NRP1-mediated Sema3A signals coordinate laminar formation in the developing chick optic tectum

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ABSTRACT

The optic tectum comprises multiple layers, which are formed by radial and tangential migration during development. Here, we report that Neuropilin 1 (NRP1)-mediated Sema3A signals are involved in the process of tectal laminar formation, which is elaborated by tangential migration. In the developing chick tectum, NRPI, a receptor for Sema3A, is expressed in microtubule-associated protein 2 (MAP2)-positive intermediate layers IV and V. Sema3A itself is a diffusible guidance factor and is expressed in the underlying layer VI. Using stable fluorescent labeling of tectal cells, we show that MAP2-positive intermediate layers are formed by the neurons that have been dispersed by tangential migration along the tectal efferent axons. When Sema3A was mis-expressed during laminar formation, local Sema3A repelled the tangential migrants, thus eliminating MAP2-positive neurons that expressed NRP1. Furthermore, in the absence of the MAP2-positive neurons, tectal layers were disorganized into an undulated form, indicating that MAP2-positive intermediate layers are required for proper laminar formation. These results suggest that NRP1-mediated Sema3A signals provide repulsive signals for MAP2-positive neurons to segregate tectal layers, which is important in order to coordinate laminar organization of the optic tectum.

KEY WORDS: Laminar formation, Optic tectum, Sema3A, NRP1, Cell migration, Chick

INTRODUCTION

The optic tectum is the main visual center for non-mammalian vertebrates. The avian optic tectum comprises 16 laminae, which are subdivided by distinct neuronal cell types (Ramón y Cajal, 1911; La Vail and Cowan, 1971a; Sernut and Alvarado-Mallart, 1986). In chick embryos, the dorsal part of the mesencephalon expands after embryonic day 3 (E3) through massive cell proliferation to form tectal swelling. After proliferating in the ventricular layer, post-mitotic neuronal precursor cells migrate radially to their destinations (Gray et al., 1988; Gray and Sanes, 1991). The process of layer formation has been extensively studied in the mammalian cerebral cortex, which comprises six layers in which post-mitotic cells migrate in an inside-out type manner (Angevine and Sidman, 1961; Rakic, 1974; McConnell, 1988). The mode of migration of tectal post-mitotic cells is very different from that of the cerebral cortex. Three waves of radial migration take place during laminar formation of the chick optic tectum. The first migration between E3 and E5 forms the inner laminae, the second migration between E4 and E7 forms the outer laminae and the third migration between E5 and E8 forms the middle laminae (La Vail and Cowan, 1971b; Sugiyama and Nakamura, 2003). In addition to radial migration, tangential migration also occurs during tectal laminar formation (Domesick and Morest, 1977; Puelles and Bendala, 1978). After E6, some radially migrating cells change direction to a tangential route and migrate dorsoventrally to differentiate into multipolar neurons (Gray and Sanes, 1991; Martinez et al., 1992). These observations suggest that tangential migration might be a key process for building tectal architecture, as in the cerebral cortex (Marín and Rubenstein, 2001). However, how tangential migration and its derivative cells are implicated in the laminar formation of the optic tectum remains elusive.

The neuropilin-semaphorin system plays diverse roles in development, including the regulation of cell migration (Tran et al., 2007). Class 3 secreted semaphorins direct the migration of various cell types, such as oligodendrocytes (Spassky et al., 2002), neural crest cells (Eickholt et al., 1999), endothelial cells (Miao et al., 1999; Serini et al., 2003; Shoji et al., 2003) and leukocytes (Delaire et al., 2001). Semaphorin-mediated guidance is bi-functional as it serves as an attractant or repellent depending upon the cell type. In laminar formation of the cerebral cortex, Sema3A attracts cortical neurons expressing Neuropilin 1 (NRP1), a receptor for class 3 semaphorins, during radial migration of the cortical neurons (Chen et al., 2008). By contrast, Sema3A and Sema3F repel cortical interneurons that express NRPI and/or NRP2 receptors, preventing tangential migration of these cells into the striatum, instead channeling them into the cortex (Marín et al., 2001).

In the developing chick optic tectum, it has been reported that NRPI is expressed in specific tectal layers that have a binding affinity with secreted Sema3A (Takagi et al., 1995; Feiner et al., 1997). We thus questioned whether NRPI-mediated Sema3A signals play a role in tectal laminar formation. In our preliminary study, we noticed that the tectal cells in the intermediate layers express NRPI and that these cells are derived from the tangentially migrating cells. These observations suggested that tangential migration might contribute to the process of laminar formation, which might be regulated by NRPI-mediated Sema3A signals.

In the present study, we aimed to assess the contribution of tangential migration to tectal layer formation and to examine the role of NRPI-mediated Sema3A signals in this process. We observed the migration of tectal post-mitotic cells through stable labeling of tectal cells with fluorescent proteins and demonstrated that tangential migration occurred along the tectal efferent axons. The cells after migration differentiated into multipolar neurons in layers IV and V (layers IV/V), which expressed MAP2 and NRP1. Mis-expression of Sema3A eliminated the multipolar neurons that expressed NRPI, resulting in the undulation of the overlying layer VI. We concluded that NRPI-mediated Sema3A signals play a role in the formation of...
the intermediate layers IV/V which is important to coordinate laminar organization of the optic tectum.

RESULTS

Sema3A and NRP1 are expressed in distinct tectal layers

It has been reported that NRP1 is expressed in the specific layers in the developing chick optic tectum (Takagi et al., 1995). Staining with an NRP1-specific antibody revealed that NRP1 was expressed in layers IV/V at E8.5 (Fig. 1A-C; Roman numerals indicate tectal layer number; Yamagata et al., 2006; La Vail and Cowan, 1971a). We found that microtubule-associated protein 2 (MAP2), a neuronal marker, was also expressed in layers IV/V (Fig. 1D-F; Yasuda and Fujita, 2003). Expression of Sema3A, a ligand for NRP1, was localized in layer VI (Fig. 1G,H). The protein localization of Sema3A was examined through binding to chick NRP1-AP, which contains extracellular domains of the receptor fused with alkaline phosphatase. Although NRP1-AP was immobilized to layer VI (Fig. 1K,L), a control alkaline phosphatase protein did not bind to any tectal layers (Fig. 1M,N). Expression of Sema3A mRNA and NRP1-AP binding suggest that the Sema3A protein is localized to layer VI, which overlays NRP1-expressing layers IV/V.

MAP2-positive neuronal layers are formed by the tangential migrants

It has been reported that some of the radially migrating cells in the developing chick optic tectum change direction to the tangential route and migrate to the intermediate layers (Gray and Sanes, 1991). We questioned whether these layers correspond to the MAP2-positive layers IV/V and examined the tangential migration in the tectum. The tectal cells were traced by using stable labeling through a transposon-mediated gene transfer system (Sato et al., 2007). The expression vectors encoding tol2 transposase (pCAGGS-T2TP) and enhanced green fluorescent protein (EGFP; pT2K-CAGGS-EGFP) were co-electroporated into a small area of the ventricular layer of the optic tectum at E4.5. At 4 days after electroporation (E8.5), in sections perpendicular to the anterior-posterior axis of the tectum, most of the GFP-labeled cells were radially arranged in the transfected area (Fig. 2A). These cells might have been labeled in the ventricular layer and radially migrated to the upper layers.
Some labeled cells were horizontally arranged in layers IV/V. These cells might have emigrated tangentially from the transfected area in ventral and dorsal directions (Fig. 2A). Staining with an antibody against MAP2 revealed that the tangential migrants were distributed in MAP2-positive neuronal layers (Fig. 2B,E). GFP-labeled cells in layers IV/V were large and multipolar, as well as MAP2-positive (Fig. 2G-I), indicating that these cells correspond to various transitional forms of ganglion cells or multipolar neurons (Domesick and Morest, 1977; Puelles and Bendala, 1978; Gray and Sanes, 1991).

We next examined the spatio-temporal sequence of the tangential cell migration. At E6.5, 2 days after electroporation, a group of GFP-labeled cells, which elongated horizontally, migrated ventrally from the transfected area into the intermediate zone (Fig. 3A,B). Cell nuclei were labeled using co-electroporation of the mCherry gene cassette that included a nuclear localization signal (mCherry-Nuc). Serial distribution of the mCherry-positive nuclei indicated that the tangential migration continued successively (Fig. 3C). The migrating cells extended the tangential process toward the ventral direction, parallel to the tectal efferent axons running dorsoventrally, which expressed NgCAM (the chick homolog of the L1 cell adhesion molecule), the marker of the tectal efferent axons (Fig. 3D; Kröger and Schwarz, 1990). At E7.5, the labeled cells migrated more ventrally and also dorsally in the intermediate zone (Fig. 3E). The migrating cells exhibited a bipolar shape, typical to the directionally migrating cells (Fig. 3H). At E8.5, the tangential migrants in the ventral and dorsal directions traveled long distances in order to be distributed throughout the whole tectum (Fig. 3F). Most of the migrants were located in layers IV/V, indicating that they had left the intermediate zone, which corresponded to layer III at E8.5, and translocated to the upper layers in order to differentiate into various forms of multipolar neurons (Fig. 3I). At E10.5, the tangential migrants eventually formed arborized multipolar neurons in layers IV/V (Fig. 3G,J). We noticed that the tangential migrants also existed in the superficial layer after E7.5 (Fig. 3E-G; arrowheads), which corresponded to the second tangential migration, which has been reported previously (Gray and Sanes, 1991).

**Movement of tangential cell migration along the tectal efferent axons**

Taking advantage of stable labeling, we observed the movement of the tangential cell migration in a flat-mount culture. First, we...
characterized the tectal efferent axons by examining their destinations. When a portion of tectal wall was labeled at E4.5 by using EGFP, the tectal efferent axons originating from the transfected area were labeled with GFP at E6.5, which elongated in parallel arrays to the ventral direction (Fig. 4A; n=3, three embryos were examined). At E10.5, the labeled axons reached the thalamic region ipsilaterally, and also contralaterally through the ventral supraoptic decussation (Fig. 4B; n=5). They were eventually destined for the rotundus nucleus bilaterally at E12.5 (Fig. 4C; n=3). The projection of the tectal efferent axons indicated that the labeled axons constitute the tectofugal pathway, and these axons had tectotortundal projections (Deng and Rogers, 1998; Wu et al., 2000; Puelles et al., 2007; Fedtsosva et al., 2008). Next, we labeled tectal cells with GFP and mCherry-Nuc to follow the tangentially migrating cells. In the isolated tectum, GFP-labeled tectal efferent axons elongated ventrally and turned anteriorly toward the diencephalon at E7.0 (Fig. 4D). Within the ventral part of the transfected area, many GFP-labeled cells with mCherry-positive nuclei were observed, indicating that these cells had migrated ventrally from the transfected area (Fig. 4E; n=3).

To monitor the dorsoventral movement of these tangentially migrating cells in a flat-mount culture, three-dimensional images of GFP, mCherry and differential interference contrast (DIC) at E6.0 were captured by using a confocal laser microscope (n=4). A number of mCherry-Nuc-positive cells moved ventrally along the GFP-labeled axon fasciculi of the tectal efferents (supplementary material Movie 1), which elongated dorsoventrally in the intermediate zone, as shown in the sections in Fig. 3B-E. The moving nuclei typically exhibited an oblong shape along the proceeding direction (supplementary material Movie 1, mCherry-Nuc). Moreover, mCherry-Nuc-positive cell nuclei proceeded in contact with the GFP-labeled tectal axon fasciculi, which were recognized in white arrays in DIC (supplementary material Movie 1, lower panels). Upon imaging at a higher magnification (supplementary material Movie 2; Fig. 5A-I), we noticed that the migrating cells held a single motile leading process, which traced pre-existing GFP-positive tectal axons (supplementary material Movie 2, left panels). Moreover, the nuclei proceeded on the tectal axons or moved adjacent to them (supplementary material Movie 2, lower panels; Fig. 5A-C,G-I). The clinging movements along the tectal axons were most evident on the tracking images of the frames (Fig. 5J-L). Trajectories of the cell (GFP) and the nuclei (mCherry) stayed adhered along the dorsoventral array of the tectal axon fasciculi (DIC), suggesting that the cells migrate on the passages of tectal axons. We further confirmed the attachment of migrating cells on tectal axons in fixed specimens. In confocal images, GFP-labeled migrating cells with mCherry-labeled nuclei were tightly attached to the bundles of tectal axons, which were also labeled by GFP (Fig. 5M). Immunostaining of NgCAM, the marker for tectal axons (Kroger and Schwarz, 1990), revealed that the migrating cell, the shape of which was marked with GFP, was attached to NgCAM-positive bundles, meaning that migrating cells proceeded in contact with the tectal axon fasciculus (Fig. 5N). We noticed that most of the GFP-positive bipolar cells had not only a leading process but also a very thin trailing process, which elongated from the soma (Fig. 5N). In the area just dorsal to the site of transfection, we found that the cells tangentially migrating in the ventral to dorsal direction also held a leading and a trailing process and attached to NgCAM-positive tectal axons (Fig. 5O,P). Finally, axophilic movement of tangentially migrating cells was confirmed by removing the axons on which the cells proceeded. After cutting the ventral tectal tissue, including the distal end of the tectal efferent axons, we observed the behavior of dorsoventral cell migration (supplementary material Movie 3). Focusing on the movement of mCherry-Nuc-positive cell nuclei, the migrating cells stopped at the distal tip of the axons and then turned back in the opposite direction (arrowheads) or restarted, proceeding in the same direction as the following axons elongated (arrows). In both cases, the migrating cells persistently traced the axonal bundles. These features of cell migration imply that the tangentially migrating cells move ventrally and dorsally along the tectal efferent axons.

Mis-expression of Sema3A disrupts tectal laminar organization

The results so far indicate that the tectal intermediate layers (layers IV/V) are formed by the cells that have migrated tangentially and differentiated into MAP2-positive neurons. Because NRP1 is expressed in layers IV/V, and Sema3A is expressed in the overlying layer VI, we sought to elucidate the roles of NRP1 and Sema3A in the tectal laminar formation. To this end, Sema3A was mis-expressed in the tectum by using the Tet-On system to limit the time of mis-expression had already started 10 h after doxycycline injection (Fig. 6C), and mis-expressed Sema3A was detected in
radially arranged cells covering the tectal layers at E8.5 (Fig. 6D). At E10.5, tectal laminae were severely undulated; in particular, layer VI expanded upward (Fig. 6E), where radially arranged columns of GFP-positive cells were located (Fig. 6F; \( n = 11 \), 11 embryos were examined). In the control, transfection of EGFP alone did not affect the laminar organization (Fig. 6G, H; \( n = 4 \)). Caspase 3 staining indicated that apoptotic cell death was not induced by mis-expression of Sema3A (Fig. 6I, J; \( n = 4 \)).

Mis-expression of Sema3A eliminates MAP2-positive neurons that express NRP1

To explore the reasons for the disorganization of the tectal laminae after Sema3A mis-expression, we focused on the MAP2-positive neurons that expressed NRP1. At E8.5, two days after induction of Sema3A expression, MAP2-positive neurons in layers IV/V were completely lacking around the radial columns where Sema3A was mis-expressed (Fig. 7A, arrows; \( n = 6 \)). The undulation of layer VI was not evident at this time point (Fig. 7A, arrowheads). At E9.5, three days after induction, undulation of layer VI became prominent (Fig. 7B, arrowheads) at the region where the underlying MAP2-positive neurons were lacking (Fig. 7B, arrows; \( n = 3 \)). This time sequence suggests that Sema3A first eliminated MAP2-positive neurons and subsequently yielded undulation of layer VI. When Sema3A was induced much later at E8.5, the mis-expression area was located in upper layers, such as layers VI, VII and VIII (Fig. 7C), and MAP2-positive neurons were partly eliminated (Fig. 7C, arrows; \( n = 3 \)). However, the upper layers VI-VIII were not severely affected (Fig. 7C, arrowheads). These results suggest that disruption of the tectal layers occurred after the elimination of the underlying layers. It was confirmed that NRP1-expressing neurons in layers IV/V were eliminated (Fig. 7D, arrows) around the region where Sema3A was mis-expressed (Fig. 7D, arrowheads; \( n = 6 \)), whereas NgCAM-positive efferent axons in layer III were unaffected (Fig. 7E; \( n = 6 \)). Mis-expression of GFP in the control did not change the distribution of MAP2- or NRP1-expressing neurons (Fig. 7F,G; \( n = 7 \)).

Sema3A repels tangential migrants that form multipolar neurons

Because MAP2- and NRP1-positive neurons after tangential migration were eliminated by the mis-expression of Sema3A, we investigated whether excess of Sema3A might have repelled the tangential migrants during their disposition to layers IV/V. To verify this, the tangentially migrating cells were pre-labeled with mCherry by electroporation at E4.5, and later Sema3A expression was induced at E6.5, as in the previous experiment. At E8.5, mCherry-labeled cells were radially arranged in the area of electroporation (Fig. 8A), and some of them migrated tangentially and localized in layers IV/V (Fig. 8A). However, mCherry-labeled tangential migrants were not arranged continuously but were partially lacking around the sites of Sema3A induction where GFP was expressed (Fig. 8A,B; \( n = 7 \)). The density of the mCherry-labeled cells was high next to the Sema3A induction site (Fig. 8A). In some cases, mCherry-labeled cells were eliminated from the area where Sema3A was induced and were mis-located, away from the induction site (Fig. 8C-E). In the control experiments, GFP mis-expression did not disturb the distribution of the mCherry-labeled cells in layers IV/V (Fig. 8F-H; \( n = 7 \)). These defects of cell disposition imply that Sema3A diffuses from the source of its mis-expression to repel tangential migrants and prevent them from forming layers IV/V.

To demonstrate the repulsive activity of Sema3A on the tangential migrants, we monitored the behavior of the mCherry-labeled cells during the induction of Sema3A in tectal slice culture. The tectal slices at E8.5 containing mCherry-labeled multipolar neurons after
tangential migration were cultured in doxycycline-containing medium to induce Sema3A and GFP expression. The multipolar neurons initially residing within the transfected radial column (arrowheads) migrated out from the area of increasing GFP signal (white box) where Sema3A was mis-expressed (supplementary material Movie 4; \( n=3 \)). These observations confirm that Sema3A repels tangential migrants that form multipolar neurons.

**Radial migration to the upper layers is enhanced after mis-expression of Sema3A**

Mis-expression of Sema3A eliminated MAP2- and NRP1-positive neurons in layers IV/V and resulted in the disorganization of tectal layers, such as layer VI. We suspected that the radial migration of the tectal cells to the upper layers might have been affected after Sema3A mis-expression, which resulted in the formation of undulated layers. After induction of the transgenes at E6.5, we injected 5-bromo-2′-deoxyuridine (BrdU) into the aqueduct of the tectal hemispheres at E7.0 and visualized BrdU-labeled cells 48 h later at E9.0. The radial distribution of BrdU-labeled cells that had migrated from the ventricular layer was compared between the radial columns of the transfected tectal hemisphere and the control untransfected hemisphere (Fig. 9A, B and Fig. 9C, D, respectively). In the control sections, most of the BrdU-labeled cells were scattered in and under layer VI but scarcely reached over layer VI (Fig. 9C, D). In the experimental sections, the labeled cells were distributed in all layers, and some of them reached the superficial layers (Fig. 9A, B). To check the different distribution of the BrdU-labeled cells along the radial axis, the cumulative proportion of each BrdU-labeled cell was plotted against a relative migration distance from the ventricular zone to the pial surface (Fig. 9E). We noticed that the plots of the experimental section (red plots; \( n=69 \)) had a longer migration distance than that of the control section (blue plots; \( n=68 \)). To quantify the overall distribution of the BrdU-labeled cells, we divided the tectal layers outside the ventricular zone into ten equally spaced bins along the radial axis (Fig. 9F, G) and obtained the proportion of the BrdU-labeled cells within each bin (Fig. 9F; each of the ten sections from five embryos that had been transfected with Sema3A plus EGFP). A line chart of the control sections (blue line) peaked in the middle layers (bins 5 and 6) and declined rapidly in the upper layers (bins 7-9), whereas that of the experimental sections (red line) sloped gently in the middle to the upper layers (bins 4-9), suggesting a shift of cell distribution toward the upper layers. Finally, we compared the cumulative proportion of the BrdU-labeled cells along the radial axis (Fig. 9G). The accumulation of the BrdU-labeled cells toward the upper layers was similar between the experimental and control sections in EGFP-transfected embryos (green and orange line, respectively; each of the ten sections from four EGFP-transfected embryos). By contrast, in embryos that had been transfected with Sema3A plus EGFP, the accumulation curve of the experimental sections (red line) was shifted distinctly toward the upper layers from that of the control sections (blue line). These analyses confirm that radial migration to the upper layers is enhanced after Sema3A mis-expression.

**DISCUSSION**

In the present study, we obtained the following results: (1) Sema3A and NRP1 are expressed complementarily, Sema3A in layer VI and NRP1 in layers IV/V; (2) MAP2-positive layers IV/V are formed from neurons that have migrated tangentially along the tectal efferent axons; (3) mis-expression of Sema3A eliminates MAP2-positive neurons that express NRP1 and results in undulated laminar organization; (4) Sema3A repels the tangential migrants; and (5)
radial migration to the upper layers is enhanced after mis-expression of Sema3A. Based on these results, a possible role of NRP1-mediated Sema3A signals in laminar formation of the developing optic tectum is indicated.

Tracing of the stably labeled cells during tectal layer formation revealed that some of the radially migrating cells change direction to the tangential route and span a very long distance. Time-lapse imaging analysis demonstrated that the tangentially migrating cells trace the bundles of pre-existing tectal efferent axons, which serve as the substrate for migration. These results confirm a previous report that associates tangential migration with axon fasciculus (Gray and Sanes, 1991). An axonal substrate for tangential migration might facilitate a successive migration stream over a long distance. It was also shown that the tangential migrants differentiate into MAP2-positive neurons in layers IV/V. Because MAP2-positive neurons were distributed over the whole tectum, tangential migration is a crucial process for the formation of layers IV/V.

We showed that Sema3A and NRP1 were expressed in the complementary tectal layers. Class 3 semaphorins can function as guidance molecules, either as an attractant or repellent, depending on the signal-receiving cells that express the receptors. The mis-expression experiment of Sema3A revealed that MAP2-positive neurons, which express NRP1, were eliminated around the site of the mis-expressed Sema3A. This result indicates that Sema3A in layer VI exerts repulsive effects on the NRP1-expressing neurons in layers IV/V. Moreover, we found that multipolar neurons, which were labeled by mCherry and displaced by tangential migration, were repelled by mis-expressed Sema3A. This suggests that mCherry-labeled cells after tangential migration differentiate into multipolar neurons that express NRP1, which in our study were repelled by locally induced Sema3A. Because the density of the mCherry-labeled cells next to the Sema3A induction site was high, repelled neurons might be excluded horizontally from the Sema3A source as shown in the repulsion analysis using tectal slice culture (supplementary material Movie 4).

In the developing tectum, Sema3A is expressed in layer VI cells, which overlie layers IV/V cells, which express NRP1 (a schematic model is shown in Fig. 10). During tectal layer formation, layer VI...
cells and layers IV/V cells have distinct birthdates and origins. Among the three waves of radial migration, the first migration between E3 and E5 forms the inner laminae, the second migration between E4 and E7 forms the outer laminae and the third migration between E5 and E8 forms the middle laminae (La Vail and Cowan, 1971b; Sugiyama and Nakamura, 2003). Cells of layers IV/V, which are in the inner laminae, derive from radially migrating cells during the first migration. After E5, these radially migrating cells change direction and migrate tangentially along the tectal efferent axons in the intermediate zone, which later corresponds to layer III. During E7.5 to E8.5, the tangentially migrating cells translocate to the upper layers, IV/V, to differentiate into NRP1-expressing neurons. By contrast, layer VI cells, which are in the middle laminae, are formed by the third radial migration after E5. These radially migrating cells pass through layers III-V, while first migrates move tangentially, and eventually form layer VI, expressing Sema3A, which overlies differentiating multipolar neurons after tangential migration. Given the repulsive activity of Sema3A on the NRP1-expressing neurons, we suspect that endogenous Sema3A in layer VI might act in the sorting of NRP1-expressing neurons. The effects of Sema3A mis-expression on the tangential migrants were examined. The tangentially migrating cells were pre-labeled by the electroporation of an mCherry construct at E4.5, and Sema3A was mis-expressed during E6.5-E8.5. (A,B) mCherry-labeled multipolar neurons, which migrated tangentially from the transplanted area (denoted with E.P.), were horizontally aligned in layers IV/V. However, they were lacking locally around the sites of Sema3A mis-expression where GFP was expressed (asterisks). The density of the mCherry-labeled cells was high next to the Sema3A induction site (arrows in A). (C-E) In some cases, mCherry-labeled cells were eliminated from the area where Sema3A was induced (asterisk) and were mis-located, away from the induction site (arrow). (F-H) Mis-expression of GFP alone did not disturb the distribution of mCherry-labeled cells in layers IV/V. Staining in combination with mCherry (magenta; A-H), GFP (green; B-D,E,G,H) and DAPI (cyan; E-H) are shown. Scale bars: 100 μm.

order to segregate these layers. Because other class 3 semaphorins are also expressed in layer VI (Y. W., unpublished), multiple inhibitory semaphorin signals might redundantly protect the segregation of the different layers.

The experiment in which Sema3A was mis-expressed revealed that the disorganization of layer VI occurred after the elimination of the underlying layers IV/V. Layer VI mainly comprises cells which became post-mitotic in the ventricular zone and then migrate radially between E5-E8 (La Vail and Cowan, 1971b; Sugiyama and Nakamura, 2003). Because undulation of layer VI was not prominent at E8.5, two days after the mis-expression of Sema3A, the absence of layers IV/V did not disturb the initial formation of layer VI. Thereafter, the thickness of layer VI became varied and deformed into undulation at E9.5, suggesting that the loss of layers IV/V might have triggered abnormal disposition of layer VI cells. In the cerebral and cerebellar cortices, the undulation of the layer structure can be caused by over-migration of the radially migrating cells (Moers et al., 2008). Similarly, in tectal layer formation after Sema3A mis-expression, proliferative tectal cells labeled with BrdU in the ventricular zone migrated radially and were distributed into the more upper layers than those of the control, suggesting that enhanced radial migration can drive layer VI undulation. We propose that the loss of NRP1-expressing cells upon Sema3A overexpression might perforate layers IV/V, which might lead to further radial migration. Supporting this idea, the loss of the tectal pial cells, which overlay tectal layers, causes over-migration of the radially migrating cells, therefore inducing undulation of the layers (McCowan et al., 2012). These results indicate that the uniform distribution of layer IV/V cells, which is elaborated by tangential migration, is required to maintain lamination of layer VI cells after radial migration.

In summary, we propose a model of tectal laminar formation in the developing chick optic tectum (Fig. 10). During laminar formation, radial migration of post-mitotic cells from the ventricular zone to the upper layers occurs in every area of the optic tectum. Some of these cells take the tangential route in the intermediate zone, which later corresponds to layer III, and proceed on the fasciculus of the tectal efferent axons running along the dorsoventral axis. Following broad dispersal over the whole tectal area, the cells, after tangential migration, translocate to layers IV/V and differentiate into MAP2-positive multipolar neurons, which express NRP1. Sema3A is expressed in layer VI, which is formed upon radial migration. Sema3A provides repulsive signals to MAP2-positive neurons that express NRP1 and prevents them from entering the upper layers. MAP2-positive multipolar neurons in layers IV/V are required for proper laminar formation. Taken together, the data of this study indicates a role for NRP1-mediated Sema3A signals in the formation of the intermediate layers, which is important to coordinate laminar organization of the optic tectum.

MATERIALS AND METHODS

Constructs pCAGGS-T2TP (Tol2 transposase), pT2K-CAGGS-EGFP (stable EGFP vector), pT2K-CAGGS-rtTA2sM2 (Tet-On activator) and pT2K-BI-EGFP (Tet-On EGFP vector) were kindly provided by Yoshiko Takahashi (Sato et al., 2007). The mCherry gene cassette of pmCherry-C1 (Clontech) and the nuclear localizing signal of pDsRed2-Nuc (BD Biosciences) were inserted into pT2K-CAGGS-rtTA2sM2. The mCherry gene cassette of pmCherry-C1 was inserted into pT2K-CAGGS-EGFP. The ECFP gene cassette of pECFP-N1 (Clontech) was inserted into pT2K-CAGGS to form pT2K-CAGGS-mCherry. The ECFP gene cassette of pECFP-N1 (Clontech) was inserted into pT2K-CAGGS to form pT2K-CAGGS-ECFP. A CDNA fragment of chick Sema3A was amplified from a mixture of E3 and E4.5 chick brain mRNA using reverse transcription PCR. The full-length coding region was inserted into pT2K-BI-EGFP (pT2K-BI-EGFP-Sema3A).
Ex ovo and in ovo electroporation
Ex ovo culture of chick embryos was performed as previously described (Nakamura and O’Leary, 1989; Luo and Redies, 2005). The contents of fertile chicken eggs at E2.5 were transferred into the tectal hemispheres at E7.0, and BrdU-labeled cells were visualized 48 h later. Sections from the hemisphere that had been transfected with Sema3A plus EGFP (A,B) were compared with those from a control untransfected hemisphere of the equivalent region (C,D). Scale bar: 100 µm. Layers above layer VI of the normal embryos are depicted by a square bracket (B,D).

Ex ovo electroporation was performed using a pre-pulse of 30 V with a pulse length of 1 ms and a pulse interval of 5 ms, which was then proceeded by four pulses of 6 V with a pulse length of 5 ms and a pulse interval of 10 ms, using a CUY21EX pulse generator (BEX, Japan).

Fig. 9. Enhanced radial migration after Sema3A mis-expression. (A-D) Radial migration of tectal cells labeled with BrdU after Sema3A mis-expression. After induction of Sema3A at E6.5, BrdU was injected into the tectal hemispheres at E7.0, and BrdU-labeled cells were visualized 48 h later. Sections from the hemisphere that had been transfected with Sema3A plus EGFP (A,B) were compared with those from a control untransfected hemisphere of the equivalent region (C,D). Scale bar: 100 µm. Layers above layer VI of the normal embryos are depicted by a square bracket (B,D). (E) Distribution of BrdU-labeled cells in the radial axis. Cumulative relative frequency of each labeled cell was plotted against a relative migration distance from the ventricular zone to the pial surface. n=69 cells (Experimental, Exp), n=68 cells (Control, Cont). (F,G) Quantitative analysis of the distribution of BrdU-labeled cells. The tectal layers outer to the ventricular zone were divided into ten equally spaced bins along the radial axis, and the proportion of BrdU-labeled cells in each bin was obtained and averaged across ten sections from five embryos (those transfected with Sema3A plus EGFP shown in F,G) or four embryos (EGFP-transfected shown in G). The relative frequency (F) or cumulative relative frequency (G) of the labeled cells in each bin was plotted. Error bars represent the s.e.m.

Fig. 10. A model for tectal laminar formation. In the tectal development, bipolar-shaped cells (red) migrate tangentially (red arrows) on the axon fasciculus of the tectal efferents (gray), which run along the dorsoventral axis in layer III, in order to broadly disperse over the whole tectal area. Cells after tangential migration differentiate into MAP2-positive multipolar neurons (magenta) in layers IV/V, which express NRP1. Sema3A (blue) is expressed in layer VI, which is formed by radial migration (cyan). Sema3A provides repulsive signals to MAP2-positive neurons that express NRP1 and segregates them from entering the upper layers.
In ovo electroporation was performed at E1.5 (stage 10-11, Hamburger and Hamilton, 1951) as described previously (Funahashi et al., 1999; Watanabe and Nakamura, 2000). For Tet-On activation of Sema3A by doxycycline, pT2K-BI-EGFP-Sema3A (3 µg/µl) or control pT2K-BI-EGFP (3 µg/µl) was mixed with pCAGGS-T2TP (1 µg/µl) and pT2K-CAGGS-rT2A2SM2 (1 µg/µl) and injected into the mesencephalon, between a pair of electrodes (LF610P4x1, Unique Medical Imada). Four pulses of 25 V, 50 ms were charged in a one-second interval by the electroporator (CUT18EDT, BEX). At 5 and/or 7 days after the electroporation (E6.5, E8.5), 100 µl of doxycycline (0.2 mg/ml) was injected into the yolk with a micropipette to induce transgene expression.

**Flat-mount culture**

The optic tectum from the embryo that had been electroporated ex ovo at E4.5 was dissected out at E6.0 in ice-cold Hanks’ balanced salt solution (HBSS). The tectum was cut transversely with a micro scalpel and laid in a flat-mount on a Millicell CM-ORG cell culture insert (Millipore), which was set on a 35-mm glass-bottomed dish (Matsunami) filled with culture medium (60% Opti-MEM, 20% F12, 10% fetal bovine serum, 10% chick serum, 50 units/ml penicillin, 50 µg/ml streptomycin). The dish was then incubated on an inverted microscope (IX81, Olympus, Japan) in a humid chamber unit with a gas flow of 40% O2 and 5% CO2 and a controlled temperature of 38°C. Images of GFP and mCherry fluorescence with DIC were captured by using a confocal laser scanning microscope (FV300, Olympus) every 5 µm along the z-axis to a depth of 60-80 µm. z-stack images at every 10 min interval during a period of 18-24 h were collected to construct a time-lapse movie.

**Immunostaining**

Cryosections were stained with the following monoclonal antibodies: MA5-12826 (anti-MAP2, Thermo Scientific), 8D9 (anti-NgCAM, Developmental Studies Hybridoma Bank (DSHB)), TB2 (anti-NRP1, kindly provided by Masahito Yamagata; Yamagata et al., 2006) or an anti-red fluorescent protein (PM005, MBL) polyclonal antibody. Alexafluor594-labeled anti-mouse or anti-rabbit IgG secondary antibodies were used (Invitrogen). The GFP signal was enhanced using an anti-GFP polyclonal (A6455; Invitrogen) or an anti-GFP monoclonal (012-20461, Wako) antibody with an AlexaFluor594-labeled secondary antibody (Invitrogen). Fluorescence images were captured by using a cooled charge-coupled device digital camera (ORCA-ER, Hamamatsu, Japan) or a confocal laser scanning microscope (FV300, Olympus).

**In situ hybridization**

In situ hybridization of cryosections was performed as previously described (Henrique, 1997; Watanabe and Nakamura, 2000). Antisense digoxigenin-labeled RNA probes were used for the coding region and the 3’-UTR of the chick Sema3A mRNA (3 kb downstream from nucleotide 2431 of the coding region) or for the coding region only (1.1 kb).

**AP-fusion protein binding assay**

Chick NR1p ectodomain fused with alkaline phosphatase at its C-terminal (NR1P-AP) was prepared as previously described (Watanabe et al., 2004). Binding of NR1P-AP to the ligands was confirmed in COS7 cells that had been transfected with expression vectors containing chick Sema3A cDNA (supplementary material Fig. S1).

**BrdU-labeling and migration analysis**

Fifteen microliters of 5-bromo-2’-deoxyuridine (BrdU: 1 mg/ml) was injected into the aqueduct of the tectal hemispheres at E7.0. The tectal hemispheres were fixed 48 h later at E9.0. Cryosections were pre-treated with 2.0 M hydrochloric acid (HCl) at 37°C for 20 min and then neutralized with 2.0 M hydrochloric acid (HCl) at 37°C for 20 min and then neutralized with 0.1 M boric acid (pH 8.5) at room temperature for 30 min to detect BrdU-labeled cells with a monoclonal antibody (010198, BioScience Products), and nuclei were stained using DAPI. Distance from the ventricular zone to the BrdU-labeled cells was measured within the transfected radial column with a width of 100 µm, using ImageJ software to analyze the radial migration after mis-expression.

**Repulsion analysis in slice culture**

In ovo electroporation of pT2K-BI-EGFP-Sema3A (3 µg/µl) or control pT2K-BI-EGFP (3 µg/µl) with pCAGGS-T2TP (1 µg/µl), pT2K-CAGGS-rT2A2SM2 (1 µg/µl) and pT2K-CAGGS-ECP (1 µg/µl) was applied to the left mesencephalon at E15. At E4.5, pCAGGS-T2TP (1 µg/µl) with pT2K-CAGGS-mCherry (5 µg/µl) was electroporated into the left optic tectum. At E8.5, the optic tectum that had been labeled with cyan fluorescent protein (CFP) and mCherry was embedded with 3% low melting agarose and sliced at 250 µm with the vibratome. The tectal slices were mounted in collagen gel (60% rat tail collagen in Dulbecco’s modified Eagle medium) in a glass-bottomed dish (Matsunami) and covered with 1 ml of the culture medium (described in flat-mount culture) supplemented with doxycycline (2 µg/ml). Prospective GFP-positive areas after doxycycline-mediated induction were identified with the co-electroporated CFP signal. mCherry-labeled multipolar cells in CFP-positive radial columns were focused under a confocal laser microscope in the culture chamber system described above. The behavior of mCherry-labeled cells during the induction of Sema3A and GFP or control GFP was monitored every 10 min for 20 h.

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Competing interests

The authors declare no competing financial interests.

Author contributions

Y.W. designed the study, analyzed the data and prepared the manuscript. Y.W. and C.S. performed the experiments. H.Y. developed the approach and edited the manuscript.

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Supplementary material

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