CNBP regulates forebrain formation at organogenesis stage in chick embryos

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Abstract

We recently demonstrated that Cellular Nucleic acid Binding Protein (CNBP)−/− mouse embryos exhibit forebrain truncation due to a lack of proper morphogenetic movements of the anterior visceral endoderm (AVE) during pre-gastrulation stage (Chen, W., Liang, Y., Deng, W., Shimizu, K., Ashique, A.M., Li, E., Li, Y.P., 2003. The zinc-finger protein CNBP is required for forebrain formation in the mouse, Development 130, 1367–1379). However, CNBP expression pattern in the mouse forebrain suggests that CNBP may have more direct effects during forebrain development. Our data show that CNBP is expressed in tissues of early chick embryo that are the equivalent to the mouse embryo. Using a combination of RNAi-silencing and Retrovirus-misexpression approaches, we investigated the temporal function of CNBP in the specification/development of the chick forebrain during organogenesis. The silencing of CNBP expression resulted in forebrain truncation and the absence of BF-1, Six3 and Hesx1 expression, but not Otx2 in chick embryos. Misexpression of CNBP induced the expression of BF-1, Six3 and Hesx1 in the hindbrain, but not the expression of Otx2. These results offer novel insights into the function of CNBP during organogenesis as the regulator of forebrain formation and a number of rostral head transcription factors. Moreover, CNBP and Otx2 may play roles as regulators of forebrain formation in two parallel pathways. These new insights into CNBP functions underscore the essential role of CNBP in forebrain formation during chick embryo organogenesis.

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Introduction

The rostral head is a topographically complex structure that comprises unique tissues within the CNS, including the cerebral cortex, basal ganglia, eye, thalamus and hypothalamus. Despite the recent progress in functional studies of rostral head genes through mouse gene knockouts, the molecular and cellular mechanisms underlying how rostral head structure formation are still largely unknown. This could be due in part to the fact that most of these genes are involved in early embryonic development during pre-gastrulation and gastrulation, preventing the study of rostral head formation during organogenesis. As the forebrain emerges relatively late in development, disruption of genes using standard gene-targeting methods can prove uninformative if phenotypes at earlier stages of embryogenesis cause lethality or disrupt the formation of the forebrain indirectly. For example, CNBP, Bmp4, Fgf8, Notch1 and Otx2 are required for normal gastrulation and/or early patterning (Acampora et al., 1995; Ang et al., 1996; Chen et al., 2003; Meyers et al., 1998; Sun et al., 1999; Swiatek et al., 1994; Winnier et al., 1995). Although these genes, as well as others, are suspected of playing roles in forebrain development during organogenesis (Chen et al., 2003; Chenn and McConnell, 1995; Furuta et al., 1997; Meyers et al., 1998; Rhinn et al., 1998; Shimamura and Rubenstein, 1997; Zhong et al., 1997), as shown by their specific anterior expression pattern, studies of their functions in forebrain development during organogenesis have been precluded by the onset of severe malformations at earlier developmental stages.
**Materials and methods**

**In situ hybridization and immunostaining**

Whole-mount in situ hybridization was performed as described (Deng et al., 2001). The full-length mouse CNBP cDNA was subcloned and linearized with NcoI and transcribed with T3-RNA polymerase. Enl and Hnf3β cDNA were linearized and transcribed with T7-RNA polymerase. Other antisense probes used were for *Otx2*, *Lim1*, *Six3*, *Dkk1*, *Goc*, *Bf-1*, and *Hes1*. At least five embryos with the same genetic background were analyzed with each probe. Immunostaining was performed as described (Chen et al., 2003).

**siRNA preparation**

siRNA against gfp (siGFP, target sequence 5′-GCAGCUGACCCUCGUAU-3′) and two 21-bp CNBP siRNAs against chick CNBP (SiCNBP1, target sequence 5′-AAGTGCGGACGCACTGGCCAT-3′ and siCNBP2, target sequence 5′-AAGGACTGTGATCTTCAGGAG-3′) were designed and synthesized as described in the protocol in Silencer™ siRNA Construction Kit (Cat#1620, Ambion) Austin, TX. We made a CNBP SiCNBP2m, which has a single nucleotide mismatch (underlined) as a useful negative control 5′-AAGTGCGGACGCACTGGCCAT-3′.

**RT-PCR**

RT-PCR was carried out as described in the protocol in AccessQuick™ RT-PCR System (Cat# A1702, Promega) Madison, WI. Chick CNBP-F primer sequence is 5′-TTCCTCCGAGACACTGTTGATC-3′, each CNBP-R primer sequence is 5′-TGGGCAAGTGAAGAGATTCC-3′. A 450bp DNA fragment was generated. As a control, we used chick GAPDH-F primer sequence, which is 5′-CATCCACCGTCTTCTGGTG-3′, and chick GAPDH-R primer sequence, which is 5′-CTCTCTGCGCAAAGTCCAAG-3′. A 480 bp DNA fragment was generated.

**Electroporation of siRNA into chick embryos**

Electroporation of pCAGIG and siRNA was done according to Pekarik et al. (2003). Before manipulation, 2 ml albumen was removed and the top of the shell was elliptically cut with scissors to open a window over the embryo. Chick embryos in Hamilton–Hamburger stage 9 (H.H. stage 9) of development were injected with a plasmid pCAGIG (a generous gift from Dr. Connie Cepko) encoding GFP into the anterior of chick embryos with or without siRNA against GFP (siGFP, target sequence 5′-GCAGCUGACCCUCGUAU-3′, spanned 120–141). The BTX electroporation generator ECM830 (BTX, San Diego, CA, USA) was used to generate electric pulses. The electrode BTX Genetron model 516 was used for electroporation of siRNA and pCAGIG into the prospective forebrain area in the study. Electrodes (2 mm × 2 mm) were placed on anterior sides of chick embryos, and electric pulses were applied (15V, 50 ms, 2.5 mm apart, 3 times). Fertilized white Leghorn eggs were incubated horizontally at 38.5°C and staged according to Hamburger and Hamilton (Hamburger and HHH, 1951). Two days after electroporation, embryos were analyzed by whole-mount fluorescence microscopy. For CNBP silencing, 0.5 µl CNBP siRNA solution (0.5 µg/µl), including 0.05% Fast Green, was injected into the prospective forebrain region. In order to determine if the severe truncation phenotype is restricted to electroporated regions, CNBP siRNA was co-electroporated with GFP expression pCAGIG as an independent marker for electroporation efficiency. After injection and electroporation (15 V, 50 ms, 2.5 mm apart, 3 times), the window in the shell was sealed with plastic tape and embryos were incubated another 48 h to reach H.H. stage 19.Embryos were harvested, washed in PBS, fixed in 4% paraformaldehyde overnight and processed for whole-mount in situ hybridization. All experimental manipulations were performed on standard specific pathogen-free white Leghorn chick embryos.

**CNBP misexpression in chick embryos**

The CNBP-retroviral vector was constructed by inserting the coding sequence of CNBP in place of the src oncogene in a RCASBP vector (a generous gift from Dr. Tabin ) as described in Logan and Tabin (1998). The proviral DNA was transfected into a primary chick embryo fibroblast cell line, as described in Logan and Tabin (1998). The transfected host cell line then produced large quantities of infectious virus particles that were secreted into the medium. This viral supernatant was harvested, concentrated and then used to directly infect embryos. A virus titer of 1–2 × 10⁶ colony-forming units/ml was used. Chick embryos at Hamilton–Hamburger (H.H.) stage 9 of development were injected with CNBP-RCASBP retrovirus into the prospective hindbrain region where CNBP is normally not expressed. Embryos were harvested 48 h after injection, washed in PBS, fixed in 4% paraformaldehyde overnight and processed for whole-mount in situ hybridization as described (Deng et al., 2001).

**Results**

**CNBP expression pattern in chick embryos**

To identify the role of CNBP in chick rostral head development, we analyzed the expression of CNBP in early chick embryos using whole-mount in situ hybridization. CNBP expression is detected in epiblast and hypoblast cells of the unincubated embryo (stages XIII/XIV) (Figs. 1A and B). Hypoblast expression continues with the elongation of the streak (stage 3c) (Fig. 1C). At stage 4, expression of CNBP is detected in the neuroectoderm of the prospective forebrain, while expression within the streak itself is down-regulated (Fig. 1D). Strong expression of CNBP is restricted to the most anterior portion of the embryos at stage H.H. stage 5 (Fig. 1E). A few hours later, at H.H. stage 8, CNBP expression is strongly...
expressed strongly in the region of the prospective forebrain region at stages 9 and 10 (Figs. 1G and H). At stages 19 and 20, the anterior part of the central nervous system has developed to show distinct telencephalic vesicles lying one on either side of the midline, in addition to the diencephalon, mesencephalon and metencephalon. Other features of development include the appearance of four vestibular clefts and 36 somites. We found that the expression of *CNBP* during organogenesis stage was dynamic. During the initial period of maxillary process (H.H. stage 19), we detected *CNBP* transcripts in the telencephalon and midbrain, with a specific presence in the developing tail (I). A few hours later, *CNBP* expression was observed in the forebrain (especially in the telencephalon) and midbrain. During this period, expression was also detected in the heart and tail (J). At late H.H. stage 19, *CNBP* transcripts began to assemble predominantly in the forebrain, with significant expression in the midbrain and more expression of *CNBP* was detected in the tail, heart and wing bud at this stage (K). At H.H. stage 20, *CNBP* transcripts displayed almost identical expression patterns, with more expression detected in the early facial prominences and less expression detected in tail (L) \( (N = 3) \).

A CNBP knockdown by CNBP siRNA during early organogenesis resulted in forebrain truncation

In order to investigate the requirement of *CNBP* for forebrain induction during organogenesis, we made two
different CNBP siRNAs (Figs. 2A–C). To test the electroporation area and efficiency, we introduced a plasmid pCAGIG encoding Green Fluorescent Protein (GFP) into the forebrain of chick embryos at H.H. stage 9 after fertilization, with or without siRNA against SiGFP (target sequence 5′-GCAGCGACCCUGAAGUUCAU-3′, spanned 120–143). The development at this stage allowed direct access for injecting siRNA into the prospective forebrain area and facilitated the study of the effects of silencing CNBP in the rostral head tissues through different organogenesis stages. Two days after electroporation (H.H. stage 19), Chick embryos were analyzed by whole-mount fluorescence microscopy. Normal morphology of the embryos electroporated with the GFP plasmid was observed (Fig. 2D). In the same position, under whole-mount fluorescence microscopy, extensive fluorescence was seen in the forebrain of the same embryo (Fig. 2E). Treatment with 1.0 mg/ml of siGFP, in contrast, greatly abrogated the fluorescent signal (Fig. 2F, G). We demonstrated that siRNAs against the gene for green fluorescent protein (GFP) could inhibit the expression of GFP. This confirms that the technique

Fig. 2. siRNA in ovo electroporation approach in chicken system. Chick embryos in H.H. stage 9 were windowed. DNA (1.0 mg/ml) and siRNA (1.0 mg/ml) were pipetted into the embryos. (A, C) Generation of CNBP siRNAs. Two 21-bp CNBP siRNAs against chick CNBP (siCNBP1 and siCNBP2) were designed (C) and synthesized (B) as described in the protocol in Silencer™ siRNA Construction Kit. (B) R1, siCNBP1; R2, siCNBP2; M, DNA marker. (D–G) GFP siRNA (siGFP) inhibited expression of GFP. Normal morphology of embryos electroporated with only the GFP plasmid (D) and extensive fluorescence was seen in the forebrain of the same embryo under whole-mount fluorescence microscopy (E, arrow). In contrast, treatment with 1.0 mg/ml of siGFP greatly abrogated the fluorescent signal (F, G). In panel F, the embryo appears normal after the injection of siGFP and under the fluorescence microscopy, no signal is detected from the forebrain (G, arrow), indicating that siRNAs against the gene for green fluorescent protein (GFP) could inhibit expression of GFP. Panels D and F show the normal morphology of the chick embryo injected with GFP and panels E and G show the exact same embryo in the same position under whole-mount fluorescence microscopy with extensive fluorescence or fluorescence silencing seen in the forebrain. (H–O) CNBP siRNAs interfere with forebrain formation. SiRNA2m were tested to document specificity (H, L). The forebrain and midbrain developed normally in embryos electroporated with SiGFP (H, L, arrow). Embryos were electroporated with 1.0 mg/ml siCNBP1 (I–K) and siCNBP2 (M–O), respectively. Chick embryos electroporated with both CNBP and siRNAs showed the forebrain truncation malformation phenotype. The forebrain truncation phenotypes of chick embryos electroporated with siCNBP2 (M–O, arrows) were more severe than those with siCNBP1 (I–K) (N = 4).
can deliver gene expression in the desired location, i.e. prospective forebrain and can effectively silence gene expression by targeting siRNA.

We next used CNBP siRNA to study the role of CNBP during different organogenesis stages. We designed two CNBP siRNAs against chick CNBP (siCNBP1 and siCNBP2, Figs. 2A–C). We electroporated 0.5 mg/ml siCNBP1 and siCNBP2 into the forebrains of chick embryos. As a negative control, we made a CNBP SiCNBP2m that has a single nucleotide mismatch to establish specificity. As expected, the rostral head developed normally in embryos electroporated with SiCNBP2m (Figs. 2H, L). Remarkably, chick embryos electroporated with both CNBP siRNAs showed forebrain truncation phenotypes (Figs. 2I–K and M–O). This result corresponds with the CNBP rostral head expression pattern described previously (Fig. 1). Interestingly, the defect phenotypes of chick embryos electroporated with both CNBP siRNAs showed a similar severity of forebrain truncation throughout this study (Figs. 2–5). The trunks and tails of the CNBP knockdown embryos were relatively well formed, which confirms the specificity of the technique in targeting CNBP in the developing forebrain only.

The siRNA data could be difficult to interpret without corresponding GFP fluorescence for siRNA electroporated embryos. To determine if the phenotype is restricted to electroporated regions, CNBP siRNAs were co-electroporated with GFP expression plasmid as an internal control marker for electroporation efficiency and localization. As can be seen from Figs. 3A–D, this GFP is remarkably localized to forebrain when CNBP SiCNBP2m, the negative control, was co-electroporated with GFP expression plasmid. Notably, when CNBP siRNA was co-electroporated with the GFP expression plasmid, the knockdown mutant appears to have a dot of residual GFP marked by the arrow at the end of the truncated head (Fig. 3C). This indicates that most of the GFP region was abolished because the electroporated forebrain was truncated.

To investigate when CNBP is lost in these embryos and to what extent reduction of CNBP correlates with the severity and frequency of the phenotypes, we examined CNBP expression silenced by siCNBP at both the mRNA and protein levels. There was a marked reduction of CNBP mRNA signal at 10 h (H.H. stage 11) after electroporation (Figs. 3E, F). However, forebrain reduction was not observed in the knockdown embryos at stage 11. By 20 h after electroporation, CNBP mRNA was silenced at the forebrain of H.H. stage 16 chick embryos (G, H, arrows). By 20 h after electroporation, CNBP protein could not be detected in the rostral head (J, arrows). RT-PCR showed that CNBP siRNA strongly reduces CNBP mRNA but not GAPDH mRNA derived from the forebrain of H.H. stage 11 chick (3K). These results are representative of 155 injected embryos that gave consistent forebrain truncation phenotypes (N = 7).
embryos (Figs. 3G, H). Whole-mount immunostaining with anti-CNBP antibody revealed that by 20 h (H.H. stage 16), CNBP expression was silenced. CNBP expression was undetectable in the forebrains of chick embryos (Figs. 3I, J). Forebrain truncation was observed in the knockdown chick embryos in stage 16. We also performed RT-PCR that indicated that CNBP siRNA strongly reduces CNBP mRNA but not GAPDH mRNA derived from forebrain of H.H. stage 11 chicks (Fig. 3K). This result confirms that RNA interference successfully silenced CNBP expression in the forebrain of the chick embryos, and that the apparent lack of expression will allow the study of the effects of CNBP loss of function in the forebrain. These data strongly suggest that the phenotype of forebrain truncation in siCNBP knockdown chick embryos resulted from CNBP silencing.

Analyses of the anterior defects in CNBP knockdown chick embryos using tissue-specific molecular markers

To demonstrate which tissues are missing in the knockdown chick embryos, we analyzed the expression of CNBP using a number of tissue specific markers in H.H. stage 19. Electroporation of CNBP siRNA was carried out in the forebrain regions where CNBP mRNA and protein are expressed (Fig. 4). After electroporation, the forebrain was truncated in H.H. stage 19 (Fig. 4B) compared to control embryos (Fig. 4A). Serial sections of the in situ sections indicated forebrain truncation and midbrain malformation in the CNBP siRNA knockdown embryos (Figs. 4C, D). Subsequently, we studied forebrain markers to check whether their expression had been affected. In CNBP knockdown embryos, expression of the telencephalon forebrain marker BF-1 (Tao and Lai, 1992) was absent (Fig. 4F) compared with the wild-type chick embryos, indicating that forebrain patterning was non-functional in the CNBP knockdown chick embryos. Similarly, Six3 expression, which marks the diencephalon, was also absent in CNBP mutant chick embryos (Figs. 4G, H), further confirming that forebrain...
ment was defective. En2, a midbrain–hindbrain junction marker, was expressed in the anterior region of both H.H. stage 19 normal and CNBP knockdown chick embryos (Figs. 4I, J), indicating that the development of the anterior hindbrain was not influenced by RNA interference.

In contrast, Otx2 (a forebrain and midbrain marker) transcripts were detected in the midbrain of both wild-type and mutant chick embryos. Anterior reduction in the telencephalon of the siCNBP knockdown embryos caused a reduction in telencephalon Otx2 expression (Fig. 4L). However, the expression in the midbrain was similar to that of the wild type (Figs. 4K, L). This result was further confirmed in serial in situ sections (Figs. 4M, N). Expression of Otx2 is lost in anterior structures, but there is still tissue there. We believe that the residual tissue is the CNS tissue that has lost identity due to the absence of CNBP. The tissue is neither midbrain nor forebrain tissue so Otx2 was not expressed there. Collectively, our marker gene expression analysis indicates that the CNBP knockdown results in forebrain truncation in chick embryos. Development of the midbrain, hindbrain, trunk, and tail of CNBP knockdown embryos was normal.

Analyses of CNBP knockdown embryos using anterior CNS markers

To further examine whether the CNBP knockdown affects formation of the anterior Central Nervous System (CNS), we analyzed the expression of anterior CNS markers: Lim1, Hnf3β, Gsc and Dkk1 at H.H. stage 19. Lim1 was expressed in the ingressing axial mesendoderm and notochord, with areas of staining in ventral midbrain and hindbrain. This pattern is seen in both knockdown and wild-type chick embryos (Figs. 5A, B). The expression pattern of Hnf3β in knockdown embryos was similar to that of wild-type embryos except there is no expression in the dorsal head mesenchyme in the mutant (Figs. 5C, D). There is a strong Hnf3β expression at the mesenchyme of dorsal and ventral forebrain and midbrain in the wild-type embryos (Fig. 5E), but no expression in the dorsal head mesenchyme in the mutant as shown in sagittal sections of the whole-mount Hnf3β in situ hybridization (Figs. 5E, F). Prechordal plate marker Gsc appeared in the mesenchyme of the forebrain and the first branchial arch in the wild type; however, it was missing in the forebrain of knockdown chick embryos (Figs. 5G, H). Dkk1 was detected in the neuroectoderm of the forebrain and midbrain, and the developing somites of the wild type, while it was absent in the forebrain and dorsal midbrain of the knockdown embryos (Figs. 5I, J). These results indicate that the CNBP knockdown affects the formation of the anterior CNS during chick embryo organogenesis.

Misexpression of CNBP induces the expression of downstream target genes in the hindbrain

The loss-of-function using siCNBP RNA approach showed that silencing of CNBP resulted in forebrain truncation in early organogenesis of the chick embryo. However, limited information about the mechanism can be derived from the loss-of-function approach since most of the forebrain tissues were absent. In order to characterize the mechanism causing the defects, we also used a gain-of-function approach to study genes downstream of CNBP that may account for the observed results. To address whether CNBP is sufficient to induce the expression of forebrain-specific gene markers, we used avian replication-competent retroviruses to misexpress CNBP in the developing chick hindbrain. All standard techniques were performed according to the method of Logan and Tabin (1998).

Chick embryos at H.H. stage 9 of development were injected with CNBP-RCASBP retrovirus in the prospective hindbrain region, where CNBP is normally not expressed. Embryos were harvested 48 h after injection (H.H. stage 21). CNBP (Figs. 6A, B) and forebrain marker genes, including BF-1 (Figs. 6C, D), Six3 (Figs. 6E, F), Hexl (Figs. 6G, H) and Otx2 (Figs. 6I, J) were used as probes for the whole-mount in situ hybridization analysis. Misexpression of CNBP in the hindbrain was observed (Figs. 6A, B). Interestingly, the misexpression of CNBP in the hindbrain induced the ectopic expression of forebrain markers BF-1, Six3 and Hex1 throughout the entire hindbrain of chick embryo (Figs. 6C–H). We had previously reported that BF-1, Six3, and Hexl were completely absent in CNBP mutant mice (Chen et al., 2003), which is consistent with the CNBP knockdown and misexpression results in chick embryos. These results suggest that BF-1, Six3 and Hexl are downstream target genes of CNBP. However, the misexpression of CNBP in the hindbrain could not induce the ectopic expression of midbrain marker, Otx2, in the hindbrain (Figs. 6I, J). The results suggest that CNBP and Otx2 may play roles as regulators of forebrain formation in two parallel pathways.

Discussion

CNBP expression in the equivalent of the mouse embryo

To find the clue of the CNBP function in chick embryonic development, we analyzed the expression pattern of CNBP in early chick embryos using whole-mount RNA in situ hybridization. As can be seen from Fig. 1, CNBP expression pattern in chick embryos is in the equivalent tissues of the mouse embryo. Our previous experiments indicated that the expression of CNBP could be detected in the AVE in the early stages of the mouse model. The Hypoblast in chick embryos has been verified embryologically to be functionally equivalent to mouse AVE. The hypoblast plays a role in directing cell movements in the adjacent epiblast. These movements distance the future forebrain region from the developing organizer (Hensen’s node). Therefore, the experiments indicated a key role for CNBP in avian rostral head development in chick embryos, expanding on a previous study and revealing that the earlier forebrain expression pattern in chicks is homologous to that in mice (Chen et al., 2003). CNBP also is strongly expressed in forebrain, indicating its possible function in forebrain formation in both chicks and mice.
Fig. 5. Molecular analysis of anterior CNS markers in CNBP knockdown embryos by whole-mount RNA in situ hybridization. *Lim1* is expressed in the ingressing axial mesendoderm and notochord and can also be detected in the ventral midbrain and hindbrain of both wild-type and mutant embryos (A, B, arrows). The expression pattern of *Hnf3β* in knockdown embryos was similar to that of wild-type embryos except there is no expression in the dorsal head mesenchyme in the mutant (C, D, arrow). There is a strong *Hnf3β* expression at the mesenchyme of dorsal and ventral forebrain and midbrain in the wild-type embryos (E, arrow and solid arrow head), but no expression in the dorsal head mesenchyme in the mutant shown in Sagittal sections of the whole-mount *Hnf3β* in situ hybridization (E, F, arrow and open arrow head). (G) Prechordal plate marker Gsc appeared in the mesenchyme of the forebrain, the ventral midbrain, and the first branchial arch of the wild type (G, arrow). Gsc expression was missing in the forebrain of the knockdown embryos (H, arrow), but was expressed in the ventral midbrain (H, arrow). *Dkk1* was detected in the neuroectoderm of the forebrain and midbrain in the wild-type embryos (I, arrow), while it was absent in the forebrain of the knockdown embryos (J, arrow) (*N* = 5).
Fig. 6. Induction of ectopic expression of forebrain markers in hindbrain by CNBP misexpression. Chick embryos in H.H. stage 9 of development were injected with CNBP expression – RCASBP virus in the prospective hindbrain region (B, D, F, H). The contrasting embryos (A, C, E, G, I) were injected with RCASBP virus. (A, B) The expression of CNBP was detected in both the forebrain and hindbrain in the misexpressed embryos in H.H. stage 21 (B, arrow), while the expression of CNBP was confined to the forebrain in contrasting embryos (A). The expression of BF-1, Six3 and Hesx1 occurred additionally in the hindbrain (D, F, H, respectively, arrows) as compared to the contrasting embryos (C, E, G, respectively). In contrast, misexpression of CNBP expression in the hindbrain could not induce ectopic Otx2 expression in the hindbrain (I, J, arrow) (N = 4).
siRNA knockdown approaches in the study of forebrain development

Silencing of CNBP expression in the forebrain resulted in forebrain truncation. From these studies, we conclude that CNBP has a direct role in forebrain development. Although the electroporation of siRNAs into midhindbrain regions approach has been reported (Nakamura et al., 2004), to our knowledge, this is the first report to study gene function in forebrain formation during organogenesis using RNAi mediated loss-of-function approach in chick embryonic development.

The ablation of CNBP function in the chick results in severe truncation of the forebrain (Figs. 2H–O). This provides direct genetic evidence that CNBP plays an essential and unique role in chick forebrain development at the early organogenesis stage (Fig. 7). Some variation in the severity of the phenotype was seen and that is possibly due to the haploinsufficiency, which suggests that the CNBP gene must be expressed above a threshold level to ensure normal development. This variation was also detected in 40% of CNBP heterozygous newborn mutant mice which exhibited multiple defects, including growth retardation and craniofacial defects (e.g. a smaller mandible and complete lack of eyes), and died shortly after birth (Chen et al., 2003). In addition, the injection and the electroporation techniques cannot guarantee the delivery of the siRNA to the exact same location within the developing prosencephalon. However, the consistent forebrain truncation phenotype that resulted from silencing CNBP proves the reliability of this technique.

CNBP is required for forebrain formation during organogenesis

The data provide direct genetic evidence that CNBP plays an essential and novel role in chick forebrain development during early organogenesis (Fig. 7). These novel results could not be obtained in our previous study due to the early defect of AVE that causes the anterior truncation during gastrulation (Chen et al., 2003). It is clear that the RNAi and in ovo methods allow for greater temporal and spatial control of the manipulation of CNBP expression. As a result of this study, we know that CNBP is essential in the development of the forebrain during organogenesis. This result is consistent with the CNBP expression pattern during organogenesis. Notably, this is the first report of CNBP playing a role in forebrain formation during organogenesis.

The forebrain begins to form midway through gestation, and null mutation of CNBP in the model leads to early phenotypes,
reduced analysis of its role at later stages. Targeted disruption of CNBP with the use of RNA interference during the early organogenesis stage of chick embryo overcomes the problems of early tissue defects. Since the knockdown occurred during early organogenesis, the defects must be the loss of CNBP’s expression in the rostral heads of chick embryos (Fig. 7).

In this study, we targeted gene knockdown at the forebrain. Throughout the study, the midbrains of several embryos appear to have some abnormal morphology that might be a result of forebrain truncation. However, in most embryos, it appears fairly normal. The expressions of midbrain marker gene Otx2 and En2 in the midbrain appear unaffected. The function of CNBP in midbrain formation could not be defined in the study.

We found that there were residual tissues on the anterior structures in the knockdown chick embryo. We believe that these residual tissues were the CNS tissue that had lost identity due to absence of CNBP. Tissue identity is determined by marker gene expression. Otx2 (marker for midbrain and forebrain) was only expressed in midbrain, but not in the anterior tissue, i.e. the midbrain and forebrain marker only extending to the midbrain, but did not extended to the anterior tissues, indicates that the midbrain is normally formatted, but not forebrain. It was further confirmed that the residual tissue was not forebrain tissue since forebrain marker gene BF1 was not expressed in the tissues (Fig. 4F).

Characterization of CNBP downstream genes using the retroviral gain of function approach

Our results suggest that one of the CNBP functions in forebrain formation is carried out by activating the expression of BF-1. BF-1 is a winged-helix transcriptional repressor that plays important roles in both progenitor cell differentiation and regional patterning in the mammalian telencephalon (Li et al., 1996). Regionalization of the vertebrate forebrain involves repression of Wnt1 expression by Six3 within the anterior neuroectoderm (Lagutin et al., 2003). Activity of the homeobox gene Hex1 is also required in the anterior neural ectoderm, and variable forebrain truncations have been observed in Hex1-null embryos (Martinez-Barbera and Beddington, 2001; Martinez-Barbera et al., 2000). Six3 and Hex1 are among the earliest genes to function in the anterior neural plate during head patterning (Lagutin et al., 2003). Therefore, CNBP may carry out its function by controlling the expression of Six3 and Hex1. Our finding suggests that CNBP plays a role in the gene regulation of important rostral head transcription activators, including BF-1, Six3 and Hex1 (Fig. 7), in forebrain formation during chick embryo organogenesis.

Misexpression of CNBP in chick embryos induced the expression of BF-1, Six3 and Hex1, but not Otx2. Otx2 is a central protein for rostral head induction and development. It was shown to be necessary for the regulation of a variety of genes involved in morphogenesis, cell migration and the acquisition of anterior neural identity (Boncinelli and Morgan, 2001). Misexpression of CNBP did not induce the expression of Otx2 in the hindbrain (Fig. 6J), this suggests that Otx2 is not a downstream target gene of CNBP. As Otx2-null mutant embryos failed to both execute the movement of the AVE from the distal end to proximal region of the embryo (Perea-Gomez et al., 2001) and lacked anterior structures (Ang et al., 1996), we suspect that the function of CNBP and Otx2 may be parallel in rostral head development. These data expand our basic understanding of the molecular mechanisms of both normal facial development and craniofacial deformity and will also aid in the development of therapeutic means for intervention in diseases involving craniofacial defects.

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