Changes of retinofugal pathway development in mouse embryos after Sonic hedgehog antibody perturbation

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Abstract: **Objective** To understand the function of Sonic hedgehog in chiasm development in mouse embryos of embryonic day 13 (E13) to E15. **Methods** Brain slices of E13-E15 mouse embryos containing the optic pathway from the eyes to the optic tract were prepared and cultured in DMEM/F12 in the presence of 10% fetal bovine serum at 37 ℃ in a rolling incubator for 5 h. The antibody to Shh was added into the culture medium of the slices in the treatment group, while no additional chemical or only normal mouse IgG was added in the control groups. After culture, the brain slices were fixed and a DiI granule was inserted into the optic disc in one eye. Seven days later, the tissue overlying the chiasm was removed to expose the DiI-labeled chiasm for observation under confocal microscope, and the images were analyzed by METAMORPH software. **Results** Shh antibody treatment produced a reduction of crossing of the earliest retinal axons at the midline of E13 chiasm, and the uncrossed axons were also influenced by Shh antibody at E15. **Conclusion** Shh executes a transient but important function in axon decussation in the early stage of mouse optic chiasm development and signals axon turning in the later stage.

Key words: Sonic hedgehog; chiasm; development

In the mammalian animals, the axons of the optic nerve from each eye merge into the optical chiasm, where some of the axons cross the midline to project into the contralateral optic tract, while a small number of others turn away and form the ipsilateral optic tract. This pattern of axon routing gives the characteristic X-shaped pathway at the optic chiasm. Three main changes of axon order take place when retinal axons grow from the optic stalk to the optic tract: i) segregation of crossed and uncrossed axons before they reach the midline; ii) age-related reorganization of axons in the chiasm and the optic tract; iii) retinotopic organization in the optic tract[1].

Sonic hedgehog (Shh), found originally in the notochord and floor plate, is a member of hedgehog family[2] involved in eye formation[3-5], retinal ganglion cell production[6], development of the optic disc and stalk neuroepithelial cells[7]. In chick embryos, ectopic Shh expression also leads to such abnormal development as expansion of the retinal pigmented epithelium, enlargement of the optic stalks and reduction of the neural retina[8]. In our previous study, we detected the expression of Shh and its receptor, Patched (Ptc), in the developing optic chiasm, which underwent changes during different periods of development[9]. In this study, we attempted to define the role of Shh in the developing chiasm of mouse embryos by disturbing Shh signaling pathway using 5E1, a monoclonal antibody to Shh.

Reveived: 2006-09-10

Supported by Research Grant Council of Hong Kong (CUHK 4417/03M)

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MATERIALS AND METHODS

Animals

The experimental procedures of the present study were approved by the Animal Experiment Ethic Committee of the Chinese University of Hong Kong. Time-mated, pigmented C57 mice were obtained from the University Animal House. The day on which the vaginal plug was found was considered as the embryonic day 0 (E0).

Preparation of brain slices

The pregnant mice were sacrificed by cervical dislocation and the mouse embryos from E13 to E15 were removed by caesarean section. The embryos were decapitated and kept in chilled Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing penicillin (1 000 U/ml) and streptomycin (1 000 μg/ml). After removing the dorsal and ventral part just above and below the eyeball, the brain slices containing the optic pathway (from the eyes to the optic tract) were obtained from E13-E15 embryos. The brain slices were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (Life Technologies, USA) at 37 ℃ in a rolling incubator for 5 h, during which a jet of oxygen

was supplied 3 times to the culture. 5E1 (mouse anti-Shh IgG_1, DSHB) was added into the culture medium at the concentration of 40 μg/ml. The control preparations included brain slices cultured in the absence of the added chemicals, or with addition of normal mouse IgG (Chemicon, USA). After culture, the brain slices were fixed in 4% paraformaldehyde at 4 ℃ overnight. A DiI (1,1-dioctadecyl-3, 3, 3', 3'-tetramethylindo-carbocyanine perchlorate, DiIC18 (3), Molecular Probes, OR, USA) granule was inserted into the optic disc in one eye and the brain slices were treated in 2% formalin. Seven days later, the tissue overlying the optic chiasm was removed to expose the DiI-labeled chiasm, which was observed under a confocal microscope.

Confocal microscopy

DiI-labeled retinal axons in the optic chiasm were examined in whole mount preparations of the optic pathway. These DiI-labeled whole mounts were examined using a confocal imaging system (Bio-Rad MR600, Hertford, UK) connected to a Zeiss Axiophot photomicroscope with a green excitation filter set (GHS, 514 nm excitation and 550 nm emission long pass). The digital images were processed using confocal assistant software (Bio-Rad, USA) and stored on Zip disk (Iomega, USA).

Analyses of retinal trajectory at the optic chiasm

Retinal axons in whole mounts of the chiasm were imaged using the Extended Focus Image command in COMOS software (Bio-Rad, USA) that captures the images at low magnification (×10, Zeiss Plan-Neofluar, Germany) and then at higher magnification (×20, Zeiss Plan-Neofluar, Germany). The effects of Shh antibody 5E1 on axon crossing at the midline of the chiasm were measured in whole-mounts of the optic pathways in E13-14 embryos, the early period when retinal axons were growing across the midline [10,11]. The pixel intensities of all labeled retinal axons and growth cones within two areas (each measuring 100 μm×200 μm) flanking both sides of the midline (Fig.1A) were measured individually using the "Region Measurements" functions in METAMORPH Software (Version 4.6r5; Universal Imaging Corp., USA). The degree of axon crossing was calculated as the ratio of adjusted pixel intensity past the midline *vs*. that before the midline.

We also observed the changes of the uncrossed axons in E15 brain slices when a substantial number of axons turned and projected to the ipsilateral optic tract in response to 5E1 treatment. We used METAMORPH software to measure the pixel intensity and area of a defined region in the projected images of the initial segment of the ipsilateral tract (Fig.3 B). The axons projecting to the ipsilateral side were represented by the average pixel intensity (accumulated pixel intensity divided by total area). The data in various experiment groups were analyzed using one-way ANOVA in the INSTAT software (GraphPad, Inc., USA).

RESULTS

5E1 disrupted the crossing of early axons in E13

In E13 mouse embryos, the first axons had arrived at and crossed the midline of the chiasm [12-14]. The effects of anti-Shh antibody 5E1 on axonal growth at the chiasm were investigated in this early stage of optic pathway development by means of brain slice culture. In the control group without addition of the chemicals, the early axons were found to enter the ventral diencephalon and grow perpendicularly towards the midline. Some axons had already crossed the midline (Fig.1A and B). The crossing in these brain slices was quantified by measuring the fluorescence intensity of DiI-labeled axons within a defined region before and after axon crossing of the midline (see the placement of grids for this measurement in Fig.1A), and presented as the ratio of pixel intensities in the post- versus pre-midline regions of the chiasm. In the brain slices treated with normal mouse IgG (40 μg/ml), no obvious change was noted in the trajectories of retinal axons at the chiasm in comparison with the slices cultured without antibody addition, suggesting that normal mouse IgG did not influence the pathfinding of the axons at the chiasm (Fig.1C and 1D). After 5E1 treatment, the number of axons crossing the midline reduced in the brain slices obtained at E13 (Fig.1E and 1F). In this experiment, analyses showed that fewer axons crossed the midline after 5E1 treatment ($P<0.05$; Fig.1G).

5E1 did not affect axon midline crossing in E14 chiasm

At E14, more retinal axons joined the mouse retinofugal pathway (Fig.2A and B). Addition of normal

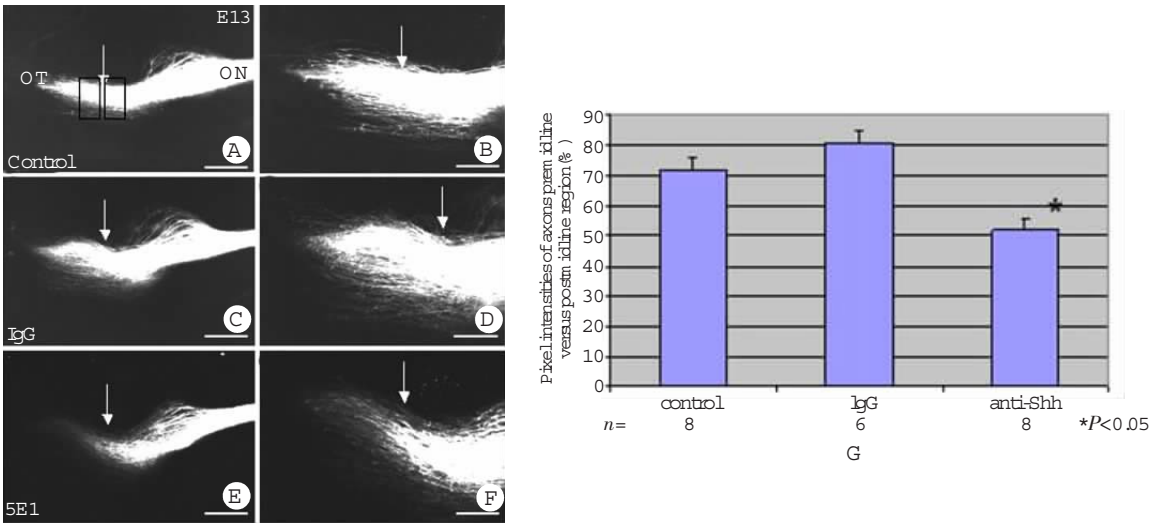

Fig.1 Confocal micrographs showing DiI-labeled retinal axons in whole-mount preparations of the E13 chiasm

The micrographs in the left column are images in lower magnification depicting the optic stalk, chiasm and optic tract, and those in the right column are in higher magnification showing the details (this arrangement is also adopted in the following similar figures). White arrows indicate the midlines. A and B: In control preparations cultured for 5 h in the absence of the chemicals, dye-filled axons in the optic nerve enter the chiasm first in a caudo-medial direction, and later turn and grow in the direction perpendicular to the midline. After crossing the midline, the retinal axons continue their path as a bundle of fibers and enter the optic tract (OT). C and D: Similar fiber trajectories in the chiasm are observed in the brain slices treated with normal mouse IgG (40 μg/ml). E and F: After perturbation of Shh with 5E1 (40 μg/ml), fewer axons cross the midline. G: Graph showing the results of different groups. White arrows indicate the midline. Scale bar=200 μm in A, C and E; Scale bar=100 μm in B, D and F. All images in this text are shown with the rostral side on the top.

mouse IgG did not affect the axon routing in the chiasm as compared with the control group (Fig.2C and D). We investigated the effects of Shh function perturbation on axon routing using anti-Shh antibody. Measurements of the pixel intensity in the post- and pre-midline regions indicated that no significant change of in axon crossing took place in the anti-Shh-treated group at E14 chiasm in comparison with the control groups (Fig.2E, F and G).

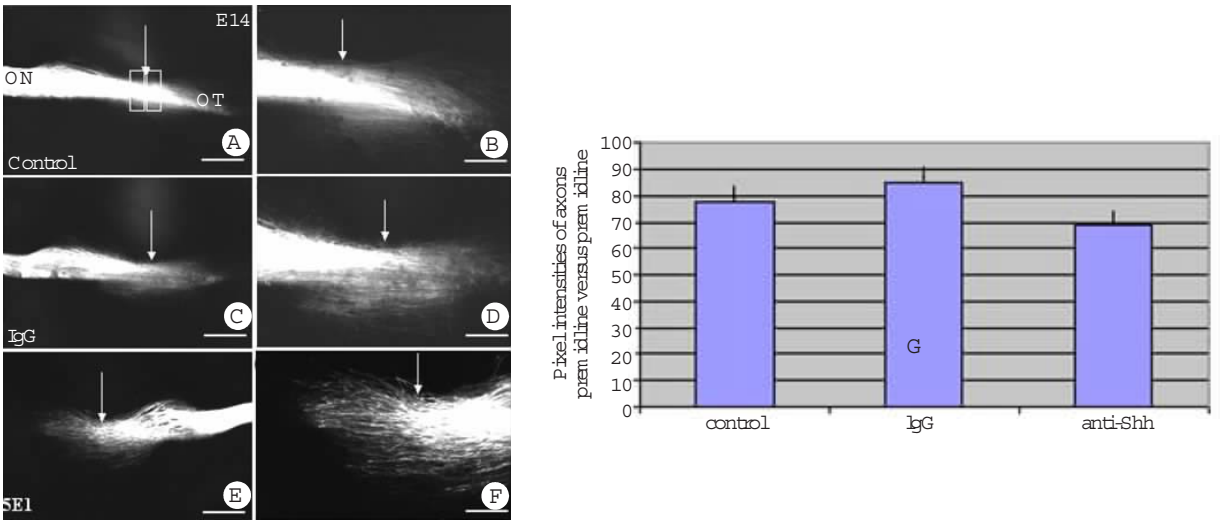

Fig.2 Confocal micrographs showing the DiI-labeled retinal axons in whole mount preparation of the E14 chiasm

A and B: In control preparations, many retinal axons have already crossed the midline and enter the optic tract (OT) in whole-mount preparations of the optic pathway, and some project into the opposite optic nerve. C and D: Similar fiber trajectories in the chiasm are observed in the brain slices treated with normal mouse IgG (40 μg/ml). E and F: After the brain slices are treated with 5E1, the axons in retinofugal pathway are reduced but no significant change is found in axon crossing. G: Graph showing the results of different groups. White arrows indicate the midline. Scale bar=200 μm in A, C and E; Scale bar=100 μm in B, D and F

5E1 influenced turning of uncrossed axons in the optic tract at E15

At E15, many axons had already passed the chiasm and entered the optic tract. In the control preparations, more numerous axons were observed to cross the midline and project into the contralateral optic tract (Fig.3A). An obvious uncrossed projection was also observed in this stage (Fig.3A and B). Fluorescence intensity of the dye-filled uncrossed axons was analyzed within a defined region (as shown in Fig.3B) in various groups. Addition of normal mouse IgG did not lead to obvious changes in the uncrossed axons (Fig.3C and D), whereas Shh antibody treatment resulted in increased number of uncrossed axons (Fig.3E, F and G, $P<0.05$).

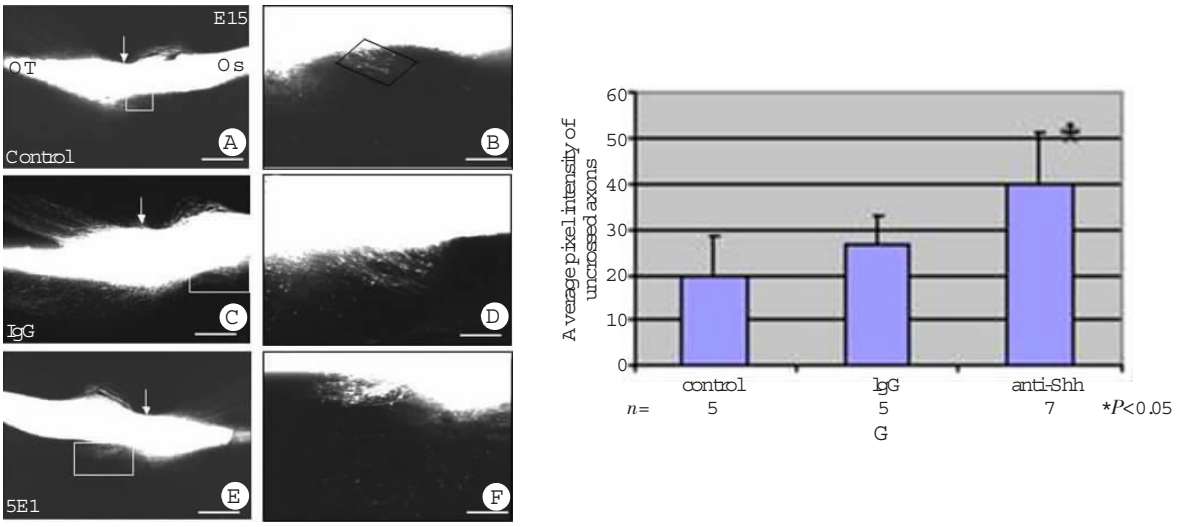

Fig.3 Confocal micrographs showing DiI-labeled retinal axons in whole-mount preparations of the E15 chiasm

A and B: In control preparations, many retinal axons have already crossed the midline and enter the optic tract (OT) in whole-mount preparations of the optic pathway, and some uncrossed axons emerge at E15. C and D: Similar fiber trajectories in the chiasm are observed in the brain slices treated with normal mouse IgG (40 μg/ml). E and F: After the brain slices are treated with 5E1, more uncrossed axons appear. G: Graph showing the effect of 5E1 on uncrossed axons. White arrows indicate midlines. Scale bar in A, 200 μm (A, C and E); in left pictures, 100 μm

DISCUSSION

In the present study, we investigated the effects of Shh perturbation with antibody on axon routing in brain slice preparation of the mouse retinofugal pathway. Shh antibody treatment resulted in a reduction in the number of the earliest retinal axons crossing the midline of chiasm at E13, but not at E14 or E15, suggesting a transient but important function of Shh signaling in axon decussation at the mouse optic chiasm. Anti-Shh also treatments caused increased number of uncrossed axons at E15 compared to the control group.

Shh regulated axon midline crossing in early chiasm development

The molecular mechanism regulating axon midline crossing at the chiasm was largely unknown. We had shown that disturbances of normal Shh function with anti-Shh produced a reduction of the early axons crossing the midline in the optic chiasm at E13, suggesting that Shh might promote axon decussation in early chiasm development. Previous studies had shown that Shh was a negative regulator of retinal axon growth in chicken. Ectopic Shh expression in the entire ventral forebrain resulted in growth retardation of retinal axons, and these axons barely reached the chiasm [15]. In Pax-2-mutant mouse, Shh overexpression in the hypothalamus was associated with projection of all retinal axons into the ipsilateral tract without crossing the midline [16]. Similar results were observed in NOI zebrafish mutants, which had alterations in pax2/5/8-like genes [3]. These observations suggested that Shh at the midline might suppress axon crossing. In this study, we observed fewer axons crossing the midline at E13 after blocking Shh, and this result seemed contradictory to descriptions of Shh functions in other studies. One explanation was that Shh played different roles in its interaction with various other molecules, For instance, Shh may serve as an

axonal chemoattractant in axon guidance at the midline when it worked with Netrin-1[17]. Also, different choices of the methods for treatment and experimental animal species may lead to the different results. In further development of the embryos, obvious effects of Shh antibody on axon crossing were no longer observed. In our previous study, we found abundant Shh and Ptc in the ventral diencephalon, mainly located close to the midline at E13. However, Shh expression was reduced at the midline with embryo development. These changes of Shh expression might explain, at least partially, why Shh only signaled the axon crossing at the midline at E13.

Shh controlled axon divergence in mouse chiasm

In addition to the effect on the axon crossing, Shh signaling disturbance also affected the development of the uncrossed projection in the mouse chiasm. At E15, the uncrossed axons from the ventral temporal retina emerged in normal embryos. In the present experiment, we observed that interference of Shh function by Shh antibody resulted in an increase in the uncrossed optic pathway, suggesting that functional blocking of Shh prompted more axons to turn away from the midline. We therefore hypothesized that Shh might serve as a chemoattractant factor for some axons and direct these axons to cross the midline in normal embryos. Once Shh function was blocked, these axons might halt at the midline, probably in the presence of other inhibitory molecules. Previous studies had shown that axon divergence in the mouse chiasm appeared to rely on the midline cues. We found previously that CD44 function inhibition led to a reduction in the uncrossed axons in the E15 chiasm [18], and chondroitin sulfate proteoglycans played an important role in the development of uncrossed pathway in the mouse[19]. These results demonstrated that axon divergence at the midline was controlled by multiple molecules.

Blocking Shh function with its antibody, as we found in this study, led more axons to halt before the midline at E13, which did not occur at E14. The axons subsequently turned away from the midline at E15 after antibody treatment. These results suggest that Shh executed different functions in various stages of embryo development. Hypothetically, Shh might play the role by interacting with other molecules in chiasm development. With embryo development, the distribution and function of this molecule undergo alterations, leading consequently to changes in the interaction between Shh and other molecules. This hypothesis, however, still needs to be tested with further experimental evidence.

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Shh对发育中小鼠视觉传导通路的影响

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摘要:目的 探察Shh对E13-E15小鼠胚胎中视觉传导通路发育的影响。**方法** E13至E15小鼠胚胎的眼至视束部分制备成脑厚片,置于含10%的小牛血清的DMEM/F12的培养液中,在37℃恒温滚动培养箱中培养5h。实验组中将Shh抗体加入培养液中。培养结束后,将脑厚片以4%多聚甲醛固定,将DiI颗粒置于视盘。7d后,在手术显微镜下,暴露被标记的视神经纤维,在激光扫描共聚焦纤维镜下观察。**结果** 用Shh抗体阻抑Shh的功能,可引起E13跨越中线的视神经纤维减少以及E15投射至同侧的视束的神经纤维的增多。**结论** 在视交叉形成的早期,Shh引导视神经纤维跨越中线。在视交叉形成的后期,Shh引导视神经转弯。

关键词: Shh;视交叉;发育
中图分类号:R329 **文献标识码:**A **文章编号:**1673-4254(2006)12-1679-06