© 2014. Published by The Company of Biologists Ltd | Development (2014) 141, 2131-2138 doi:10.1242/dev.102905

RESEARCH ARTICLE

Role of En2 in the tectal laminar formation of chick embryos

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ABSTRACT

The chick optic tectum consists of 16 laminae. Here, we report contribution of En2 to laminar formation in chick optic tecta. En2 is specifically expressed in laminae g-j of stratum griseum et fibrosum superficiale (SGFS). Misexpression of En2 resulted in disappearance of En2-expressing cells from the superficial layers (laminae a-f of SGFS), where endogenous En2 is not expressed. Misexpression of En2 before postmitotic cells had left the ventricular layer indicated that En2-misexpressing cells stopped at the laminae of endogenous En2 expression and that they did not migrate into the superficial layers. Induction of En2 misexpression using a tetracycline-inducible system after the postmitotic cells had reached superficial layers also resulted in disappearance of En2-expressing cells from the superficial layers. Time-lapse analysis showed that En2-misexpressing cells migrated back from the superficial layers towards the middle layers, where En2 is strongly expressed endogenously. Our results suggest a potential role of En2 in regulating cell migration and positioning in the tectal laminar formation.

KEY WORDS: Laminar formation, Optic tectum, Engrailed-2, Chick

INTRODUCTION

The optic tectum is a visual center of non-mammalian vertebrates, and receives axons of retinal ganglion cells in a retinotopic manner. The chick optic tectum consists of 16 laminae: stratum opticum (SO), laminae a-j in stratum griseum et fibrosum superficiale (SGFS), stratum griseum centrale (SGC), stratum album centrale (SAC), stratum griseum periventriculare (SGP), stratum fibrosum periventriculare (SFP) and the ependymal layer (LaVail and Cowan, 1971a; Mey and Thanos, 2000). Neuronal precursor cells proliferate in the ventricular layer, and stop proliferation to differentiate into neurons. Postmitotic neurons migrate to their final destination depending on their birthdate in the ventricular layer. In the mammalian cerebral cortex, neuronal migration has been well analyzed; it has been shown that neurons migrate in an inside-out manner, i.e. later-born neurons migrate beyond earlier-born neurons to form outer layers (Leone et al., 2008; Molyneaux et al., 2007). In *Reeler* mutant mice, neurons cannot migrate in an inside-out manner, and consequently formation of the laminar structure is disrupted (Pearlman et al., 1998; Rice and Curran, 1999). Analysis of tectal laminar formation is behind that of the cerebral cortex. Nonetheless, it has been shown that the neuronal migration pattern in the developing tectum is unique and different from that in the cerebral cortex. In the chick optic tectum, neurons born in earlier stages form the outer and the inner layers, and later-born neurons form the middle layers (LaVail and Cowan, 1971a; Sugiyama and Nakamura, 2003). How the cell migration pattern is regulated and how the laminae are specified remain unclear.

Engrailed-2 (En2) is a transcription factor that plays crucial roles in various developmental processes of both vertebrates and invertebrates. In brain development, it has been shown that En2 is involved in tectal polarity formation by conferring posterior positional information on the tectum for the retinotopic projection (Itasaki et al., 1991; Itasaki and Nakamura, 1992, 1996). En2 misexpression caused nasal retinal fibers to project to the En2-misexpressing site (Friedman and O'Leary, 1996; Itasaki and Nakamura, 1996; Logan et al., 1996; Shigetani et al., 1997). It has been shown that En2 can be secreted and has activities in transcription and protein translation (Joliot and Prochiantz, 2004; Prochiantz and Joliot, 2003). En2 also appears to contribute directly to retinotectal map formation by attracting nasal retinal fibers *in vitro* (Brunet et al., 2005).

In order to determine whether En2 attracts nasal retinal fibers *in vivo*, we carried out misexpression of En2. We did not observe En2-misexpressing cells near the surface of the tectum, though cells that had been transfected with only enhanced green fluorescent protein (EGFP) were present near the surface. This observation led us to study the roles of En2 in the tectum laminar formation. We show here that En2 is expressed in laminae g-j of the SGFS at later stages of tectum development, and that En2-misexpressing cells could not migrate into the superficial layers, where En2 is not expressed endogenously. Induction of En2 misexpression in cells in the superficial layers using a tetracycline-inducible system showed that cells that were induced to express En2 moved out of the superficial layers and migrated back into the layers that express endogenous En2. Our results suggest that En2 has a potential role in neuronal cell migration in the tectal laminar formation.

RESULTS

Endogenous En2 is expressed in a lamina-specific manner in the E10 optic tectum

En2 plays a crucial role in tectal axis formation in early stages of chick embryo development (Nakamura et al., 1994). In order to study whether En2 is involved in tectal development in later stages, we first examined its expression. Because we had been interested in contribution of En2 in retinotectal map formation, we examined En2 expression immunohistochemically in tecta around embryonic day (E) 10, when the retinotectal projection is being formed. En2 was expressed in a layer-specific manner in the optic tectum at E10 (Fig. 1). En2 was strongly expressed in laminae g-j of SGFS, expression being particularly strong in lamina i. En2 was not expressed in superficial layers (i.e. laminae a-f of SGFS) (Fig. 1B,D).

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Received 25 August 2013; Accepted 20 March 2014

Development (2014) 141, 2131-2138 doi:10.1242/dev.102905

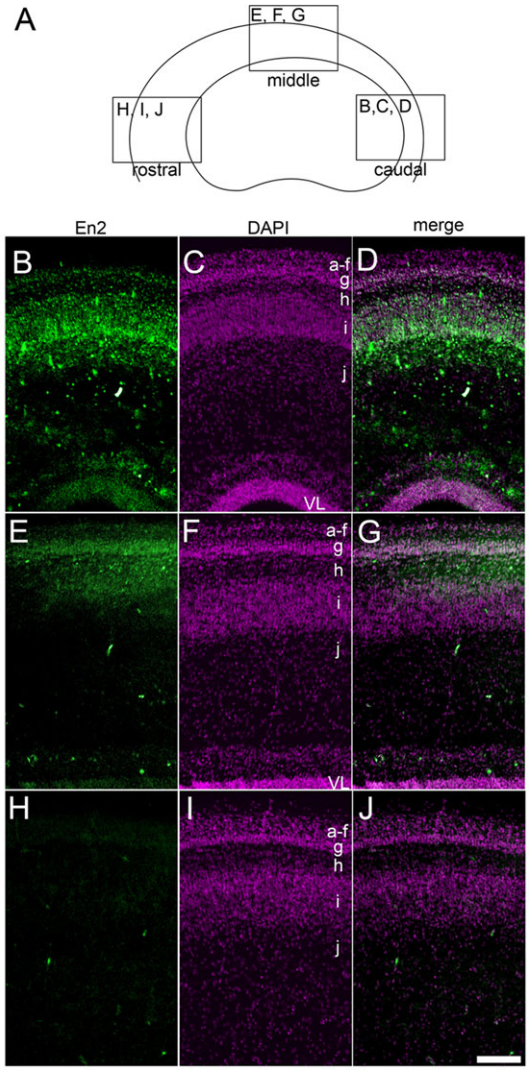

Fig. 1. En2 expression pattern in the E10 optic tectum. Immunostaining of En2 protein in the E10 optic tectum. (A) Illustration of the optic tectum to indicate approximate positions of the figures. (B-J) En2 immunostaining (B,E,H), DAPI staining (C,F,I) and merged images (D,G,J) in the caudal (B-D), middle (E-G) and rostral (H-J) tectum. At the caudal level, En2 protein is expressed in laminae g-j of SGFS (B,D), expression being particularly strong in lamina i. En2 protein is expressed in a caudal-to-rostral gradient (B,E,H). At the rostral level, En2 is hardly expressed (H,J). Scale bar: 100 μm.

Punctate expression of En2 was seen in the deeper layers of the caudal tectum (Fig. 1B,D). En2 expression retained the caudal-to-rostral gradient as that observed at earlier stages (Fig. 1E,G,H,J).

Neurons transfected with En2 are not found in the superficial layers

Because En2 expression retained its caudal-to-rostral gradient at ~E10, we first speculated that En2 is involved in retinotectal map formation by attracting nasal retinal fibers. For this purpose, En2 was misexpressed in the chick tectum. Misexpression of En2 in the tectum at early stages may perturb the tectal axis (Araki and Nakamura, 1999; Friedman and O'Leary, 1996; Itasaki and Nakamura, 1996; Logan et al., 1996; Shigetani et al., 1997), so it was necessary to misexpress En2 after the establishment of the tectal axis. Because tectal neurons that are born after E6 migrate into the middle layers (laminae h-j of SGFS) (Sugiyama and Nakamura, 2003), a tetracycline-inducible expression system was adopted. Midbrain neuroepithelial cells were transfected with pT2K-BI-En2-EGFP, pT2K-CAGGS-rtTA-M2 and pCAGGS-T2TP by electroporation at E1.5. The bidirectional tetracycline-responsive element (BI) vector assures bidirectional expression, so that both En2 and EGFP are induced in the transfected cells by doxycycline (Dox, an analog of tetracycline) administration. At E1.5, all the mesencephalic neuroepithelial cells proliferate as stem cells so transfected cells are expected to be distributed in all the layers, including the superficial layers. En2 and EGFP expression was induced by administration of Dox every 24 h from E8.5 to E12.5, and the tecta were fixed at E13.5. In this system, we expected that neurons that had been transfected at E1.5 and had left the neuroepiethelial layer before E6 would misexpress En2 in the superficial layers. In the control tecta, which had been transfected with pT2K-BI-EGFP for misexpression of only EGFP, we could see sharp EGFP fluorescence on the surface of the tectum at E13.5 (n=13), as we had expected (Fig. 2A,C). In addition to dotted fluorescence, fiber-like fluorescence was observed. In the experimental tecta that had been transfected with pT2K-BI-En2-EGFP (n=19), fluorescence was blurred when we focused on the surface of the tectum (Fig. 2B,D), suggesting that the transfected cells were not distributed on the surface. We then made cryosections of the tecta to see the distribution of transfected cells. In the control, transfected cells were distributed in almost all layers, including the superficial layers (laminae a-f) (14 sections from three tecta; Fig. 2E). EGFP-transfected cells beneath the surface differentiated into horizontal cells, which are characteristic of the superficial layers of the tectum (laminae a-d of SGFS) (Hilbig et al., 1998; Hunt and Brecha, 1984; Luksch and Golz, 2003) (Fig. 2E, yellow arrowheads). Fiber-like fluorescence from the surface view may be that of the horizontal cells. Surprisingly, in the experimental group, En2-misexpressing cells were not located in the superficial layers (20 sections from three tecta) (Fig. 2F), where En2 is not expressed endogenously. Consequently, the thickness of the superficial layers (laminae a-f) was reduced in En2-misexpressing tecta (Fig. 2E,F). We could not detect fluorescent horizontal cells. As the transfected cells may have been located in the superficial layers when En2 misexpression commenced at E8.5 by Dox administration, the result suggests that En2-misexpressing cells may disappear from the superficial layers after E8.5.

Next, we wondered whether neurons that are induced to express En2 before they leave the ventricular layer could reach superficial layers. In order to answer this question, we electroporated the tectum at E5.5 by injecting the DNA solution in the aqueductus, and administered Dox at the same time as electroporation and then every 24 h to induce En2 misexpression. At this stage, many cells in the tectum are postmitotic (LaVail and Cowan, 1971b) and postmitotic cells accumulate over the ventricular layer (Sugiyama and Nakamura, 2003). Under our experimental conditions, only neuroepithelial cells that face the aqueductus are transfected (Sugiyama and Nakamura, 2003). Postmitotic cells do not face the aqueductus so they are not transfected (Sugiyama and Nakamura, 2003). Embryos at this stage are submerged in the albumen *in ovo* so a shell-less culture system was adopted for easier access to the optic tectum at electroporation. The embryos were cultured until E10.5. In the control, many transfected cells were found in the superficial layers above lamina g (23 sections from three tecta; Fig. 2G). In the experimental group, En2-misexpressing cells were not found in the superficial layers in any of the sections examined (29 sections from three tecta; Fig. 2H). The results indicate that En2-misexpressing cells cannot migrate into the superficial layers.

If the cells that would have destined to migrate into superficial layers were prevented from migrating there owing to En2

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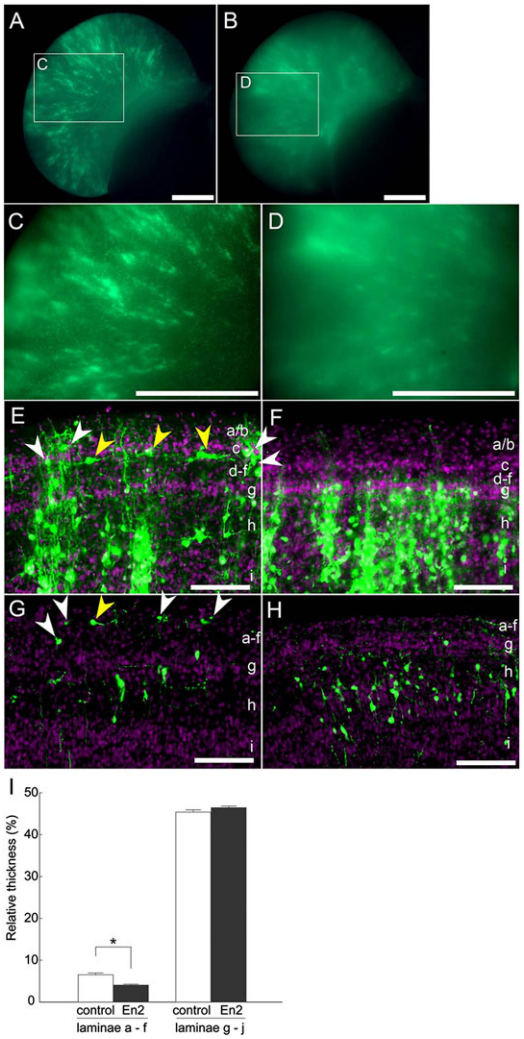

Fig. 2. En2-misexpressing cells are not located in the superficial layers. (A-F) E13.5 tectum. pT2K-BI-En2-EGFP, pT2K-CAGGS-rtTA-M2 and pCAGGS-T2TP were electroporated at E1.5, and Dox administration was commenced at E8.5. (A,C) Dorsal view of a control tectum. pT2K-BI-EGFP was used instead of pT2K-BI-En2-EGFP. C shows a higher magnification of the boxed area in A. On the surface of the control tectum, clear radial bundles of transfected cells were seen. There were also many scattered transfected cells on the surface (C). (B,D) Dorsal view of an En2-misexpressing tectum. D shows a higher magnification of the boxed area in B. Radial bundles of the transfected cells appeared unclear and there were no scattered cells on the surface (D). (E,F) Cryosections of an E13.5 tectum. In the control (E), transfected cells were distributed in the superficial layers (white and yellow arrowheads) as well as in the middle layers. Horizontal cells, which are characteristic of the superficial layers, were also seen (yellow arrowheads). By contrast, En2-misxpressing cells were not located in the superficial layers, but were located in the middle layers (F). (G,H) E10.5 tectum. The expression vectors were electroporated to the tectum at E5.5 and Dox administration was commenced at the same time as electroporation in order to induce gene expression immediately. (G) In the control, transfected cells are located in the superficial layers (arrowheads), including horizontal cells (yellow arrowhead), as well as in the middle layers. (H) En2-misexpressing cells were distributed in the middle layers, but not in the superficial layers. (I) Relative thickness of laminae a-f and laminae g-j against that of all the layers. Relative thickness of the superficial layers (laminae a-f) in the En2-misexpressing tecta was less than that in the control. By contrast, relative thickness of laminae g-j in the En2-misexpressing tecta was slightly greater than that in the control. Error bars represent s.e.m. **P*<0.001. Control, 23 sections from three tecta; En2-misexpressing tecta, 29 sections from three tecta. Student's *t*-test. Scale bars: 1 mm (A-D); 100 μm (E-H).

misexpression, the thickness of superficial layers would be reduced, and that of the layers of endogenous En2 expression (laminae g-j) would be increased. Indeed, comparison of Fig. 2G and Fig. 2H reveals that the thickness of the superficial layers of the En2-misexpressing tectum is reduced. We quantified this by measuring the thickness of all the layers together, that of the superficial layers (laminae a-f) and that of laminae g-j in the E10.5 tecta that were transfected at E5.5 and continuously treated with Dox from just after transfection. In the control, the thickness of all the layers was 879.51 ±4.67 μm, and that of the superficial layers and of laminae g-j was 57.29±3.59 μm and 400.44±4.24 μm, respectively (23 sections from three tecta). In the En2-misexpressing tecta, the thickness of all the layers was 783.56±6.49 μm, and that of the superficial layers and of laminae g-j was 31.96±1.96 μm and 364.33 ±4.49 μm, respectively (29 sections from three tecta). Then we calculated the relative thickness of the superficial layers and of laminae g-j against that of all the layers. As expected, the relative thickness of the superficial layers in En2-misexpressing tecta was thinner than that in the control (control, 6.52±0.41%; En2-misexpressing tecta, 4.06±0.24%; $P<0.001$; Fig. 2I). The relative thickness of laminae g-j appeared to be slightly thicker in the En2-misexpressing tecta but this was not statistically significant (control, 45.53±0.40%; En2-misexpressing tecta, 46.48±0.37%; $P<0.1$). These results strongly support our view that En2-misexpressing cells could not migrate into the superficial layers and stopped in the layers that express En2 endogenously.

From these results, it was not possible to determine whether En2 attracts nasal retinal fibers *in vivo* because retinal fibers pass the most superficial layer of the tectum, but we were able to infer that En2 might be involved in cell migration and cell positioning in tectal laminar formation.

En2-misexpressing cells disappear from the superficial layers

In the tecta in which En2 had been misexpressed from E8.5 to E13.5 by Dox administration after electroporation at E1.5, En2-misexpressing cells were not located in the superficial layers. It was expected that some portion of transfected cells may have migrated to the superficial layers at the onset of En2 misexpression, and those that were induced to express En2 in the superficial layers disappeared from there. Thus, we performed time course analysis. Embryos were electroporated at E1.5 and Dox administration was commenced at E8.5. It was reported that weak expression of the transgene is observed from 3 h after Dox administration (Watanabe et al., 2007). At 7 h after Dox administration, we could detect En2-misexpressing cells throughout all tectal layers, even in the superficial layers above lamina g, in all the sections examined (15 sections from two tecta) (Fig. 3B). By 12 h after Dox administration, En2-misexpressing cells diminished in the superficial layers (12 sections from two tecta) (Fig. 3D). By 24 h after Dox administration, En2-misexpressing cells in the superficial layers were dramatically decreased (14 sections from two tecta) (Fig. 3F). In the control, transfected cells could be continuously seen in the superficial layers at 7 h (17 sections from two tecta), 12 h (nine sections from two tecta) and 24 h (12 sections from two tecta) after Dox administration (Fig. 3A,C,E). Then, we quantified the cell number in laminae a-f relative to that in all the layers in the middle region of the tectum (Fig. 3G). Relative cell number of En2-misexpressing cells in the superficial layers was 7.90±0.30% (15 sections from two tecta), 3.32±0.47% (12 sections from two tecta) and 0.51±0.17% (14 sections from two tecta) at 7, 12 and 24 h after Dox administration, respectively. In the control tecta, the relative cell number of EGFP-expressing cells in the superficial layers was 10.30±0.90% (17 sections

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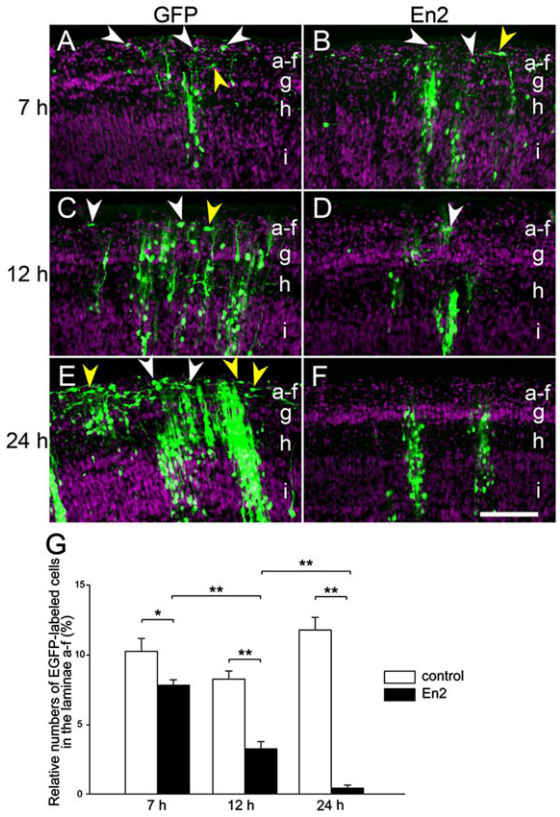

Fig. 3. En2-misexpressing cells disappear from the superficial layers. Time-course analysis for distribution of En2-misexpressing cells. (A,C,E) In the control, transfected cells, shown in green, were seen in the superficial layers at all time points examined. (B,D,F) En2-misexpressing cells (green) were seen in the superficial layers at 7 h after Dox administration (B) but diminished in the superficial layer at 12 h after the administration (D). En2-misexpressing cells were no longer seen in the superficial layer at 24 h after the administration (F). Arrowheads (white and yellow) indicate cells distributed in the superficial layers. Yellow arrowheads indicate horizontal cells. (G) Relative numbers of the EGFP-positive cells in the superficial layers. The number of En2-misexpressing cells decreased with time, and was remarkably reduced by 24 h after Dox administration. Error bars represent s.e.m. **P*<0.05, ***P*<0.001. Control: 584 cells in 17 sections from two tecta for 7 h; 352 cells in nine sections from two tecta for 12 h; 2753 cells in 12 sections from two tecta for 24 h. En2-misexpressing tecta: 1179 cells in 15 sections from two tecta for 7 h; 1848 cells in 12 sections from two tecta for 12 h; 4193 cells in 14 sections from two tecta for 24 h. Student's *t*-test. Scale bar: 100 μm.

from two tecta), 8.30±0.56% (nine sections from two tecta) and 11.84±0.90% (12 sections from two tecta) at 7, 12 and 24 h after Dox administration, respectively. Quantification revealed that even 7 h after Dox administration the relative cell number was slightly smaller than that of the control ($P<0.05$). There were statistically significant differences between En2-misexpressing tecta at each time point. The results indicate that En2-misexpressing cells cannot stay in the superficial layers and disappeared from the superficial layers by 24 h after induction of En2 misexpression.

It is important to confirm that the distribution of transfected cells is constant among the control tecta. So, we quantified the relative number of EGFP-expressing cells in layers in two control tecta (four sections each). In one tectum, relative numbers of the transfected cells in laminae a-f, g, h-j and in the laminae from SGC to the ventricular layer were 12.81±1.24%, 7.98±1.78%, 56.14±1.15% and 23.07±1.10%, respectively. In the other tectum, they were 9.77±0.43%, 8.53±0.92, 54.36±0.91% and 27.34±1.53%, respectively. This result shows a constant distribution pattern of the transfected cells in the control tecta.

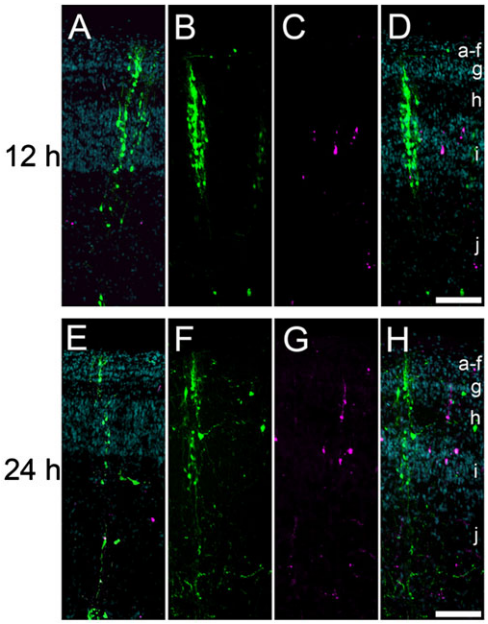

Fig. 4. Disappearance of En2-misexpressing cells from the superficial layers is not due to cell death. Cell death was examined at 12 (A-D) and 24 (E-H) h after Dox administration by immunostaining of active caspase-3, which is a molecular marker of apoptosis (magenta). (A,E) Control in which only EGFP (green) was expressed. The figures are merges of EGFP, active caspase-3 (magenta) and DAPI (blue). (B-D,F-H) En2 misexpression. (B,F) En2-misexpressing cells (green), (C,G) active caspase-3 (magenta) and (D,H) merged images of En2 misexpression, active caspase-3 and DAPI. A small number of apoptotic cells were detected but those apoptotic cells were not overlapped with the transfected cells, indicating that En2 misexpression did not induce cell death. Scale bars: 100 μm.

En2-misexpressing cells migrate out of the superficial layers

There are two possibilities that can explain the disappearance of En2-misexpressing cells: one is death of En2-misexpressing cells in laminae a-f of SGFS, the other is migration of En2-misexpressing cells away from laminae a-f.

Active caspase-3 is a molecular marker of apoptosis (Nicholson et al., 1995; Srinivasan et al., 1998). We checked the possibility of cell death by examining active caspase-3 by immunohistochemistry (Fig. 4). In both the control and En2-misexpressing tecta, only a few cells were positive both for EGFP and active caspase-3. At 12 h after Dox administration, the numbers of EGFP-positive cells that were also caspase-3-positive were 0.26±0.17% (eight sections from two tecta) and 0.19±0.13% (ten sections from two tecta) in the control and in En2-misexpressing tecta, respectively. At 24 h after Dox administration, the relative number of caspase-3-positive cells was not changed: 0.26±0.13% in the control and 0.24±0.12% in the En2-misexpressing tecta (ten sections from two tecta each). There was not a significant difference among the relative numbers of caspase-3-positive cells. The results indicate that cell death is not involved in disappearance of En2-misexpressing cells from the superficial layers.

Next, we used time-lapse analysis to investigate the possibility that En2-misexpressing cells migrated away from the superficial layers. For analysis, the tecta were taken out at 7 h after the first Dox administration (at E8.5) and sliced at a thickness of 250 μm. The slices were cultured and time-lapse images were recorded under a confocal laser microscope. In the control, transfected cells migrated towards the surface of the tectum or tangentially. Inwards migration was not observed in any of the three cultures examined (Fig. 5A; supplementary material Movie 1). By contrast, En2-misexpressing

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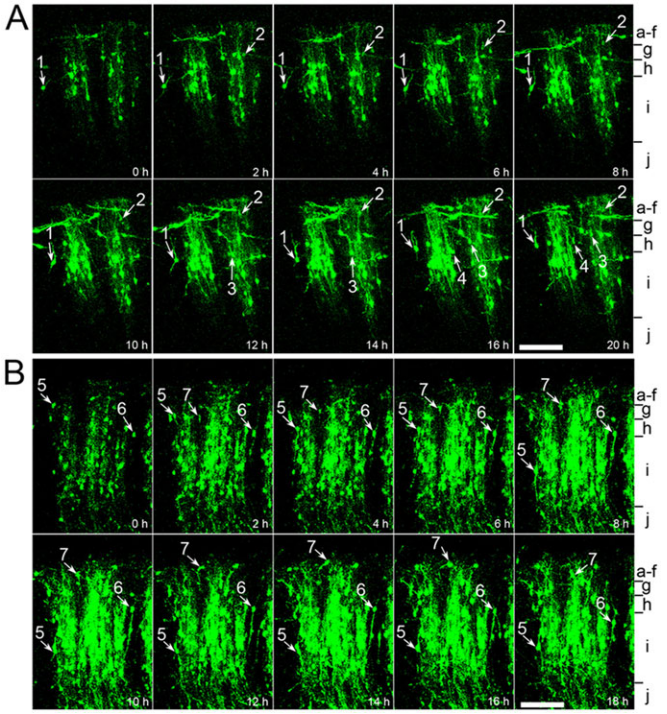

Fig. 5. En2-misexpressing cells migrate back from the superficial layers towards the middle layers. Time-lapse analysis for migration of En2-misexpressing cells. (A) Control. Cells transfected with only EGFP migrated towards the superficial layers (arrows 1-4) but no cells migrated back towards the middle layers. (B) En2-misexpressing cells migrated back towards the middle layers (arrows 5-7). Some cells (arrows 6, 7) initially migrated to the superficial layers but they changed direction and migrated back towards the middle layers. Scale bars: 100 μm.

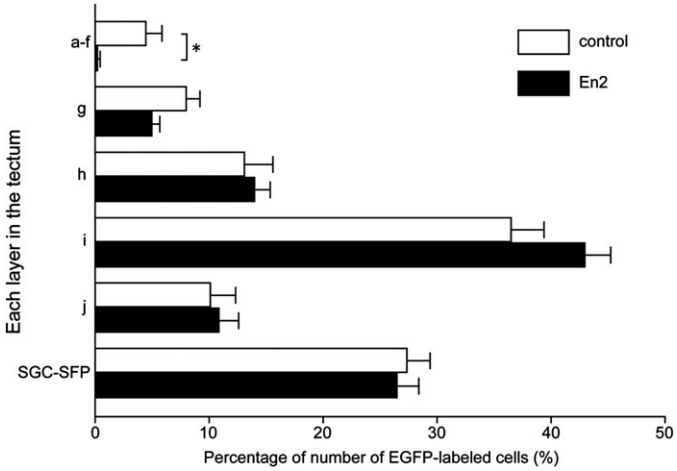

Fig. 6. En2-misexpressing cells migrate back mainly to lamina i of SGFS. Percentage of the number of transfected cells in laminae of the optic tectum at E10.5 that had been electroporated at E1.5 and administered Dox at E8.5 and E9.5. The percentage of En2-misexpressing cells was less than that of the control in the superficial layers (laminae a-f). By contrast, En2-misexpressing cells accumulate in lamina i of SGFS, where endogenous En2 is strongly expressed. Graph shows mean±s.e.m. calculated from seven sections of one tectum (control, 358 cells; En2-misexpressing tecta, 1177 cells). *P<0.01. Student's t-test.

cells migrated back towards the middle layers from the superficial layers (Fig. 5B; supplementary material Movie 2). Backwards migration was observed in all the four cultures that were recorded. Some neurons stayed in lamina i after they migrated inwardly (Fig. 5B, arrows 5, 6; supplementary material Movie 2). Interestingly, some neurons migrated towards the surface of the tectum once, but turned back inwardly (Fig. 5B, arrows 6, 7; supplementary material Movie 2). The results indicate that En2-misexpressing cells could not stay in the superficial layers and migrated back towards the middle layers.

En2-misexpressing cells accumulate in the laminae that express endogenous En2

Finally, we examined which laminae the En2-misexpressing cells migrated back to. To analyze the distribution of the transfected cells, we quantified the number of the transfected cells in the laminae at 48 h after commencement of Dox administration at E8.5 (Fig. 6). We counted the number of EGFP-positive cells in laminae a-f, g, h, i, j and SGC-SFP in the transfected area, and expressed this relative to the total number of EGFP-positive cells in the area (seven sections of a tectum transfected with pT2K-BI-En2-EGFP or pT2K-BI-EGFP). As was seen previously, there were very few En2-misexpressing cells in the superficial layers (laminae a-f) so the numbers of cells in the laminae a-f were summed. The statistical analysis clearly shows that the relative number of En2-expressing cells in laminae a-f was reduced compared with that of the EGFP-expressing cells in the control (control, 4.53±1.36%; En2-misexpressing tecta, 0.29±0.15%; P<0.01). There was a trend towards an increase in the relative cell numbers in laminae h, i and j in the En2-misexpressing tecta compared with those in the control (Fig. 6). Increase was largest in lamina i (control, 36.60±2.82%; En2-misexpressing tecta, 43.05±2.23%; P<0.1) indicating that En2-misexpressing cells might have migrated from laminae a-f to lamina i, but these increases were not statistically significant.

DISCUSSION

How neuronal cells migrate and determine their destinations during the laminar formation of the tectum has been challenged. In this study, we showed that En2 protein is expressed in a lamina-specific manner in the developing optic tectum. Then, we have shown that En2-misexpressing cells could neither reach superficial layers (laminae a-f of SGFS) nor stay there, and that En2-misexpressing cells migrated away from the superficial layers inwardly and accumulated mainly in the lamina i of SGFS, where endogenous En2 is strongly expressed.

Endogenously, En2 protein was expressed in laminae g-j of SGFS, expression being particularly strong in lamina i. By contrast, En2 was not expressed in the superficial layers. We speculated that En2 is involved in the formation of specific laminae, and carried out En2 misexpression experiments. Because En2 affects tectum polarity, we adopted a tetracycline-inducible system to induce En2 misexpression after the tectum polarity was established. In electroporation experiments with the tetracycline-inducible plasmid set at E1.5, administration of Dox from E8.5 to E12.5, and fixation at E13.5, all the mesencephalic neuroepithelial cells are stem cells at electroporation so transfected cells were expected to be distributed in all the layers of the tectum, as was shown in the control tecta. Although En2 misexpression was induced after some portion of the transfected cells may have reached the superficial layers, En2-misexpressing cells were not found in the superficial layers at fixation. In electroporation experiments at E5.5 with commencement of Dox administration just after electroporation, transfected cells would be expected to leave the neuroepithelium as neurons, and some proportion of these neurons to migrate to the superficial layers, as was shown in the control tecta. However, En2-misexpressing cells were not found in the superficial layers. Based on the observation that En2 misexpression did not induce

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cell death, we interpreted the result of the former experiments as that En2-misexpressing cells could not stay there, and the latter as that En2-misexpressing cells could not migrate into the superficial layers.

Then we were interested in the behavior of the cells in which misexpression of En2 was induced after they had migrated into the superficial layers. As En2 misexpression did not induce cell death, we speculated that En2-misexpressing cells cannot stay in the superficial layers and are forced to migrate out of these layers. In order to test this hypothesis, we carried out time course and time-lapse analyses. In the time course analysis, embryos were electroporated with the tetracycline-inducible plasmid set at E1.5, treated with Dox at E8.5, and fixed at 7, 12 or 24 h after Dox administration. We could see En2-misexpressing cells in the superficial layers at 7 h after Dox administration. The cells in the superficial layers gradually diminished and disappeared from there by 24 h after Dox administration. Time-lapse analysis revealed that En2-misexpressing cells migrated away from the superficial layers back to the middle layers. Quantitative analysis indicated that En2-misexpressing cells had migrated back to lamina i, where endogenous En2 expression is most conspicuous. The results suggest that cells that were induced to misexpress En2 in the superficial layers could not stay there and were forced to migrate to the laminae in which En2 is endogenously expressed. Thus, our results strongly suggest that En2 is implicated in migration and positioning of neuronal cells in tectal laminar formation. When En2 misexpression was induced at E8.5, horizontal cells, which are characteristic of the superficial layers, disappeared. Together with the pattern of endogenous En2 expression and the disappearance of horizontal cells, our results also suggest that En2 may cause neurons to form the middle layers, which consist of later-born neurons. Furthermore, our observation unraveled another interesting phenomenon that neuronal cells were transferred from the false layers to the right destination layer.

How En2 regulates neuronal cell migration and positioning still remains unclear. En2 misexpression affects the migration of En2-misexpressing cells; that is, the effects are cell-autonomous, suggesting that En2 acts as a transcriptional factor. This result does not rule out the possibility that En2 acts as a secreted protein to attract nasal retinal fibers indicated by Brunet et al. (Brunet et al., 2005), but, if we consider the fact that retinal fibers run through SO, which lies on lamina a of SGFS and far from the laminae in which En2 is expressed endogenously, it is more plausible to think that En2 acts as a cell-autonomous transcriptional factor rather than as a secreted protein to attract nasal retinal fibers. It has been also reported that knockout of engrailed in mice affected migration and positioning of neurons cell-autonomously in developing dorsal raphe nucleus in the mid-hindbrain (Fox and Deneris, 2012). Alteration of molecules for cell adhesion and/or cell recognition by misexpression of En2 is one possibility that could account for such cell migration (Suzuki and Takeichi, 2008; Valiente and Marin, 2010). Indeed, several cell adhesion molecules, such as cadherins, ephrin/Eph family and some extracellular matrices, are expressed in a lamina-specific manner (Braisted et al., 1997; Kenny et al., 1995; Marin et al., 2001; Yamagata et al., 1995, 2006; Yamagata and Sanes, 2005), suggesting that a variety of cell adhesion properties may play important roles in the laminar formation of the developing tectum by providing cells with a distinct surface affinity. There may be another possibility that repulsive interaction acts between En2-misexpressing cells and non-expressing cells in the superficial layers, and that En2-misexpressing cells may be expelled away from the superficial layers. During tectal axis formation and retinotopic projection, En2 regulates expression of ephrinA2 and ephrinA5 (Logan et al., 1996; Shigetani et al., 1997), which mediates a repulsive signal (reviewed by Kullander and Klein, 2002), supporting the possibility that En2 may regulate ephrin/Eph signaling during the laminar formation as well.

Which molecules directly regulate tectal neuronal cell migration is still unknown. Further studies are required to elucidate intracellular signaling downstream of En2. But our findings suggest that the final destination of the neurons is determined by transcription factors, such as En2, and their downstream molecules. Sugiyama and Nakamura (Sugiyama and Nakamura, 2003) have reported that the majority of neuronal cells arising at later stages contribute mainly to laminae h-j of SGFS. The results that En2 is strongly expressed in lamina i, and that En2-misexpressing cells were transferred mainly to lamina i from the superficial layers in this study suggest that En2 may give a property of later migratory cell fate to the neuronal cells. Sugiyama and Nakamura (Sugiyama and Nakamura, 2003) have also shown that misexpression of Grg4 (Groucho related gene) gives late migratory cell fate to neuronal cells that form laminae h-j. As the engrailed protein family has been reported to interact with Groucho protein as a transcriptional factor (reviewed by Chen and Courey, 2000; Courey and Jia, 2001; Morgan, 2006), there may be a possibility that En2 cooperates with Grg4 for the formation of the middle layers.

MATERIALS AND METHODS

Expression vectors

Vectors used are pT2K-BI-EGFP, pT2K-BI-En2-EGFP, pT2K-CAGGS-rtTA-M2 and pCAGGS-T2TP. Chicken *En2* was amplified from pMiw-En2-HA (Araki and Nakamura, 1999) with primers 5′-TCTGC-TAGCGCCACCATGGAGGAGGGCGGCCGCAG-3′ and 5′-CCCGATA-TCTTATGCGTAGTCTGGGACGTC-3′ and subcloned into *Nhe*1-*Eco*RV site of pT2K-BI-EGFP (Clontech, Mountain View, CA). pT2K-CAGGS-rtTA-M2 and pCAGGS-T2TP were provided by Dr. Y. Takahashi (Sato et al., 2007; Watanabe et al., 2007). Gene expression cassettes in pT2K vectors are flanked by Tol2 transposable elements so that they can be transferred and integrated into cell genome by Tol2 transposase encoded by T2TP. As pT2K-BI vector contains BI, transgenes can be expressed only in the concomitant presence of tetracycline and reverse tetracycline-controlled transcriptional activator (rtTA), which is expressed continuously under the control of CAGGS, and transfected cells can be detected by EGFP fluorescence.

Electroporation

In ovo electroporation was carried out as described previously (Funahashi et al., 1999; Momose et al., 1999; Nakamura and Funahashi, 2001). Fertilized chicken eggs were incubated at 38°C to reach stage 10-11 (Hamburger and Hamilton, 1951). The DNA solution of a mixture of pT2K-BI-En2-EGFP or pT2K-BI-EGFP, pT2K-CAGGS-rtTA-M2 and pCAGGS-T2TP was injected into the lumen of the midbrain. The final concentration of each plasmid was 1 µg/µl. Fast Green (Wako, Osaka, Japan) was added to the DNA solution at the final concentration of 0.05% for better visualization of the solution. Addition of Fast Green raises viscosity and keeps the solution in the tube. A rectangular pulse of 25 V, 50 ms/s was charged four times by an electroporator (CUY21, Bex, Tokyo, Japan).

For electroporation of E5.5 chick embryos, embryos were moved to shell-less culture system at E2.5 for easier access to the optic tectum at E5.5 (Auerbach et al., 1974; Luo and Redies, 2005). The DNA solution was injected in the aqueductus mesencephali. A single pulse of 30 V, 1 ms was charged, then a rectangular pulse of 8 V, 5 ms/s was charged four times by an electroporator (CUY21EX, Bex, Tokyo, Japan).

Expression of the transgene was induced and sustained by application of a solution of doxycycline (Dox, an analog of tetracycline; 100 µl of 0.2 µg/µl in PBS) to the yolk sac every 24 h until embryos reached appropriate stages. Administration of Dox was commenced at E8.5 for the electroporation experiments at stage 10-11. In the case of electroporation to E5.5 embryos, administration of Dox was commenced immediately after electroporation.

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Immunohistochemistry

Embryos were fixed with 4% paraformaldehyde overnight at 4°C. The fixed midbrain was transferred to 30% sucrose overnight, embedded in OCT compound (Sakura Finetek, Torrance, CA), and cryosectioned at 14 µm. The tissue was blocked with 3% bovine serum albumin (Sigma) for 1 h, and incubated in the primary antibody diluted in the blocking solution overnight at 4°C. The tissue was then incubated in the secondary antibody for 1 h at room temperature counterstained with 1 µg/ml DAPI (Dojindo, Kumamoto, Japan). Anti-En2 monoclonal antibody 4D9 (1:100) (Patel et al., 1989) and anti-active caspase-3 polyclonal antibody (1:500; Sigma, C8487) were used as primary antibodies. The latter antibody detects apoptosis (Nicholson et al., 1995; Srinivasan et al., 1998). Alexa Fluor 488-conjugated anti-mouse IgG antibody and Alexa Fluor 594-conjugated anti-rabbit IgG antibody (Invitrogen) were used as secondary antibodies.

Time-lapse imaging analysis

For time-lapse imaging analysis, slice culture of the optic tectum was performed based on the method previously described (Placzek and Dale, 1999). The optic tectum was excised and embedded in 3% low-melting agarose gel (NuSieve GTG Agarose, Lonza, Rockland, ME) in Hanks' Balanced Salt Solutions (Life Technologies), and cut at 250 µm using a vibrating blade microtome (Leica). The tectal slices were placed on a glass-bottom dish (Matsunami) and embedded in collagen gel (Placzek and Dale, 1999). After embedding, 1 ml of culture medium [60% Opti-MEM (Life Technologies), 20% F12 (Life Technologies), 10% fetal bovine serum, 1% chick serum, 1×penicillin-streptomycin (Life Technologies)] was added. The tectal slices on the glass-bottom dish were placed under confocal laser microscope with culture chamber system (Olympus). Images were taken every 20 min for 20 h.

Acknowledgements

We thank for Prof. Y Takahashi and Prof. K Kawakami for pCAGGS-T2TP and pT2K-CAGGS-EGFP vectors.

Competing interests

The authors declare no competing financial interests.

Author contributions

M.O. and H.N. designed the research and developed the approach; M.O., H.H., Y.W. and J.F. performed experiments; M.O. and H.N. wrote the paper.

Funding

This study was supported partly by a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan [17023003]; and by a grant from the Japanese Society for the Promotion of Science [22570199].

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.102905/-/DC1

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