directly correspond to the actual units.

We initially compared the difference between the following three conditions, Netrin is an attractant, repellent, and both. When we assume that Netrin always acts as an attractant, neuron and glia are mixed with each other due to glia attraction by neuron (Figure S1A). In contrast, if Netrin always acts as a repellent, neuron and glia are separated by a gap between them due to glia repulsion by neuron (Figure S1B). Thus, if Netrin is a simple attractant or repellent, the sharp boundary cannot be established. We next tested the third condition in which the attraction and repulsion of glia by Netrin proportionally change according to Netrin concentration based on the results of in vitro culture study (Figure 4C) (Taylor et al., 2015). Intriguingly, neuron and glia show distinct domains with a very small overlap and a short distance forming a sharp boundary (Figure 4E), suggesting that the above-mentioned assumptions are sufficient to explain the boundary formation. The formation of the sharp boundary can be explained by the following dual functions of Netrin. When the peaks of neuron and glia are distant, a low level of Netrin causes the attraction of glia toward neuron (Figure 4F). When they are close to each other with an overlap, a high level of Netrin causes the repulsion of glia from the neuron cluster, whereas a low level of Netrin still causes attraction of the glia cluster (Figure 4G).

We tested the robustness of this result in different parameter sets for migration speed, diffusion speed, and degree of attraction and repulsion by plotting the degree of overlap and the distance between neuron and glia (Figures 4H and 4I). Here, we define that the two clusters of cells form a sharp boundary when their overlap and distance are sufficiently small. The white dotted lines encircle the area in which the degree of overlap is less than 1.2 based on the left panels, whereas the black dotted lines indicate the area in which the distance between two cell clusters is less than 3.5 based on the right panels. These threshold values were chosen according to the control result (Figure 4E). The red lines indicate the intersections between white and black lines showing the conditions for sharp boundary. (H) Diffusion of A and R (dA = dR) is changed between 5 and 20, whereas migration of N and G (x = x = x = x) are changed between 0.5 and 1.5. (I) Attraction and repulsion of G (N = N = N = N) are changed between 0.5 and 1.5. (J–N) Mutant conditions: (J) fra (g = 0), (K) unc5 (g = 0), (L) slit (g = 0), (M) Netrin mutant (g = 0) and Netrin expression in glia (g = 0). See also Figure S1.
invasion of IPC cells into the OPC, because active proliferation of IPC NBs located within the lamina glia or OPC area would further enhance their invasion. Indeed, the invading IPC cells contain NBs in mutant backgrounds (Figures 2F–2H).

In contrast, large gaps are formed between neuron and glia in the mutant conditions for fra (ag = 0) and Netrin (na = 0; Figures 4J and 4M). These outcomes can be explained by the lack of glia attraction by Netrin. A similar gap is found when Netrin is ectopically expressed in glia (ga = 0.1; Figure 4N). An increase in A induced by ectopic Netrin production might reduce its attraction (and enhance its repulsion), which eventually causes a large gap between neuron and glia. However, in the real brain tissue, this kind of gap containing no cell does not exist. Surrounding cells would penetrate to fill the gap. We speculate that IPC NBs are somehow forced to fill the gap and eventually invade the OPC through the lamina glia layer in vivo (Figure 2). This assumption needs to be validated in the future study. Although our model does not directly demonstrate the boundary defects found in fra and NetAB mutants, the switch between attraction and repulsion in Netrin signaling clearly explain the mechanism of the boundary formation.

Netrin Signaling Dysfunction Disrupts the Medulla-Lobula Complex Boundary in the Adult

Netrin signaling suppression caused disordered arrangement of the medulla neurons and intrusion of IPC cells into the OPC in the larval brain (Figures 2 and 3). We examined the effects of these early defects on the structure of the adult optic lobe. Note that the medulla, lobula, and lobula plate are 90° rotated in a clockwise manner compared with the larval stage (compare Figures 1A3 and 5F). Lamina wide field 2 (Law2) neurons project their dendrites throughout the medulla (from layer M1 to layers M9-10 of the medulla) and can be used as a specific marker for the structure of the medulla (Figure 5A) (Hasegawa et al., 2011; Tuthill et al., 2014; Suzuki et al., 2016). Law2 neurons visualized by R11D03-Gal4 UAS-IVS-CD8GFP do not project to the lobula and lobula plate in control brains (Figure 5A) (Tuthill et al., 2014). In contrast, Law2 processes projected to the lobula region (Figure 5B, white arrow; n = 9/14), and the medulla and lobula were obviously intermingled as visualized by Ncad staining in NetABΔ brains (Figure 5B; n = 43/67). In addition, the lobula and lobula plate were incompletely separated, and the border between these ganglia was vague in NetABΔ brains (Figure 5B, yellow arrow; n = 27/31). Similar disorganization was also observed in fra3/fra4 and unc5Δ brains (Figures 5C–5E). The medulla and lobula were combined (Figures 5D and 5E; white arrows), and the lobula complex was incompletely separated (Figures 5D and 5E; yellow arrows). Thus, Netrin signaling is required for the compartmentalization between the medulla, lobula, and lobula plate.

**DISCUSSION**

The brain is subdivided into multiple distinct regions that consist of many different types of neurons, and each region plays unique roles to carry out complex high-order functions. During development, the brain is subdivided into individual compartments that are defined by sharp borders that inhibit cell migration between different compartments to prohibit the mixing of cells and to contribute to the functional specification of each domain. In the present study, we demonstrate that Netrin signaling is essential for establishing the sharp border between the OPC and IPC in the optic lobe of *Drosophila*. Although we and another group previously reported that Slit-Robo signaling is involved in the regulation of the border formation between the OPC and IPC (Suzuki et al., 2016) and also between the lamina and IPC (Tayler et al., 2004), Netrin signaling is also required for the formation of these borders (Figure 2) and for proper organization of the adult optic lobe (Figure 5). The importance of axon guidance signaling, especially Ephrin-Eph signaling, in border formation has been emphasized in vertebrate brain development (Xu et al., 1995, 1999; Batlle and Wilkinson, 2012; Cayuso et al., 2015). The present study reveals that multiple regulatory mechanisms establish the compartmental subdivision in brains from invertebrates to vertebrates.

**Molecular Mechanisms of Netrin Signaling-Mediated Regulation of Border Formation between OPC and IPC**

We found that dysfunction of Netrin signaling caused severe defects in compartmental subdivision of the fly visual center. In NetAB, fra, and unc5 mutant brains, IPC cells were strikingly extruded, which results in incomplete separation of the medulla, lobula, and lobula plate in the adult optic lobe (Figure 5). Because the IPC produces lobula and lobula plate neurons, it is plausible that the early defects in the boundary between the OPC and IPC eventually cause the boundary defects between the medulla and the lobula complex.
Our expression analyses indicate that both Fra and Unc5 are localized in the GPC-derived neurons and the lamina glial cells (Figures 1E and 1F), suggesting that Netrin signaling in these neurons and/or lamina glial cells is essential. Although neuron-specific suppression of Netrin signaling also caused IPC extrusion (data not shown), suppression of Netrin signaling in a subset of lamina glial cells was sufficient to disrupt the border (Figures 3J and 3K), suggesting that Netrin signaling in the lamina glial cells needs to be activated to establish the proper arrangement of lamina glial cells and proper compartmental subdivision of the visual center.

Netrin signaling is broadly accepted as a classic guidance signaling pathway (Kennedy et al., 1994; Serafini et al., 1994; Kolodziej et al., 1996; Keleman and Dickson, 2001; Brankatschk and Dickson, 2006; Timofeev et al., 2012), and the arrangement of lamina glial cells can be regulated by its attractive or repulsive activity. It is possible that the disordered distribution of lamina glial cells causes the failure of the compartmental subdivision. Lamina glial cells are neatly arranged within the lamina, and their glial fibers surround the IPC via Netrin-mediated cell attraction or repulsion. This raises a possibility that this glial enclosure contributes to the maintenance of sharp borders around the IPC. This enclosure itself is likely to be formed in the absence of Netrin signaling because the ectopic IPC cells in NetAB and fra mutants were also surrounded by glial cells (Figures 2J and 3K).
DCC was initially identified as a factor that is deleted in colorectal carcinoma and has been thought to be related to cancer metastasis (Keino-Masu et al., 1996; Rodrigues et al., 2007). Although it has been demonstrated that DCC controls apoptosis induction in p53-deficient tumor cells, the mechanism of metastasis caused by the DCC mutant remains unclear (Krimpenfort et al., 2012). Since the mutant of Fra, a fly DCC homologue, causes penetration of IPC cells into neighboring compartments, future studies based on our results may be able to address the mechanism of cancer metastasis found in patients carrying DCC mutations.

**The Switch between Attraction and Repulsion Found in Various Guidance Molecules**

According to our mathematical model, the switch between attractant and repellent of Netrin at least partially explains its role in the boundary formation. Although it is technically very difficult to prove if the switch indeed happens in vivo, Netrin-1 has been demonstrated to have similar switching functions by in vitro culture experiments (Taylor et al., 2015). In addition, the structural analysis of the Netrin1-DCC complex revealed that Netrin binds to two DCC molecules and most likely acts as an attractant when its concentration is low, whereas it binds to one DCC at high concentration (Finci et al., 2014). At higher Netrin concentration, Unc5A may replace DCC to switch from attraction to repulsion. It has been demonstrated that Unc5 is able to regulate repulsion in the absence of Fra in the fly embryonic nervous system (Keleman and Dickson, 2001). Since both Fra and Unc5 are expressed in lamina glial cells in the fly optic lobe, the above-mentioned findings are consistent with the binary function of Netrin assumed in our mathematical model. Since BDNF also shows a similar switching function (Mai et al., 2009), similar strategies may be used in many other biological systems.

Recent findings challenge the classical view of Netrin-dependent long-range attraction in the commissural axon guidance (Dominici et al., 2017; Varadarajan et al., 2017; Yamauchi et al., 2017). In addition, it was shown that Netrin signaling is not required for long-range attraction but promotes adhesion to the target layer (Akin and Zipursky, 2016). However, it is still possible to assume short-range attraction and repulsion by Netrin signaling. Indeed, the phase diagram in Figure 4H shows that small diffusion coefficients of the ligands are compatible with sharp boundary formation when the migration coefficients are small.

**An Interrelationship between Netrin Signaling and Slit-Robo Pathways in Compartmental Subdivision**

The present results and a previous report (Taylor et al., 2004) suggest that lamina glial cells regulate the integrated development of each ganglion in the visual center. An important role of lamina glial cells as a source of axon guidance ligands for the formation of the border between the lamina and the IPC has been discussed (Taylor et al., 2004). We conducted glial-cell-specific knockdown of Slit and observed the ectopic appearance of IPC cells in OPC upon induction of sli RNAi under the control of repo-Gal4 or R25A01-Gal4 (Figure 3D and data not shown). This result suggests that the lamina glial cells are the essential sources of Slit required for the compartmental subdivision. Thus, the lamina glial cells play key roles in controlling the compartmental subdivision by activating Netrin signaling as well as by producing Slit.

In addition to the lamina glial cells, Netrin signaling may also be directly linked with the Slit-Robo pathway in the GPC-derived medulla neurons, given that fra, unc5, and sli are co-expressed in these cells (Figure 1) (Suzuki et al., 2016). Indeed, inactivation of Netrin signaling in neurons also caused the boundary defects (data not shown). A defect in either one of the signaling pathways disrupts the compartmental boundary, implying that both of these signaling systems are indispensable for the compartmental subdivision in the fly optic lobe. The idea that Netrin signaling activates the transcription of sli in the lamina glial cells and GPC-derived neurons is attractive because Fra has been shown to act as a transcription factor (Neuhaus-Follini and Bashaw, 2015). However, we have not yet been able to observe such a serial relationship between these two signaling systems.

Nevertheless, Netrin and Slit pathways are broadly conserved from invertebrates to vertebrates. It would be interesting to investigate the details of the molecular mechanisms of the Netrin and Slit-Robo dual regulation system during boundary formation in the brain.

**Limitations of the Study**

Although we propose that a simple combination of Slit-dependent repulsion and dual functions of Netrin (an attractant when its concentration is low and a repellent when its concentration is high) is sufficient to
explain their roles in boundary formation, our mathematical model is very simplified from the real phenomenon found in the fly brain. Further improvement of the mathematical model and biological experiments will be necessary to address how the mechanism proposed in this study can be applied to developing organisms in vivo.

**METHODS**

All methods can be found in the accompanying Transparent Methods supplemental file.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Transparent Methods and one figure and can be found with this article online at https://doi.org/10.1016/j.isci.2018.09.021.

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**AUTHOR CONTRIBUTIONS**

Takumi Suzuki, C.L., S.K, K.N., and M.S. designed research; Takumi Suzuki, C.L., and R.T. performed biological experiments; H.T., S.H.-S., and Takashi Suzuki designed and generated the unc5 mutant fly strain; S.K, K.N., and M.S. performed mathematical modeling and numerical simulations; Takumi Suzuki and C.L. analyzed data; Takumi Suzuki, T.Y., and M.S. wrote the paper.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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Supplemental Information

Netrin Signaling Defines
the Regional Border
in the Drosophila Visual Center

Takumi Suzuki, Chuyan Liu, Satoru Kato, Kohei Nishimura, Hiroki Takechi, Tetsuo Yasugi, Rie Takayama, Satoko Hakeda-Suzuki, Takashi Suzuki, and Makoto Sato
**SUPPLEMENTAL FIGURES**

**Figure S1**

(A) Netrin always acts as an attractant. \( \frac{\partial G}{\partial t} = -a_g \nabla \cdot (G \nabla A) + p_g (C_{\text{max}} - G - N)G \)

(B) Netrin always acts as a repellent. Equations of \( G \) are shown at the bottom.

\( \frac{\partial G}{\partial t} = r_g \nabla \cdot (G \nabla A) + p_g (C_{\text{max}} - G - N)G \)

**Figure S1. Numerical results of attractant only and repellent only conditions, Related to Figure 4.**

(A) Netrin always acts as an attractant. (B) Netrin always acts as a repellent. Equations of \( G \) are shown at the bottom.
### TRANSPARENT METHODS

#### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| guinea pig anti-Bsh (1:1600) | Hasegawa et al., 2011 | N/A |
| rabbit anti-Fra (1:1000) | Kolodziej et al., 1996 | N/A |
| mouse anti-LacZ (1: 250) | Promega | |
| chick anti-LacZ (1:1000) | Abcam | |
| mouse anti-GFP (1:400) | clontech | |
| rabbit anti-c-Myc (1:100) | | |
| Rabbit anti-NetA (1:200) | University of Mainz | Benjamin Altenhein |
| rabbit anti-NetB (1:200) | University of Mainz | Benjamin Altenhein |
| rabbit anti-Unc5 (1:200) | University of Mainz | Benjamin Altenhein |
| Guinea pig anti-Dpn (1:1000) | Washington University | James Skeath |
| mouse anti-Eya (1:8) | Developmental Studies Hybridoma Bank (DSHB) | AB_528232 |
| mouse anti-Repo (1:10) | DSHB | AB_528448 |
| rat anti-Ncad (1:20) | DSHB | AB_528121 |
| anti-Fas3 (1:10) | DSHB | AB_528238 |
| anti-guinea pig Cy5 (1:200) | Jackson ImmunoResearch Laboratories | 706-175-148 |
| anti-guinea pig Alexa 647 (1:200) | Jackson ImmunoResearch Laboratories | 706-605-148 |
| anti-mouse Cy3 (1:100) | Jackson ImmunoResearch Laboratories | 715-165-151 |
| anti-mouse Cy5 (1:200) | Jackson ImmunoResearch Laboratories | 715-175-151 |
| anti-mouse FITC (1:200) | Jackson ImmunoResearch Laboratories | 715-096-151 |
| anti-rat Dylight 649 (1:200) | Jackson ImmunoResearch Laboratories | 112-495-175 |
### Experimental Models: Organisms/Strains

| Strain | Organism/Strain | Genotype | Stock Center/Resource Center | Source |
|--------|-----------------|----------|------------------------------|--------|
| **UAS-CD8GFP** | | | | |
| UAS-IVS-CD8GFP | | | BDSC | 32186 |
| UAS-dicer2 | | | BDSC | 24650, 36510 |
| R11D03-Gal4 | | | BDSC | 48453 |
| R25A01-Gal4 | | | BDSC | 49102 |
| dll-Gal4 | | | | |
| repo-Gal4 | | | BDSC | 7415 |
| sli<sup>++</sup> | | | BDSC | 31467 |
| sli<sup>–</sup> | | | BDSC | 31468 |
| sli<sup>++</sup> | | Vienna Drosophila Resource Center (VDRC) | v20210 |
| sli<sup>+</sup> | | | | |
| sli<sup>–</sup> | | Massachusetts Institute of Technology | Paul A. Garrity |
| **NetAB**<sup>+</sup> | | | | Janelia Research Campus | Barry J. Dickson |
| **NetA**<sup>+</sup> | | | | Janelia Research Campus | Barry J. Dickson |
| **NetB**<sup>+</sup> | | | | Janelia Research Campus | Barry J. Dickson |
| **NetB-myc** | | | | Janelia Research Campus | Barry J. Dickson |
| **fra-lacZ**<sup>++</sup> | | | | | Kyoto Stock Center | 122067 |
| **fra**<sup>+</sup> | | | BDSC | 8813 |
| **fra**<sup>–</sup> | | | BDSC | 8743 |
| **UAS-fra**<sup>++</sup> | | | | BDSC | 40826 |
CRISPR/Cas9-mediated mutagenesis

unc5, a novel unc null allele, was generated by CRISPR/Cas9 technology (Chen et al., 2014; Kondo and Ueda, 2013). Two gRNA vectors (pBFv-U6.2) that recognize the sequences immediately downstream and upstream of the translational start and stop sites, respectively (GCTGAAGCTTAACCAGGAGG and GACATCATAGTTGAAACCATAGG), were injected to eggs carrying vas-Cas9 (BDSC 55821). A large deletion that removes almost all of the unc5 ORF was confirmed by sequencing (translational start site-ATGGCGGTGATTAATAAAAGCCGAAATGTGATTGCCCCTCCT – break point – CATAGGCCCTTTTGATTTA -translational stop site).

Immunohistochemistry

Immunohistochemistry was performed as described (Hasegawa et al., 2011). Confocal images were acquired using Zeiss LSM510 or LSM880, and were processed using Zeiss ZEN image browser and Adobe Photoshop. In situ hybridization was performed as described previously [15]. The boundary defects were quantified by comparing the number of brains showing abnormal fusion and/or disruption of the neuropils as visualized by Fas3 and Ncad staining.

Mathematical modeling

The differential equations were calculated using the explicit finite difference method
with the zero-flux boundary condition in one dimension ($1 \leq x \leq 100$). The mesh size and time step size are 1 and 0.01, respectively ($dx=1$, $dt=0.01$). The upwind differencing scheme was used to calculate the advection terms. $G$, $N$, $A$ and $R$ represent the density of glia, neuron, Netrin and Slit, respectively (Fig. 4B). As an initial condition, the two cell types form partially overlapping but separated clusters (Fig. 4D; $A=R=0$). The rate of change in $A$ is influenced by its diffusion ($dA$), degradation ($kA$) and production by neuron ($NA$) and glia ($GA$). Similarly, the rate of change in $R$ is influenced by its diffusion ($dR$), degradation ($kR$) and production by glia ($GA$). The attraction and repulsion of neuron ($N$) and glia ($G$) are formulated according to the Keller-Segel model of chemotaxis. Since the areas of neuron and glia significantly expand during larval development, logistic growth terms are included so that the maximum cell density becomes $C_{\max}$. Thus, the rate of change in $N$ is influenced by its repulsion by Slit $r \nabla \cdot (N \nabla R)$ and by its growth $(p_{\max}(C_{\max}-G-N)N)$. We assume that the coefficient for the attraction and repulsion of glia ($G$) by Netrin ($A$) proportionally changes according to Netrin concentration as shown in Fig. 4C. Namely, the attraction coefficient becomes $a$, when $A=0$, while the repulsion coefficient becomes $r$, when $A_{\max}$, the upper limit of $A$. Thus, the rate of change in $G$ is influenced by its attraction and repulsion by Netrin $((r+a)A/A_{\max}-a) \nabla \cdot (G \nabla A)$ and by its growth $(p_{\max}(C_{\max}-G-N)G)$. Constant attraction and constant repulsion of glia by Netrin were calculated by $-a \nabla \cdot (G \nabla A)$ and $r \nabla \cdot (G \nabla A)$, respectively (Fig. S1).

We assume that the corresponding parameters are largely equivalent between Netrin and Slit signalings and between neuron and glia. The diffusion coefficients of the ligands ($d_A=d_R=10$) are significantly greater than the migration coefficients of the cells ($r=n=a=1$). The production and degradation rates of ligands are set to modest values to stabilize the ligand distributions ($k=n=g=0.2$). Netrin is not produced in the glial cells except for the ectopic Netrin condition ($g=0$). For simplicity, the maximum values for cell density ($C_{\max}$) and ligand concentrations ($A_{\max}$ and $R_{\max}$) are set to 1. $A$ and $R$ do not exceed 1.0 in our parameter settings. To focus on the roles of the attraction and repulsion, the effects of cell growth are limited ($p_{\max}=0.01$). The initial distributions of $N$ and $G$ are as shown in Fig. 4D ($A=R=0$). The following results are based on the above settings at $t=100,000$. The overlap between neuron and glia is the total area in which $G>0$ and $N>0$. Since the peak values of $G$ and $N$ tend to be $C_{\max}$, the distance between neuron and glia is the minimal distance between the points of $G=N=C_{\max}/2=0.5$.  

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