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Ectopic bone morphogenetic proteins 5 and 4 in the chicken forebrain lead to cyclopia and holoprosencephaly

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ABSTRACT **Proper dorsal–ventral patterning in the developing central nervous system requires signals from both the dorsal and ventral portions of the neural tube. Data from multiple studies have demonstrated that bone morphogenetic proteins (BMPs) and Sonic hedgehog protein are secreted factors that regulate dorsal and ventral specification, respectively, within the caudal neural tube. In the developing rostral central nervous system Sonic hedgehog protein also participates in ventral regionalization; however, the roles of BMPs in the developing brain are less clear. We hypothesized that BMPs also play a role in dorsal specification of the vertebrate forebrain. To test our hypothesis we implanted beads soaked in recombinant BMP5 or BMP4 into the neural tube of the chicken forebrain. Experimental embryos showed a loss of the basal telencephalon that resulted in holoprosencephaly (a single cerebral hemisphere), cyclopia (a single midline eye), and loss of ventral midline structures. *In situ* hybridization using a panel of probes to genes expressed in the dorsal and ventral forebrain revealed the loss of ventral markers with the maintenance of dorsal markers. Furthermore, we found that the loss of the basal telencephalon was the result of excessive cell death and not a change in cell fates. These data provide evidence that BMP signaling participates in dorsal–ventral patterning of the developing brain *in vivo*, and disturbances in dorsal–ventral signaling result in specific malformations of the forebrain.**

Recent studies have begun elucidating the molecular basis of dorsal–ventral patterning along the neural tube (see ref. 1 for review). In the caudal neural tube signals emanating from the notochord and floor plate control ventral fates, whereas signals from the surface ectoderm and roof plate specify dorsal identity. The secreted protein Sonic hedgehog (Shh) is expressed in the notochord and floor plate and is both necessary and sufficient for specifying ventral identity (refs. 2–5 and reviewed in ref. 6).

Though the molecules responsible for specifying dorsal neural tube identity are incompletely defined, bone morphogenetic proteins (BMPs) are excellent candidates for dorsal specification. Several BMPs are first expressed in the nonneural ectoderm adjacent to the neural plate and later by the dorsal midline (roof plate) of the neural tube (ref. 6 and references therein). Furthermore, "gain-of-function" studies have shown that BMP4 is sufficient for dorsal patterning of the caudal neural tube (7, 8). Unfortunately, "loss-of-function" studies do not show a neural tube patterning defect for several candidate *BMP* genes in homozygous mutant mice (9–11). These data probably reflect a functional redundancy between the multiple *BMP* genes that are expressed in the dorsal central nervous system (12).

Both BMPs and Shh are expressed in the rostral neural tube and may play roles in dorsal–ventral patterning analogous to those in the caudal neural tube. As in the spinal cord, Shh participates in ventral patterning of the midbrain (13) and the forebrain (14–16). Shh is expressed in two domains of the ventral prosencephalon (2, 16, 17). Ectopic expression of Shh in the forebrain (13, 16, 18, 19) is sufficient to induce the expression of genes and proteins characteristic of markers of the hypothalamus and basal telencephalon (13–16). *Shh*–null mice lack ventral forebrain structures and have cyclopia and holoprosencephaly (20). Shh has also been shown to be necessary and sufficient for forebrain development in zebrafish (21), demonstrating the presence of evolutionary conservation of Shh signaling in the forebrain.

The pathway(s) involved in dorsal induction and patterning of the prosencephalon are less well defined than those for ventral induction. As in the developing spinal cord, BMPs have been implicated in dorsal forebrain patterning (1, 12, 18). BMPs 4, 6, and 7 are expressed in the mouse dorsal forebrain (12). In forebrain tissue explants, BMPs can induce genes that are expressed in the roof plate (12, 18) and repress genes normally expressed in the dorsal lateral forebrain (12).

To test the hypothesis that BMPs regulate dorsal forebrain development *in vivo*, we inserted beads containing recombinant BMP5 and BMP4 (rBMP5 and rBMP4) into the developing chicken prosencephalon. This resulted in the loss of the ventral forebrain secondary to massive cell death localized to that region. Furthermore, *in situ* hybridization with a panel of probes to developmentally regulated genes in the forebrain indicates that dorsal–ventral patterning was disrupted. Finally, the chicken embryos showed a remarkable phenotype that included holoprosencephaly (a single cerebral hemisphere), a single midline eye (cyclopia), and associated craniofacial defects.

MATERIALS AND METHODS

Chicken Embryo Incubation and Bead Implantation Surgery. Specific-pathogen-free eggs (SPAFAS, Preston, CT) were incubated in a humidified rotating incubator until stages 9–12 (22). The eggs were windowed, as previously described (23), the vitelline membrane was cut open, and a small incision was made in the dorsal rhombencephalon by using a 30 gauge needle. An Affi-Gel Blue gel bead (Bio-Rad), approximately 100–300 μm in diameter, was passed into the lumen of the

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Abbreviations: BMP, bone morphogenetic protein; rBMP, recombinant BMP; Shh, Sonic hedgehog; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; E*n*, embryonic day *n*.

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neural tube by using no. 55 forceps and was manipulated into the prosencephalon. rBMP5 was used at 324 μg/ml, 3.24 μg/ml, and 32.4 ng/ml, while rBMP4 was used at 514 μg/ml, 5.14 μg/ml, and 51.4 ng/ml. The two higher doses gave similar results, whereas the lowest dose produced no observable attenuation in the phenotype (data not shown). rBMP7 was used at 812 μg/ml, 81.2 μg/ml, 8.12 μg/ml, 812 ng/ml, and 81.2 ng/ml with some variation in the phenotype induced, depending on the dose (A.B. and J.A.G., unpublished data). Although these data indicate a dose response to BMPs, the actual dose delivered to the embryo from the bead remains unknown. All of the results depicted herein were obtained by using embryos treated with rBMP5 or rBMP4 at either of the higher two doses. Our results did not differ with either of the higher doses. The bead was previously soaked in rBMP4, rBMP5, rBMP7 (Genetics Institute, Cambridge, MA), Noggin protein (a gift from R. Harland, Univ. of California at Berkeley), bovine albumin (Sigma), or 0.1 M pH 7.4 sodium phosphate buffer alone. Noggin is a secreted protein with a known function of inhibiting BMP signaling (24). After implantation, the egg was sealed with transparent packaging tape (3M) and returned to a nonrotating humidified incubator. The eggs were allowed to incubate for 1–16 days, after which time the embryos were harvested, fixed in 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4) overnight, washed three times in 0.1 M phosphate buffer with 0.1% Tween 20 (PBT), and then dehydrated through graded washes into 100% methanol (MeOH). Once in MeOH the embryos were stored at −20°C for up to 6 months.

***In Situ* Hybridization.** Whole-mount *in situ* hybridization, including riboprobe production, was performed according to previously published protocols (25). All hybridizations were performed overnight at 70°C in a rotating water bath (Bellco). Embryos were photographed on a Leica MZ12 dissecting microscope with Kodak 160T film. Kodachrome slides were scanned into Adobe PhotoShop on a Macintosh Power PC 8500 by using a Kodak RFS 2035 slide scanner.

Histology. Embryos to be sectioned, either before or after *in situ* hybridization, were prepared by transferring the embryos to 30% sucrose in PBT. The embryos were incubated overnight in 30% sucrose at 4°C, frozen in OCT on dry ice, and sectioned on a Reichert–Jung 2800 cryostat at either 10 μm or 30 μm. Embryos were oriented to be sectioned in the coronal, horizontal, or sagittal plane. Sections of embryos that had undergone *in situ* hybridization were directly coverslipped in glycerol, while untreated embryos were stained prior to coverslipping with eosin and hematoxylin according to standard protocols. Slides were viewed with a Zeiss Axioplan microscope.

Assays for Programmed Cell Death [Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling (TUNEL)] and Cell Proliferation (BrdUrd Incorporation). TUNEL assay with 4′,6-diamidino-2-phenylindole (DAPI) counterstain was performed on 20-μm coronal cryosections as previously described (26). BrdUrd incorporation into dividing cell nuclei was accomplished by injecting approximately 50 μl of 10 μM BrdUrd (dissolved in H_2O) under the amnion. The embryos were harvested 1 hr after BrdUrd injection, fixed, and sectioned as described for the TUNEL assay, and immunohistochemistry for BrdUrd was performed according to previously published protocols (27).

RESULTS

Forebrain Expression of BMPs Is Predominantly Localized to the Roof Region and the Eye. On the basis of the role of BMPs in dorsal specification of the spinal cord and the expression of *BMP2*, *-4, -6,* and *-7* in the dorsal mouse forebrain (12), we investigated the expression patterns of *BMP* family members in the developing chicken forebrain. The expression of *BMP2*, *-4, -5*, and *-7* was studied in chicken embryos from stage 9 (36-hr gestation) to 30 [embryonic day 6 (E6)] by whole-mount *in situ* hybridization. As shown in Fig. 1, *BMP5* is expressed in a thin stripe along the dorsal midline of the forebrain (telencephalon and diencephalon) beginning at stage 10 and persisting through stage 30, the latest time point examined.

BMP7 expression was also detected in the dorsal midline of the telencephalon and in the ventral midline of the diencephalon and mesencephalon (data not shown), a pattern closely resembling that in mouse embryos (12). The expression of *BMP7* in the dorsal telencephalon was not detectable until stage 20, whereas its expression in the ventral diencephalon was present from stage 17.

BMP4 and *-7* were expressed in reciprocal gradients in the developing eye. *BMP4* was expressed at highest levels dorsally, whereas *BMP7* showed higher expression ventrally (data not shown). We did not detect *BMP4* or *BMP2* expression when we used whole-mount *in situ* hybridization between stages 10 and 20 in the dorsal forebrain (data not shown) or in the lateral forebrain through E5. The expression of *BMP2*, *-4, -5,* and *-7* was observed in the embryo outside the central nervous system as described by other investigators (28–32).

Introduction of rBMP5 or rBMP4 Protein into the Forebrain Results in Holoprosencephaly, Cyclopia, and Associated Craniofacial Defects. To test whether BMPs are sufficient to affect dorsal–ventral patterning in the forebrain we implanted beads soaked in rBMPs into the rostral neural tube. Eighty-three percent (45/54) of embryos exposed to rBMP5 and 94% (96/102) of embryos exposed to rBMP4 showed various degrees of cyclopia (a single midline eye), holoprosencephaly (a single forebrain vesicle), and anomalies of craniofacial development (Fig. 2 *B*, *C*, *F*, and *G*) when harvested on various days from E3 to E17. In contrast, beads soaked in BMP5 or BMP4 placed in more caudal regions of the neural tube (mesencephalon or rhombencephalon regions) gave a distinct phenotype (J.A.G. and K.A.M., unpublished data) and never showed cyclopia or holoprosencephaly. Embryos exposed to rBMP7 protein had a distinct phenotype with two small eyes, no or only rudimentary forebrain structures, and an enlarged midbrain (tectum) (A.B. and J.A.G., unpublished data). Embryos implanted with beads soaked in PBS, bovine albumin, or Noggin resulted in no altered or unique phenotype, and embryos implanted with PBS-soaked beads were used as controls.

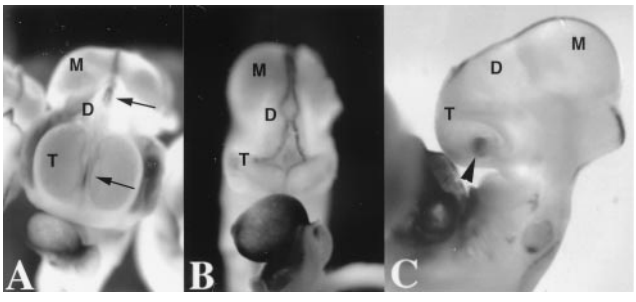

FIG. 1. Whole-mount *in situ* hybridization with a probe to chicken *BMP5*. (*A*) Staining of E4.5 embryos (implanted with a bead soaked in buffer alone) shows the normal expression pattern of BMP5 along the dorsal aspect of the brain, including the diencephalon (upper arrow) and the telencephalon (lower arrow). The expression was through the entire wall of the neural tube (data not shown). No expression was seen in the ventral forebrain (data not shown). Expression persists in embryos exposed to rBMP4 protein (*B* and *C*) and may actually be slightly increased (arrowhead points to implanted bead in *C*; see *Materials and Methods*), with staining extending laterally in the telencephalon (*B*). T, telencephalon; D, diencephalon; M, mesencephalon (tectum).

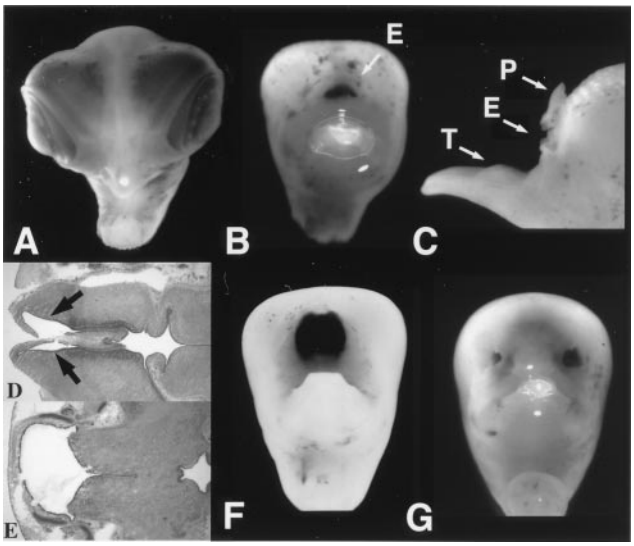

FIG. 2. Brain phenotype and spectrum of cyclopia. An E9 control chicken (*A*) is compared with E9 chicken embryos exposed to rBMP5 (*B, C*, and *G*) or rBMP4 (*F*). Variation from a single midline eye (black region) to small slightly separated eyes was seen in embryos exposed to BMP5 and BMP4. (*C*) Lateral view of the embryo in *B* highlights the position of the eye (E) relative to the superiorly placed proboscis (nasal anlagen, P) and virtually absent upper beak, resulting in the easy visualization of the tongue (T). The control E9 chicken brain (*D*) sectioned in the horizontal plane shows a single third ventricle (right) and two lateral ventricles (arrows, left). A horizontal section from an E9 cyclopic chicken shows a single third ventricle (right) and a single holosphere instead of two lateral ventricles (left). (*D* and *E*, ×25.)

Histologic sections of embryos exposed to BMPs showed a dramatic effect on forebrain and craniofacial development. Hematoxylin- and eosin-stained sections of coronally, horizontally, and sagittally sectioned E16 and E17 BMP-exposed embryos revealed the presence of a single forebrain ventricle instead of the two telencephalic lateral ventricles (compare Fig. 2 *D* and *E*). The third ventricle was normally positioned. The forebrains were very immature, showing a decreased thickness of the neuroepithelium and virtually no mantle zone. While the histological and morphological characteristics of basal telencephalon were absent, dorsal telencephalon structures, such as the Wulst, were identifiable (data not shown). The single midline eye was predominately composed of pigmented epithelium and little neural retina (data not shown), similar to the eye in the Shh homozygous mutant mouse (20). The cyclopic eye was located in the nasal sinuses. Additional craniofacial findings include a severely hypoplastic maxilla (Fig. 2*C*), midline clefts of the palate and maxilla, and a proboscis (a rudimentary nasal anlagen located above the single midline eye, seen in four of the mutant chicken embryos exposed to rBMP5, Fig. 2*C*).

Dorsal–ventral Patterning Is Disrupted in Mutant Embryos. To understand BMP effects at the molecular level, we performed *in situ* hybridization with a panel of probes that recognize dorsal-specific or ventral-specific forebrain gene expression. During the early stages of nervous system development, many genes conferring apparent positional information within the nervous system are expressed in spatially and temporally restricted patterns. To determine whether the anatomical alterations resulting from ectopically administered rBMPs were related to the altered expression of these genes, the expression patterns of several genes were assayed by whole-mount *in situ* hybridization. We first studied several members of the *Wnt* family that are specifically expressed in the dorsal prosencephalon, predominately in the diencephalon (33). Embryos exposed to beads soaked in BMP4 and BMP5 did not show alterations in the expression of *Wnt-1*, *Wnt-3a*, *Wnt-4*, *Wnt-5b*, and *Wnt-7a* in E3, E4, and E5 embryos (15 of 26 embryos), or the domain of expression was slightly increased (11 of 26 embryos) compared with control embryos (Figs. 3 and 4 for *Wnt-1* and *Wnt-4*; data not shown for the other *Wnt* genes). Furthermore, BMP4 and BMP5 induced *BMP5* expression in the dorsal forebrain (Fig. 1 *B* and *C*), but *BMP4* expression was never seen in the dorsal forebrain.

Since *Wnt-4* expression was slightly expanded in some embryos treated with BMP5 or BMP4, we tested the possibility that BMP5 could induce *Wnt-4* expression. To remove the source of endogenous BMP5, the dorsal forebrain of stage 9 or 10 embryos was extirpated with an insect pin. A grid reticule was placed in the ocular of the dissecting microscope and used to control for the surface area removed from each embryo. A BMP5-soaked bead was placed in the remaining telencephalon. Control embryos either were treated with a bead soaked in PBS or were left untreated. The embryos were harvested 24 hr after the experimental manipulations, and *in situ* hybridization with a *Wnt-4* probe was performed. Embryos that had their dorsal forebrain removed showed a slightly small head, two normal size eyes, and closed neural tubes. One of 8 embryos in which the dorsal forebrain was removed and no bead was implanted showed some expression of *Wnt-4* (Fig. 5*A*), and 2 of 12 embryos implanted with a PBS bead showed *Wnt-4* expression. In contrast, all 8 of 8 embryos that had the dorsal forebrain removed followed by the application of BMP5-soaked bead showed some expression of *Wnt-4* (Fig. 5*B*), indicating that BMP5 may regulate *Wnt-4* expression in the dorsal forebrain.

We next studied the effect of the rBMP4 beads on expression of *Pax-6*, a homeobox gene whose expression is restricted to dorsal tissues in the telencephalon and diencephalon. *Pax-6* was expressed in all embryos studied ($n = 7$, Fig. 3), although the small size of the brains in treated embryos resulted in a proportionately reduced domain of *Pax-6* expression. *Otx-1* and *Otx-2* are also expressed in the forebrain, both dorsally and ventrally (34). As with *Pax-6*, *Otx-1* expression was preserved, although again, the domain of expression was reduced in area (data not shown). These data indicate that although the size of the forebrain is reduced after treatment of embryos with rBMP, dorsal structures are not lost, and in fact, some dorsal tissues may even have expanded.

We next studied four genes expressed in the basal forebrain, *Pax-2*, *Nkx-2.1*, *Dlx-2*, and *Shh. Pax-2* is normally expressed in the ventral retina and ventral forebrain (Fig. 3; refs. 20 and 35) and was completely lost in the embryos implanted with rBMP5 or rBMP4 protein-soaked beads ($n = 12$, Fig. 3). *Nkx-2.1* is a homeobox-containing gene that is expressed in two forebrain domains: the ventral diencephalon and the basal telencephalon (preoptic area, medial ganglionic eminence, and part of the septum) (17). *Nkx-2.1* expression in the basal telencephalon was markedly reduced by the rBMP5 treatments ($n = 8$, Fig. 3), whereas the ventral diencephalic expression was moderately reduced. *Dlx-2* expression, also a genetic marker of the basal forebrain, was found to be down-regulated after exposure of the embryo to BMP5 ($n = 6$, data not shown). The expression of *Shh* in the rostral chicken embryo is dynamic, first in the prechordal plate, and later in the basal forebrain after approximately stage 20 (17). Embryos treated with BMP5 or BMP4 showed normal expression of *Shh* in the prechordal plate, but expression in the basal telencephalon was never seen ($n = 10$, Fig. 3). In summary, the expression patterns of genes normally expressed in the dorsal or ventral forebrain indicate a loss of ventral molecular markers with preservation of dorsal molecular markers.

Ectopic BMP5 Leads to Cell Death in the Ventral Forebrain. The genetic and morphologic alterations induced by rBMP5 and rBMP4 could result from tissue respecification, selective cell death, lack of proliferation, or a combination of these. The TUNEL assay was used to evaluate whether rBMP5 and

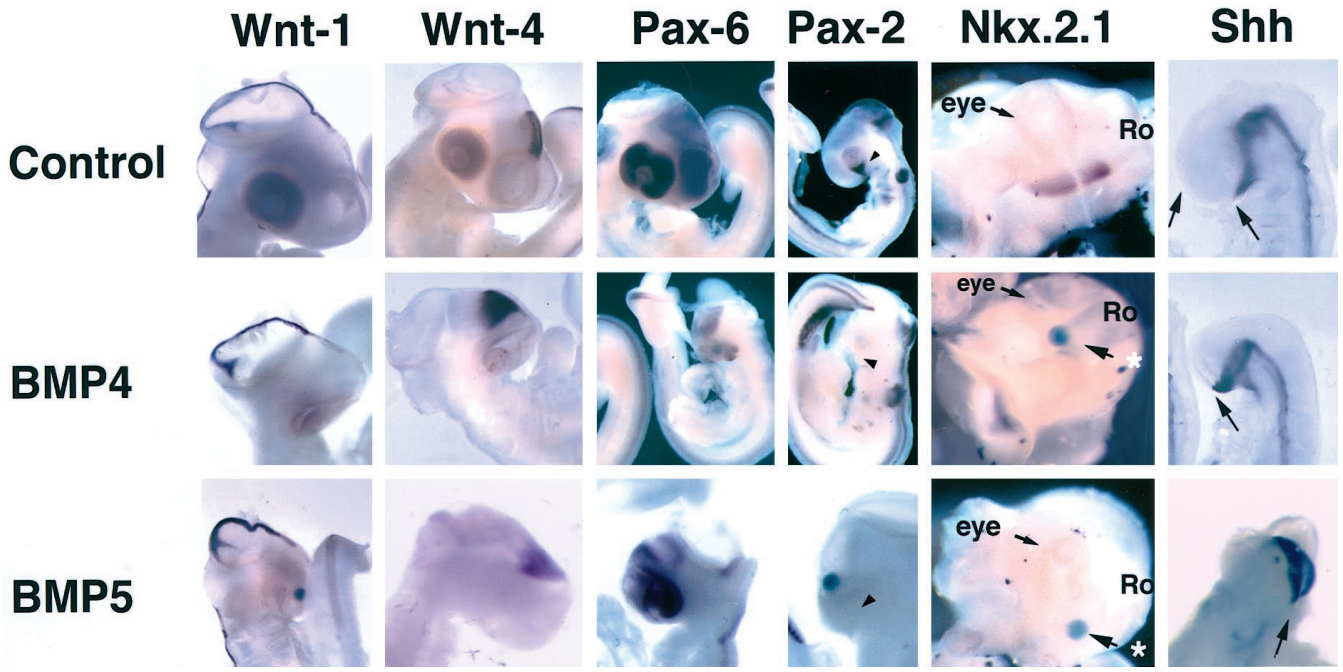

FIG. 3. *In situ* hybridization for dorsal and ventral markers in the forebrain of E4 embryos. *Wnt-1* is expressed over the dorsal midline of the mesencephalon and diencephalon. Expression of *Wnt-1* is normal or increased in embryos exposed to rBMP4 or rBMP5. *Wnt-4* expression in the dorsal diencephalon expands ventrally in rBMP4-treated embryos compared with the control embryo. *Pax-6*, normally expressed in the dorsal telencephalon, is still expressed in the embryo exposed to rBMP4 and rBMP5, although the domain of expression is reduced in embryos exposed to rBMPs. In contrast to the dorsally expressed genes above, *Pax-2* expression is completely absent in the ventral eye and ventral forebrain (arrowheads point to same region in each embryo). *Nkx-2.1* expression normally seen in the basal forebrain is absent in embryos treated with rBMP4 and rBMP5. The embryonic head has been isolated and photographed from the ventral side. The eye (labeled) can be seen in each image and the rostral (Ro) end of the head is to the right in each image (* denotes the implanted bead). *Shh* expression in the ventral forebrain ends just rostral to the optic chiasm. The morphologically distinct basal telencephalon (arrows) is present rostral to the *Shh* expression domain. Embryos implanted with beads soaked in rBMP4 or rBMP5 show no morphologically identifiable basal telencephalon; the *Shh* expression domain comes up to the rostral limit of the ventral brain. All embryos were sectioned to confirm the whole-mount *in situ* hybridization staining patterns.

rBMP4 proteins in embryos treated at E1.5 induced cell death. Extensive cell death was seen in the basal telencephalon on E3 (Fig. 6*B* and *D*) and E4, but not at E5. The ventral diencephalon (data not shown) and dorsal telencephalon (Fig. 6C) did not show increased cell death. Nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) showed similar results, as fragmented nuclei were seen in the basal telencephalon (Fig. 6*F*) but not in the dorsal telencephalon or the diencephalon (Fig. 6*E*).

We considered the possibility that BMP5 and BMP4 initiated cell death by means of the Msx-2 transcription factor, as BMP4 and BMP2 initiate cell death in neural crest cells by the up-regulation of Msx-2 (36–38). However, expression of *Msx*-2 in the basal telencephalon was not detected in brains exposed to rBMP5 or rBMP4 (data not shown). So, the cell death pathway being induced by these BMPs must be independent of Msx-2. An alternative explanation is that the cell death we observed does not reflect a BMP-initiated cell death pathway, but rather results from cells receiving conflicting developmental signals specifying dorsal fate (BMP5) and ventral fate (Shh), as has been postulated for the pathogenesis of cell death in other paradigms (39).

To address the possibility that diminished cell proliferation contributed to the alterations seen in BMP5- and BMP4-treated embryos, we injected BrdUrd into the amniotic fluid 1 hr before harvesting embryos. Cells that incorporate BrdUrd during S phase of the cell cycle were labeled with an anti-BrdUrd antibody (Boehringer Mannheim). Adjacent sections

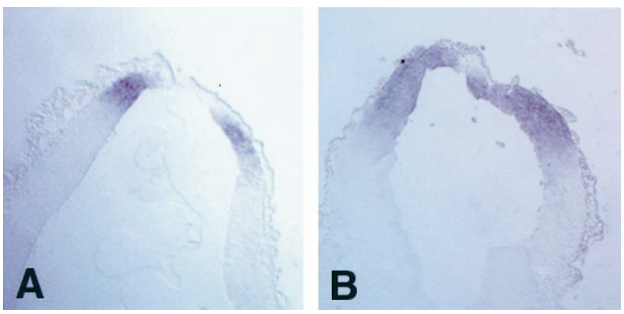

FIG. 4. Coronal sections of the forebrain showing the expression of *Wnt-4* by *in situ* hybridization. (*A*) Expression in a control embryo on E4.5. (*B*) Expression in an embryo on E4.5 after implanting a bead soaked in rBMP5. Note the expanded domain of expression after exposure to BMP5. (×200.)

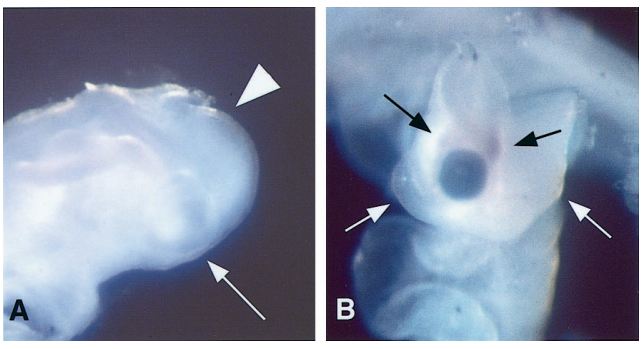

FIG. 5. Expression of *Wnt-4* mRNA after dorsal forebrain extirpation. (*A*) No expression of *Wnt-4* is seen after dorsal forebrain removal alone at stage 9 (lateral view; arrow, eye; arrowhead, site where *Wnt-4* should be expressed). (*B*) *Wnt-4* expression is seen after implantation of bead soaked in rBMP5 (blue bead seen in neural tube; white arrows, eyes; black arrows, expression of *Wnt-4*).

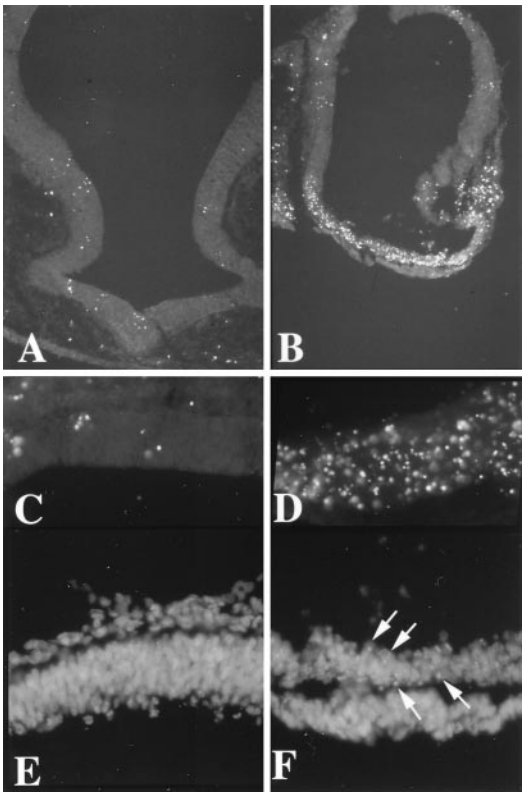

FIG. 6. TUNEL assay for cell death. Relatively few cells were labeled by TUNEL in unoperated embryos or embryos implanted with a bead soaked in buffer (*A*, coronal section of forebrain, dorsal is up and ventral down; ×100). In contrast, a selective and extensive labeling of cells, indicating cell death, was found in the ventral telencephalon after exposure to rBMP5 or rBMP4 proteins (*B*, coronal section of E3 telencephalon, dorsal is up and ventral down, a bead soaked in rBMP4 protein was implanted at stage 11; ×100). At higher power, the difference between dorsal (*C*) and ventral (*D*) telencephalon is striking (both *C* and *D* from an embryo implanted at stage 9 with a bead soaked in rBMP5 protein and harvested on E4; ×400). 4′,6-Diamidino-2-phenylindole (DAPI) nuclear stain confirmed the TUNEL findings. (*E*) Field of dorsal neural tube of embryo exposed to rBMP4 protein shows all intact nuclei. (×400.) (*F*) A field from the ventral telencephalon of the same section as *E* shows numerous condensed nuclei (arrows) characteristic of cells undergoing apoptosis. (×400.)

were stained with the TUNEL method to determine the location of cell death. The number of proliferating cells was dramatically decreased specifically in the same region of the basal forebrain that contained TUNEL-positive cells on E3 (data not shown). These data are consistent with the possibility that BMP5 and BMP4 expression leads to cell death in progenitor cells specifically in the ventral forebrain.

DISCUSSION

Our results indicate that the role of the BMPs in dorsal–ventral patterning of the rostral neural tube is similar to their role in the development of the caudal neural tube. BMP5 is spatially and temporally expressed in a domain consistent with its role in dorsal forebrain patterning in the chicken. By ectopically administering rBMP5 and rBMP4 protein in the prosencephalon we show that molecular markers of the ventral telencephalon are reduced or absent, whereas dorsal telencephalon markers are preserved. These data are similar to those from experiments in which the addition of exogenous BMP4 is sufficient to induce dorsal markers in the caudal neural tube after ablation of the dorsal region (7, 8). Similarly, beads soaked in BMP5 and placed in the lumen of the prosencephalon cause an up-regulation of at least one dorsally expressed gene, *Wnt-4*. BMP5 and BMP4 also appear to up-regulate BMP5 expression in the dorsal forebrain. These data suggest that BMPs are sufficient to specify at least some aspects of dorsal forebrain development.

Our data from an *in vivo* system also complement the *in vitro* data indicating BMPs participate in dorsal specification of the rostral neural tube. *In vitro* explant studies from the developing mouse lateral telencephalon have shown that BMP4 and BMP2 can induce the dorsal molecular marker *Msx-1* (12, 18) and suppress the lateral marker *Bf-1* (12) of the telencephalon. Using an *in vivo* model, we have determined that BMPs not only act locally on dorsal markers but have broader effects on dorsal–ventral patterning of the rostral forebrain. Furthermore, our data in chicken embryos closely parallel expression data in mice (12), suggesting that the BMP pathway is a mechanism of brain patterning conserved between avian species and mammals.

Our data indicate that ectopic expression of BMP5 or BMP4 resulted in the loss of ventral markers. The down-regulation of *Pax-2* (*Paxb* in zebrafish) was particularly interesting because this gene is also down-regulated in the zebrafish mutant *cyclops* (21), in which *Shh* expression is absent from the prechordal plate. In contrast, in the chicken embryos exposed to BMP5 and BMP4, *Pax-2* expression is also absent, but *Shh* expression is maintained in the prechordal plate. On the basis of the zebrafish data, the down-regulation of *Paxb* expression was hypothesized to be a failure of *Shh* to initiate or maintain *Paxb* expression (21). Given that *Shh* expression is maintained in our cyclopic chicken embryos, we would hypothesize that the BMP signaling pathway and the Shh signaling pathway either interact in the regulation of *Pax*-2 expression or function in parallel pathways. Alternatively, the cells normally responding to *Shh* (by expressing *Pax-2*) are dying (see below). Mutations in the human *PAX2* gene have been identified, and the phenotype includes ventral eye defects (40). However, the absence of the many other features found in the rBMP-treated chickens indicates that the loss of *Pax-2* expression is not responsible for the full phenotype we observe in the chicken embryos exposed to BMPs. Furthermore, mice homozygous for a *Pax*2 mutation show defects at the midbrain-hindbrain junction and in the ventral eye (41), but they do not have a forebrain or facial phenotype similar to that of the chickens with ectopic rBMP5 or rBMP4.

It has been previously demonstrated that mutations in *Shh* can lead to a holoprosencephaly phenotype. Mice homozygous for a *Shh* mutant gene have cyclopia and brain anomalies that resemble holoprosencephaly (20). Recently zebrafish nodal-related genes (42, 43) were found to be mutated in the zebrafish *cyclops* mutant. *Shh* is not expressed in the prechordal plate of these embryos, but injection of *znr-1* mRNA into *cyclops* mutant embryos restores *Shh* expression and results in a phenotypic rescue (42). Given that Shh is both necessary and sufficient for ventral induction, these data indicate that a failure in ventral induction can result in the cyclopic phenotype. Further support for a role of Shh in the pathogenesis of cyclopia and holoprosencephaly comes from the identification of mutations in the human *SHH* gene in the autosomal dominant form of holoprosencephaly linked to markers on chromosome 7q21 (44).

In contrast, our data suggest that interrupting dorsal–ventral patterning independent of disruptions in Shh signaling can generate a holoprosencephaly phenotype. Our results indicate that dorsal–ventral patterning was disrupted not by a failure of ventral induction, but by the loss of the ventral forebrain secondary to apoptosis. Given the many genomic sites at which human holoprosencephaly genes have been mapped (45), and our chicken data, we predict that additional genes not necessarily involved in the Shh signaling pathway will be identified as causes of holoprosencephaly and cyclopia.

Holoprosencephaly phenotypes have been observed in a variety of animal models resulting from both environmental and genetic anomalies (reviewed in ref. 46). Our results provide a particularly useful model for studying the sequence of molecular and cellular events that lead to holoprosencephaly malformations. The ectopic expression of BMP5 and BMP4 resulted in a dramatic phenotype that included holoprosencephaly, cyclopia, and specific craniofacial anomalies. In holoprosencephaly, a spectrum of eye defects ranging from cyclopia to narrowly separated eyes (compare our Fig. 2 to figure 1 in ref. 44), the location of the eyes in the nasal sinuses, anomalies of the maxilla but not the mandible, and midline facial clefts are all common to both our chicken model and the human phenotype. Furthermore, the advanced development of the chicken embryo at E17 compared with the relative immaturity of the developing mouse at the end of gestation (*Shh*-null mutant mice die on the day of birth or earlier), will offer an opportunity to study some of the later events in the pathogenesis of this disorder.

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