Control of chick tectum territory along dorsoventral axis by Sonic hedgehog

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SUMMARY

Chick midbrain comprises two major components along the dorsoventral axis, the tectum and the tegmentum. The alar plate differentiates into the optic tectum, while the basal plate gives rise to the tegmentum. It is largely unknown how the differences between these two structures are molecularly controlled during the midbrain development. The secreted protein Sonic hedgehog (Shh) produced in the notochord and floor plate induces differentiation of ventral cell types of the central nervous system. To evaluate the role of Shh in the establishment of dorsoventral polarity in the developing midbrain, we have ectopically expressed Shh unilaterally in the brain vesicles including whole midbrain of E1.5 chick embryos in ovo. Ectopic Shh repressed normal growth of the tectum, producing dorsally enlarged tegmentum region. In addition, the expression of several genes crucial for tectum formation was strongly suppressed in the midbrain and isthmus. Markers for midbrain roof plate were inhibited, indicating that the roof plate was not fully generated. After E5, the tectum territory of Shh-transfected side was significantly reduced and was fused with that of untransfected side. Moreover, ectopic Shh induced a considerable number of SC1-positive motor neurons, overlapping markers such as HNF-3β (floor plate), Isl-1 (postmitotic motor neuron) and Lim1/2. Dopaminergic and serotonergic neurons were also generated in the dorsally extended region. These changes indicate that ectopic Shh changed the fate of the mesencephalic alar plate to that of the basal plate, suppressing the massive cell proliferation that normally occurs in the developing tectum. Taken together our results suggest that Shh signaling restricts the tectum territory by controlling the molecular cascade for tectum formation along dorsoventral axis and by regulating neuronal cell diversity in the ventral midbrain.

Key words: Midbrain, Tectum, Tegmentum, Dorsoventral, Sonic hedgehog, Chick

INTRODUCTION

Midbrain consists of two major components along dorsoventral (DV) axis, the tectum and the tegmentum. It has been shown in chick-quail chimera that the optic tectum derives exclusively from the midbrain alar plate (Senut and Alvarado-Mallart, 1987). After day 3 of incubation (E3), the alar plate undergoes rapid cell proliferation to form an enlarged tectum. In contrast, the basal plate gives rise to the midbrain tegmentum. Instead of massive cell proliferation, neural differentiation takes place in this ventral area to produce many nuclei related to motor function. Although these two structures undergo quite different developmental programs, it is largely unknown how the differences are molecularly controlled along the DV axis.

Many transcription factors and growth factors are involved in midbrain pattern formation along anteroposterior (AP) axis (reviewed by Lumsden and Krumlauf, 1996). The factors so far reported are: En-1/2 (Wurst et al., 1994; Millen et al., 1994), Pax-2/5 (Krauss et al., 1992; Urbánek et al., 1994; Braud et al., 1996; Funahashi et al., 1999; Okafuji et al., 1999), Fgf8 (Crossely et al., 1996) and Otx-2 (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). Misexpression of one of these factors can induce ectopic midbrain structure in the diencephalon or in the hindbrain (Araki and Nakamura, 1999; Okafuji et al., 1999; Funahashi et al., 1999; Crossely et al., 1996; Shamim et al., 1999; Broccoli et al., 1999; Katahira et al., 2000). In such cases, ectopic tectum emerges exclusively from the alar plate, i.e., DV polarity of the induced structure is conserved. This suggests that a distinct mechanism exists for determining regional identity of midbrain structure along DV axis that is independent of that of the AP axis.

Sonic hedgehog (Shh) is produced in the notochord and floor plate, and is implicated in inducing ventral cell types in CNS (Tanabe and Jessell, 1996). In neural explants, aminoterminal cleavage product of Shh protein (Shh-N) can induce both floor plate, Isl-1 (postmitotic motor neuron) and Lim1/2. Dopaminergic and serotonergic neurons were also generated in the dorsally extended region. These changes indicate that ectopic Shh altered the fate of the mesencephalic alar plate to that of the basal plate, suppressing the massive cell proliferation that normally occurs in the developing tectum. Taken together our results suggest that Shh signaling restricts the tectum territory by controlling the molecular cascade for tectum formation along dorsoventral axis and by regulating neuronal cell diversity in the ventral midbrain.

Key words: Midbrain, Tectum, Tegmentum, Dorsoventral, Sonic hedgehog, Chick
can induce floor plate cells in dorsal neural tube (Echelard et al., 1993; Roelink et al., 1994). However, it has not been elucidated how the ventral cell types other than floor plate cells are regulated in vivo along DV axis by Shh signal.

In this study, we focus on the role of Shh in DV pattern formation of the midbrain by Shh misexpression in the embryonic brain. We examined the expression of the tectum-related genes in Shh-transfected midbrain and followed tectal development. Drastic changes in morphology along DV axis led to an investigation of cell proliferation and neural differentiation, which normally occur

**Fig. 1.** In ovo electroporation into chick embryos. pEGFP-N1 alone (C,E,G) or pEGFP-N1 plus pMiwII-Shh (D,F,H) was transfected. (A) Dorsal view of brain vesicle. Plasmid DNA (red) was injected into the central canal at stage 10. (B) DNA was electroporated by pulses charged between a pair of electrodes placed besides of the head. (C,D) Efficiency of transfection was monitored in ovo by GFP expression 1 day after electroporation. Lateral view of the right side of the head is shown. Rostral is to the right. Introduced GFP was abundantly observed in diencephalon, midbrain and hindbrain. There is no significant difference in GFP localization between C and D. (E,F) GFP localization 2 days after electroporation. (E, right) Dorsal view shows GFP is exclusively localized in unilateral half of the brain vesicle. (E, left) Note GFP is dispersed in whole midbrain. (F) GFP is not observed in dorsal midbrain. Dorsal limit of GFP-positive region is indicated with an arrow. (G,H) Expression of Shh mRNA in the brain vesicles isolated from the embryos shown in E and F, respectively. Lateral views (left) and anterior views (right). (G) Endogenous Shh expression is detected in the normally developed brain vesicle. (H) Ectopic expression of Shh is introduced in lateral region. Note that the tectum is not properly developed in Shh-transfected side. Dorsal limit of GFP-positive region shown in F is colocalized with that of introduced Shh expression indicated with an arrow. Abbreviations: tel, telencephalon; di, diencephalon; mid, midbrain; hind, hindbrain. Bars, 500 μm.

**Fig. 2.** Expression of the tectum-related genes in Shh-transfected brains at E3.5. Localization of Shh mRNA is detected in red except in D. Expression of Pax-7 is shown in black (F) and other gene expression is stained dark blue (B-E, G-Q). The level of the isthmus is indicated with an arrow in A. Expressions of Shh-transfected side (right) were compared with untransfected side (left) after bisecting the brain at the midline (B-I). Lateral (left) and dorsal (right) views are shown in J-M. Expression of normal (J,L,N,P) and Shh-transfected embryos (K,M,O,Q) are shown. Abbreviations: tel, telencephalon; di, diencephalon; mid, midbrain; hind, hindbrain. Bar, 500 μm.
Control of tectum territory by Shh in the developing tectum and tegmentum, respectively. Moreover the localization of the ventral cell types was determined to analyze the effects of Shh in cell type specification. The results of these studies indicated that Shh is involved in the patterning of the tectum territory by controlling the molecular cascade for tectum formation along DV axis and by regulating the neuronal cell diversity in ventral midbrain.

MATERIALS AND METHODS

In ovo electroporation

In ovo electroporation method (Muramatsu et al., 1997) was modified to obtain efficient transfection into brain vesicles (Funahashi et al., 1999; Nakamura and Funahashi, 2000).

Fertile chick eggs were incubated at 38°C in a humidified atmosphere for 1.5 days. Embryos were staged according to Hamburger and Hamilton (1951). Chick Shh-coding region (Riddle et al., 1993) was inserted into pMiwII expression vector derived from pMiwSV, which carries chick β-actin promoter and RSV enhancer (Wakamatsu et al., 1997). This Shh construct (pMiwII-Shh) was mixed with GFP expression vector (pEGFP-N1; Clonetech) or lacZ expression vector (pMiwZ; Suemori et al., 1990) in a final concentration at 1 μg/ml. pEGFP-N1 or pMiwZ alone was used for the control experiment. For the experiment shown in Fig. 7, Shh-coding region was inserted into another expression vector (pIRES2-EGFP; Clonetech) containing the internal ribosomal entry site of the encephalomyocarditis virus, which permits Shh and GFP genes to be translated from a single bicistronic mRNA.

DNA solution of 0.1-0.2 μl in Tris-EDTA buffer was injected into the central canal with a micropipette at stage 10 as described in Fig. 1A. A pair of electrodes (0.5 mm diameter, 1.0 mm length and 4 mm distance between the electrodes) were put beside brain vesicles on the vitelline membrane as shown in Fig. 1B. A rectangular pulse of 25 V, 50 mseconds was charged 4 times in 1 second intervals by the electroporator (CUY21, Tokiwa Science, Fukuoka, Japan; T820 and OPTIMIZER™, BTX, San Diego, CA, USA). Efficiency of electroporation was monitored by GFP expression under a fluorescence dissection microscope (MZ FLIII, Leica).

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Expression of the transfected gene can be detected 2-3 hours after electroporation and strong expression was observed during the following 24-48 hours (Funahashi et al., 1999; Fig. 1). Transfection of GFP or lacZ construct alone did not cause any morphological abnormalities. DNA was transfected into the right side unless detailed otherwise. Details of the procedure are also described elsewhere (Nakamura and Funahashi, 2000).

In situ hybridization of whole mounts and sections

For whole-mount staining, embryos were fixed in 4% formaldehyde in PBS at 4°C for 2 hours to overnight and the brains were dissected out. They were rinsed twice with PBT (PBS-0.1% Tween 20) and dehydrated in a graded series of methanol in PBS to store in 100%
methanol at −20°C. For cryostat sections, fixed embryos were immersed in 20% sucrose in PBS overnight, and embedded in Tissue-Tek OCT compound. Cryosections of 10-15 μm were transferred to Vector Bond (Vector)-coated slides.

In situ hybridization on whole-mount and cryostat sections were performed as described by Stern (1998) and Henrique (1997), respectively. Production of RNA probes of Shh (Riddle et al., 1993), HNF-3β (Ruiz i Altaba et al., 1995), Pax-6 (Araki and Nakamura, 1999), Otx-2 (Katahira et al., 2000), En-2 (Itasaki and Nakamura, 1996), Pax-2 (Okafuji et al., 1999), Pax-5 (Funahashi et al., 1999), Fgf8, Wnt-1 (Sugiyama et al., 1998) and Mxi-1 (Suzuki et al., 1991) was described elsewhere. Digoxigenin- or FITC-labeled RNA probes were synthesized according to the manufacturer's protocol (Promega).

For double in situ hybridization on whole-mount specimens, alkaline phosphatase (ALP)-conjugated anti-FITC (Boeringer Manheim) was used for the first detection of Shh mRNA with FAST Red TR/Naphthol AS/MX (Sigma FAST™; Sigma Chemical Co., St Louis, MO, USA). ALP-conjugated anti-DIG (Boeringer) was used for the second detection with 4-nitroblue tetrazorium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). The ALP for the first staining was inactivated by incubating in 0.1 M glycine-HCl pH 2.2 for 15 minutes at room temperature. The untransfected side was regarded as a control after bisecting the embryo in the midline (Fig. 2B-I).

Whole-mount immunostaining and immunohistochemistry
En2 and Pax7 proteins on whole-mount specimens were detected with 4D9 (Patel et al., 1989) and anti-Pax7 monoclonal antibody (Kawakami et al., 1997; Developmental Studies Hybridoma Bank, Iowa University, IA, USA), respectively. After in situ hybridization, embryos were postfixed in 4% paraformaldehyde in PBS, washed in PGT (PBS-0.2% gelatine-0.25% Triton X-100) and incubated overnight at 4°C in half-diluted hybridoma supernatant with PGT containing 5% heat-inactivated goat serum. The embryos were washed with PGT several times for 1 hour, reacted with HRP-conjugated anti-mouse IgG (Jackson, West Grove, PA, USA) and colored with 3,3′-diaminobenzidine (DAB) substrate containing 0.03% CoCl2. Nerve fibers were detected with anti-neurofilament antibody 3A10 (DSHB, 1/2 supernatant) after treatment of fixed embryos with 0.25% trypsin in PBS.

For immunohistochemistry, cryosections were washed several times in PBS. For E7.5 and E5.5 embryos, endogenous peroxidase was inactivated in 0.3% H2O2-0.1% NaNO3 in PBS for 10 minutes. Monoclonal antibodies against SC1 (a gift from Dr Tanaka, 1/2 supernatant), Shh-N (SE1, DSHB, 1/2 supernatant), Isl-1 (40.2D6, DSHB, 1/4 supernatant), Lim1/2 (4F2, DSHB, 1/100) and tyrosine hydroxylase (Chemicon, 1/100) or antiserum against GFP (Molecular Probes, 1/200) and 5-HT (IncStar, 1/500) were used, followed by HRP- or Cy3-conjugated goat anti-mouse and HRP- or Alexa488-conjugated goat anti-rabbit secondary antibodies.

**BrdU incorporation**
BrdU was injected into the central canal of the midbrain of E3.5 or E5.5 transfected embryos. The embryos were fixed in 4% paraformaldehyde in PBS at 2 hours after BrdU injection. Incorporation of BrdU was subsequently detected using in situ cell proliferation kit, AP (Boehringer Mannheim) with Fast Red staining. For sections, immunohistochemistry with anti-Hu antibody (16A11, a gift from Dr Wakamatsu, 1/500 dilution) was performed on cryosections, followed by staining for β-gal and BrdU detection.

**Labeling of retinal fibers with HRP**
PBS solution containing 30% HRP (horseradish peroxidase) was injected into the right eye ball of E11.5 chick embryos with a glass micropipette. The next day embryos were killed and the brain was isolated in PBS. The HRP-positive retinal fibers were stained using p-cresol-diaminobenzidine method (Streit and Reubi, 1977). The reaction was stopped in 4% paraformaldehyde in PBS and the whole brain was stored in the same fixative.

**RESULTS**
***Transfection of ectopic Shh in ovo electroproporation***

Misexpression of Shh was achieved by in ovo electroporation performed on chick embryos at stage 10, with GFP as an indicator for efficiency of transfection. (Fig. 1A,B; see Materials and Methods).

In the control experiment, GFP-expression vector alone was introduced and GFP was detected abundantly in the unilateral side of the diencephalon, midbrain, isthmus and rostral hindbrain 1 day after electroporation (Fig. 1C). This expression was sustained on the 2nd day exclusively in the unilateral half of the brain (Fig. 1E). As the dorsal midbrain grows rapidly from E3 onward, the whole enlarging tectum of the transfected side was labeled with GFP.

When pMiwi-II-Shh was electroporated together with pEGFP-N1, no obvious difference in GFP expression from the control was discerned until E2.5 (1 day after electroporation; compare Fig. 1C,D). The difference became apparent in subsequent 24 hours; GFP was positive in the midbrain but did not cover the dorsal side of the midbrain (compare Fig. 1E,F). Since GFP was localized in all of the lateral half of the tectum in the control embryo, the result suggests that the GFP-positive domain of Shh-transfected embryo did not contribute to the tectum formation.

After isolation of the brain vesicle, whole-mount in situ hybridization with Shh probe was carried out. Introduction of GFP alone did not affect endogenous Shh expression or tectal development (Fig. 1G). In contrast, in Shh-transfected embryos, ectopic expression of Shh was detected in lateral region where GFP was co-localized (Fig. 1F,H). Moreover, tectal development was significantly repressed, resulting in a flat, undeveloped midbrain on the transfected side (Fig. 1H). No significant difference in the number of cell deaths was observed between the control and the experimental side after staining with Nile Blue Sulfate (data not shown).

**Shh suppresses the expression of the tectum-related genes**

As ectopic Shh affected tectal development, we have subsequently analyzed the expressions of genes related to the tectum formation in Shh-transfected embryos at E3.5.

The transcription factor HNF-3β required for the differentiation of floor plate cells is expressed in the midline of the neural plate after stage 5 (Ruiz i Altaba et al., 1995). At E3.5 HNF-3β expression is observed in the zona limitans intrathalamica (ZLI) and the floor plate posterior to the diencephalon (Fig. 2B left). On the transfected side, ectopic expression of HNF-3β was exclusively induced in the introduced Shh-positive region of diencephalon, midbrain and hindbrain (Fig. 2A,B right).

In diencephalon Pax-6 is expressed at the dorsal side complementary to Shh expression at ventral side (Fig. 2C left). Pax-6 expression totally disappeared in the ectopic Shh-positive region, making a distinct boundary between Shh- and Pax-6-positive domains as in ZLI (Fig. 2C right).

Otx-2 transcripts are restricted anterior to the isthmus in an
of Shh in our experiment did not affect the Otx-2 expression domain and its posterior limit was conserved (Fig. 2D right).

En-2 is also involved in midbrain and hindbrain development together with En-1 (Hanks et al., 1995). Gradient of En-2 expression is a determinant of AP polarity in the midbrain (Itasaki and Nakamura, 1996; Logan et al., 1996; Shigetani et al., 1997). AP gradient of En-2 expression in the midbrain and isthmus was diminished in Shh-transfected area (Fig. 2E). A similar pattern was observed after 4D9 antibody staining to detect En-2 protein localization (data not shown).

PAX-7 localization is detected in the pretectum, tectum and dorsal hindbrain (Fig. 2F left; Nomura et al., 1998). It was shown that PAX-7 expression in the dorsal midbrain is suppressed by the heterotopic grafting of the midbrain floor plate, producing tegmentum-like structure instead of the tectum. (Nomura et al., 1998). Ectopic Shh extinguished expression of PAX-7 in dorsal midbrain (Fig. 2F right).

Pax-2 and Pax-5 are involved in the organization of the tectum (Okafuji et al., 1999; Funahashi et al., 1999). Expression of both genes in the isthmus was inhibited by Shh transfection (Fig. 2G,H). Another gene of isthmic organizer, Fgf8, is localized in the narrow band at the midbrain-hindbrain boundary (Fig. 2I left; Crossley et al., 1996). Interestingly, in the transfected embryo, the band of Fgf8 expression shifted to the rhombic lip while it was almost extinguished in the isthmus (Fig. 2I right).

We have further examined the expression of Wnt-1 and Msx-1, whose transcripts are localized on the dorsal midline from posterior diencephalon to midbrain (Fig. 2J left; Holliday et al., 1995). In the Shh-transfected embryos, causal expression of both genes in the midbrain was totally absent with diminished expression rostral to it (Fig. 2K,M).

These changes caused by Shh transfection were already detected at E2.5 (data not shown), indicating that Shh can suppress the expression of genes involved in early tectal development. Moreover, the loss of dorsal midline markers suggests that the roof plate might not have been fully generated when the ectopic Shh was introduced in the lateral hemisphere.

We have finally examined the expression of Shh-target genes Ptc and Gli-1. Both genes are expressed in the neural tube adjacent to Shh-positive domain, which reflects the Shh signal reception (Marigo and Tabin, 1996; Goodrich et al., 1996). At midbrain level, Ptc is expressed in a longitudinal line complementary to Shh expression (Fig. 2N). In Shh-transfected embryos, Ptc expression was induced in the domain where Shh was introduced. Moreover, another line of ectopic Ptc expression appeared at the dorsal side complementary to the introduced Shh expression (Fig. 2O). In contrast, the Gli-1 transcripts that are observed in the ventral half of the midbrain in the normal embryos (Fig. 2P; Platt et al., 1997) were absent with ectopic Shh expression (Fig. 2Q).

**Shh inhibits the tectum formation and its subdivision into optic lobes**

To investigate the long-term effects of Shh transfection in the tectal development, the embryos were kept alive until E5.5. From E3 onward, dorsal midbrain continues rapid expansion to form the optic tectum. In the E5.5 embryo, the tectum has just started to subdivide into right and left hemispheres at the dorsal midline (Fig. 3A). In Shh-transfected embryos, reduction of the tectum size was apparent (Fig. 3B). Subdivision of the tectum into two hemispheres was not observed at the dorsal midline. Most importantly, the transfected tectum was significantly reduced in size, while ventral tegmentum region was dorsally enlarged.

Thereafter the tectum becomes completely separated into the right and the left hemispheres to form optic lobes, which later rotate between E7 and E12 so that the rostrocaudal axis of the tectum accords with the ventrodorsal axis of the body (Goldberg, 1974; Itasaki and Nakamura, 1992). At E12.5, the rotation is almost complete and the two optic lobes are situated dorso-laterally to the tegmentum (Fig. 3C). In contrast, the tectum of Shh-transfected embryos was not separated no matter on which side Shh was introduced. Rotation of the tectum was also incomplete; the fused tectum was located at the dorsal side of the tegmentum (Fig. 3D,E). Histologically, the fused tectum still had the laminar structure of the tectum (data not shown). At E18.5, total brain size was reduced to some extent in transfected embryos and the fused tectum was in a dorsal position (Fig. 3F,G).

To determine to what extent each compartment of the tectal primordium has contributed to form the fused tectum, retinal fibers were traced with HRP. In normal E12.5 embryos, retinal fibers are distributed throughout the contralateral tectum in an anteroposterior direction (Fig. 3C). When HRP was injected into the eye ipsilateral to the transplanted side, most of the fused tectum was stained, although the number of fibers was reduced (Fig. 3D). In contrast when HRP was injected into the eye contralateral to the transplanted side, a limited number of the retinal fibers were detected on the lateral portion of the fused tectum in the transplanted side (Fig. 3E). These staining patterns confirm that the fused tectum arises mostly from the untransfected side, while the transfected tectal anlagen has only poorly enlarged.

**Shh controls cell proliferation-differentiation in the midbrain**

Changes in gene expression and morphogenesis of Shh-introduced embryos suggest that ectopic Shh extended the tegmentum territory and thus reduced the tectum territory. It remains unclear how these changes take place at the cellular level. Since the transfected tectum is significantly reduced, it is possible that Shh-positive cells did not proliferate enough to form the tectal hemisphere. To test this idea, cell proliferation was examined by BrdU incorporation. This time, lacZ expression vector was co-transfected with Shh expression vector. BrdU was injected into the midbrain ventricule of at E3.5 or E5.5 embryos, which were subsequently fixed after 2 hours to detect β-galactosidase activity and BrdU incorporation.

In the control embryo in which lacZ alone was introduced, β-gal staining covered whole tectum and tegmentum at E5.5 (Fig. 4A), showing that the alar and basal plates where lacZ was transfected have differentiated into the tectum and the tegmentum, respectively. This is consistent with the result that GFP was detected throughout the unilateral midbrain at E3.5 when GFP alone was introduced (Fig. 4E). In contrast, β-gal staining was restricted to the tegmentum region of Shh-transfected embryos even though lacZ must also have been
introduced into the alar plate (Fig. 4B). While the tectum expands as a result of extensive cell proliferation revealed by active BrdU incorporation in tectal region, much less incorporation was detected in the lacZ-positive tegmentum area, making a clear boundary of the signal between them (Fig. 4B). It indicates that, after Shh transfection, not only the basal plate but also the alar plate ceased active cell proliferation and developed into the tegmentum without contributing to the tectum formation.

The assumption that Shh represses cell proliferation and enhances neural differentiation in the midbrain is confirmed histologically in E3.5 embryos. Double staining with anti-BrdU and anti-Hu antibody was carried out (Fig. 4C). It has been shown that anti-Hu antibody 16A11 recognizes HuC and HuD proteins, which are detected in postmitotic cells in the spinal cord (Marusich et al., 1994; Wakamatsu and Weston 1997). At the untransfected side, many BrdU-positive cells were found near the pial surface and Hu protein was localized at the level of oculomotor nucleus (Fig. 4C). On the transfected side where β-gal staining was positive, there were only a small number of BrdU-positive cells, but many Hu-positive cells were found.

**Shh regulates cell type differentiation along DV axis**

Differentiation of the tectal region and enhanced neural differentiation in Shh-transfected midbrain prompted us to examine the neuronal cell types along DV axis.

First, nerve fibers were stained with anti-neurofilament antibody 3A10 and observed on whole mounts. On the control side, fibers in subcutaneous positions such as trigeminal nerves are visible (Fig. 5A). On the experimental side, many ectopic nerves emerged from dorsolateral midbrain (Fig. 5B). Since it was suggested that tegmental region extended dorsally, we wondered if these fibers were motor fibers or not. Cryosections were stained with SC1 antibody, which specifically stains motor neurons and their fibers (Tanaka and Obata, 1984). On the control side, SC1-positive cells were located just lateral to the floor plate in the oculomotor nucleus (Fig. 5D). On the experimental side, the SC1-positive zone extended to the alar plate where Shh was introduced and ectopic nerves were also SC1 positive (Fig. 5C,D).

As ectopic Shh induced additional motor neurons in dorsal region, we next examined ventral cell markers in the normal and Shh-transfected midbrain. In the normal embryo, Shh mRNA is present in the floor plate colocalized with HNF-3β expression (Fig. 5E; data not shown). Shh protein detected with 5E1 antibody against Shh-N diffuses from Shh mRNA-positive domain to form a weak gradient laterally (Fig. 5F). In the
mantle layer laterally to the Shh-positive domain, oculomotor nuclei are stained with anti-Isl-1 antibody, which labels postmitotic motor neurons (Fig. 5G; Ericson et al., 1992). More laterally Lim1/2-positive neurons are localized (Fig. 5H). In the Shh-transfected midbrain, Shh mRNA was ectopically expressed in the ventricular layer and Shh-N was dispersed to the mantle layer of the corresponding region (Fig. 5I,J). Interestingly, both Isl-1- and Lim1/2-positive neurons appeared ectopically in a much more lateral domain than their normal localization (Fig. 5K,L). Both types of neurons were colocalized with Shh-N in a dorsally extended position. Thus, in spite of the normal cell diversity ordered along DV axis, ectopic Shh induced these ventral markers in overlapping regions.

We also examined the neuronal cell types in the later stages of development. It has been reported that Shh is required for differentiation of midbrain dopaminergic and hindbrain serotonergic neurons in rat neural explants (Ye et al., 1998). Shh can also induce dopaminergic neurons in rat and chick midbrain explants in vitro (Hynes et al., 1995; Wang et al., 1995). Localization of dopaminergic and serotonergic neurons was detected in normal and Shh-transfected midbrain-hindbrain using antibodies against tyrosine hydroxylase (TH) and serotonin (5-HT), respectively. In normal E7.5 midbrain, abundant TH immunoreactivity is detected in the area corresponding to substata nigra (Fig. 6A). In Shh-transfected midbrain, the distribution of TH-positive cells was extended dorsally compared with the control side (Fig. 6B). In E5.5 hindbrain, 5-HT neurons were present at the ventral midline adjacent to the floor plate, especially in the hindbrain rostral to the pontine flexure (Fig. 6C). In Shh-transfected hindbrain, the region of 5-HT-positive cells was dorsally extended (Fig. 6D).

Enlarged distribution of both early and late neuronal cell markers suggests that Shh regulates neuronal cell diversity along DV axis in the midbrain.
Shh promotes cell differentiation non-cell autonomously

As Shh promotes cell differentiation in ectopic sites, it is possible that differentiation proceeded cell autonomously in the cells into which Shh expression vector was transfected. We have tested this possibility by transfection with another expression vector containing IRES sequence between Shh and GFP gene, which enables both genes to be expressed in the same cells (see Materials and Methods).

GFP was detected in the cells of unilateral midbrain hemisphere (Fig. 7A-C). GFP-positive cells were found at all levels between the ventricule and the outer surface of the midbrain, indicating that both mitotic and postmitotic cells are included (Fig. 7D-F). While Shh-transfected cells correspond to the GFP-positive cells, Shh protein was localized ubiquitously in the transfected domain (Fig. 7A,D). Isl-1- and Lim1/2-positive cells were found in the same domain (Fig. 7B,C). Although some of Isl-1- and Lim1/2-positive cells coexpress GFP, many of them were GFP negative, suggesting that they were not the cells into which Shh-expression vector was transfected (Fig. 7E,F). These results demonstrate that the differentiation into ventral cell types by transfected Shh proceeded non-cell autonomously. Since ubiquitous expression of Shh mRNA is detected in the transfected domain (Figs 1H, 5C,I), it may be induced by Shh signal from the transfected cells to promote cell differentiation in surrounding cells.

DISCUSSION

In the developing midbrain, the alar plate and basal plate undergo distinct developmental processes to form the tectum and the tegmentum, respectively. From E3, midbrain alar plate cells proliferate rapidly, resulting in considerable expansion of the tectal wall with little change in its thickness. On the contrary, many nuclei related to motor function form in the tegmentum. To evaluate the role of Shh in the control of the differences along DV axis in the midbrain development, we have generated embryos in which Shh was transfected unilaterally in the brain vesicles. In this study, we have demonstrated that, in Shh-transfected midbrain: (1) the expression of the genes crucial for the tectum formation was repressed and the roof plate markers were inhibited, (2) the tectum territory was significantly reduced and replaced by the dorsally extended tegmentum, generating a fused tectum, (3) neural differentiation was promoted and cell proliferation was suppressed, and (4) ventral cell types were ectopically induced in the dorsal domain through cell non-autonomous process. All these results suggest that ectopic Shh inhibited the molecular cascade for tectum formation and thus the fate of the mesencephalic alar plate for the basal plate derivatives. Taken together, we conclude that Shh negatively regulates tectal development along the DV axis by repressing tectal gene expression, which results in the promotion of neural differentiation to restrict the tectum territory from the ventral side.

It is noteworthy that many of the tectum-related genes such as En-2, Pax-7, Pax-2, Pax-5 and Fgf8 were inhibited by ectopic Shh, whereas the Otx-2-positive domain was not affected. It is suggested that Otx-2 is involved in the early determination of midbrain region along AP axis (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). Moreover, Otx-2 interacts with Gbx-2 at the midbrain-hindbrain boundary to determine the posterior limit of the tectum territory (Broccoli et al., 1999; Katahira et al., 2000). Our observation suggests that Otx-2 is controlled independently of DV polarity directed by Shh. In fact, as the boundary of dopamine neurons and serotonin neurons along AP axis was not changed in Shh-transfected embryos (data not shown), Shh may not disturb the AP patterning of cell types in the tegmentum area.

It has previously been reported that the induction of dopaminergic neurons in the midbrain is dependent on Shh and Fgf8, and serotonergic neurons appear posterior to the site of Fgf8 expression (Ye et al., 1998). In our experiments, however, although the expression of Fgf8 was repressed in the isthmus and shifted to the rhombic lip at E3.5, both dopaminergic and serotonin neurons were well induced at the ectopic sites (Fig. 6B,D). We found that, in the early stages of Shh-transfected embryos, the shift of Fgf8 expression is detected at 24 hours but has not commenced at 12 hours after electroporation (data not shown). This suggests that early exposure to Fgf8 signal is crucial for the induction of dopaminergic neurons. Consistent with this, early blocking of FGF8 by the application of FGFR3-IgG in the rat midbrain explant can completely inhibit the development of dopaminergic neurons, while it partially succeeds or fails with later application (Ye et al., 1998).

At E5.5, Shh-transfected embryos had reduced tectum territory and showed dorsal extension of the tegmentum (Fig. 3B). This suggests that the alar plate changed its fate to develop into the tegmentum by Shh-misexpression. Therefore, it is possible that Shh-transfected alar plate transformed to basal plate and contacted the contralateral alar plate in the dorsal region. We suspect that this is a major reason why the roof plate structure was not formed, leading to fusion of the tectum at the dorsal side of the tegmentum (Fig. 3D,E,G). This idea is supported by the result of the surgical ablation experiment. When unilateral midbrain alar plate was removed at stage 9-11, the roof plate region was absent in the regenerated tectum, producing a fused tectum similar to the Shh-transfected embryo in our experiment (Cowan and Finger, 1982). Direct contact of the alar and basal plates may disturb the formation of the roof plate.

Dorsal extension of ventral cell types by Shh-misexpression is summarized in Fig. 8A. On the control side, as in the normal embryo, Shh mRNA is expressed in the floor plate region. At the level of midbrain, Shh-positive domain is broader than at the trunk level. It is suggested that Shh-N diffuses from floor plate to form a ventrodorsal gradient (Tanabe and Jessell, 1996), although it is not clear how far Shh-N can diffuse in the neural tissue. Isl-1-positive cells at the oculomotor nucleus are located at the lateral limit of Shh mRNA-positive region. Lim1/2-positive cells are localized more laterally to Isl-1 domain but they do not overlap. On the contrary, on the Shh-transfected side, misexpressed Shh mRNA is positive in the ventricular layer and Shh-N diffused to the mantle layer in the correspondent neural tissue. Isl-1- and Lim1/2-positive domains extend dorsally and coexist in the same area in the mantle layer, although the ventral limit of each cell type is maintained.

This is inconsistent with the results of culture of trunk-level neural plate. Lim1/2-positive cells can be generated from naive neural plate without exposure to Shh while high concentrations of Shh induced exclusively Isl-1/2-positive cells (Ericson et al., 1995; Ang et al., 1996). Moreover, Otx-2 interacts with Shh and is suggested as a key factor for the maintenance of dopaminergic neurons (Takahashi et al., 1997). Thus, it is possible that Otx-2 may play a role in the maintenance of Shh-dependent dopaminergic neurons in the midbrain.
In our experiment, introduced Shh was so high that HNF-3β- and Isl-1-positive cells were generated. Coexistence of Isl-1- and Lim1/2-positive domain in Shh-transfected embryos suggests that the mode of determination of these cell types at rostral level may not be the same as that at the trunk level. Consistent with this idea, in the diencephalon, Isl-1-positive neurons are generated in Shh-positive domain, and they coexpress Lim-1 (Ericson et al., 1995). In addition, MNR2, a transcription factor that directs Shh-mediated differenciation of somatic motor neurons, is not expressed in oculomotor and trochlear motor neurons (Tanabe et al., 1998). Therefore, although Shh acts as a common ventralizing signal, midbrain has distinct properties for ventral cell type determination from the trunk neural tube.

Before the Shh-transfection at stage 10, cell types may not be fixed in the basal plate, as both Isl-1- and Lim1/2-positive cells were induced by ectopic Shh. Thus Shh signaling after stage 10 may be critical for these ventral cell type determination. On the contrary, Lim1/2- and Isl-1-positive cells were never induced in oculomotor nucleus and floor plate, respectively. In these regions, the cell fate of motor neuron or floor plate progenitor was already fixed before the transfection. These observations support the idea that the ventral cell type determinations occur sequentially from ventral to dorsal direction as in the spinal cord (Ericson et al., 1996). In contrast, both TH- and 5-HT-positive cells, normally located at the most ventral position, were induced dorsally by ectopic Shh. Since the initial expressions of these cell markers are relatively late, after E3, it is possible both cell types should normally be determined much later.

Whereas anti-Shh antibody stains limited ventral region of CNS, the following suggest that Shh can diffuse over large distances. Functional blocking of Shh with antibody can prevent the induction of ventral cell types in neural explant (Ericson et al., 1996). The Shh target genes Ptc and Gli-1 are expressed in the neural tube adjacent to Shh-positive domain, which may reflect the Shh signal reception (Marigo and Tabin, 1996; Goodrich et al., 1996). In fact, at the midbrain level, Gli-1 transcripts are observed in the ventral half of the midbrain and Ptc is expressed in ventral longitudinal line complementary to Shh expression (Fig. 2N,P), suggesting Shh can act as a long-range signal within the basal plate.

Finally, Fig. 5B proposes a possible role for Shh in midbrain development. In the basal plate, Shh regulates ventral cell fate, which results in the promotion of neural differenciation to form the tegumentum. On the contrary, the alar plate does not receive Shh signaling and so continues massive cell proliferation to form the enlarging tectum. There may be some factors or gene network to support this proliferative property in the developing tectum.

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