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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.

Keywords: CNBP; Gene expression; Forebrain development; Organogenesis stage; RNAi; Misexpression; Chick embryos

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.

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CNBP encodes a 19 kDa protein containing seven tandem zinc finger repeats of 14 amino acid residues (Cys-X2-Cys-X4-His-X4-Cys) (Covey, 1986). The amino acid sequence of *CNBP* is highly conserved. This striking conservation, coupled with the fact that homologous genes have been found in various organisms, suggests that *CNBP* plays an essential biological role across different species (Shimizu et al., 2003). The disruption of *CNBP* caused severe forebrain truncation due to a lack of specification and/or proper morphogenetic movements of the anterior visceral endoderm (AVE) during pre-gastrulation (Chen et al., 2003). Since *CNBP* is also expressed in the forebrain during organogenesis, we hypothesized that CNBP also plays a direct role in forebrain formation.

To explore the function of CNBP in forebrain development at organogenesis stage, we needed to use methods that allowed for greater temporal and spatial control of the manipulation of CNBP expression. Here we report investigation of the role of CNBP in rostral head formation using a combination of RNAi and in ovo techniques. Using this approach, *CNBP* expression at the prospective forebrain and forebrain was knocked down during organogenesis in chick embryos. To characterize genes downstream of CNBP, we also performed misexpression of *CNBP* in the developing chick hindbrain using *CNBP-recombined* retrovirus. *CNBP* acts as a regulator of the forebrain in chick rostral head development during organogenesis by regulating other rostral head transcription factors.

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 *Not*I and transcribed with T3-RNA polymerase. *En1* and *Hnf3β* cDNA were linearized and transcribed with T7-RNA polymerase. Other antisense probes used were for *Otx2*, *Lim1*, *Six3*, *Dkk1*, *Gsc*, *BF-1*, and *Hesx1*. 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′-GCAGCUGACCCUGAAGUUCAU-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′-AAGTGCGAACGCACTGGCCAT-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′-TCTCCCGGACATCTGTTACC-3′, chick CNBP-R primer sequence is 5′-TTGGCCAGTGAAGAGGATTC-3′. A 450bp DNA fragment was generated. As a control, we used chick *GAPDH-R* primer sequence, which is 5′-CATCCACCGTCTTCTGTGTG-3′, and chick *GAPDH-F* primer sequence, which is 5′-CCTCTCTGGCAAAGTCCAAG-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′-GCAGCUGACCCUGAAGUUCAU-3′, spanned 120–143). The BTX electroporation generator ECM830 (BTX, San Diego, CA, USA) was used to generate electric pulses. The electrode BTX Genetrode 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 HHL, 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 × 10^8 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

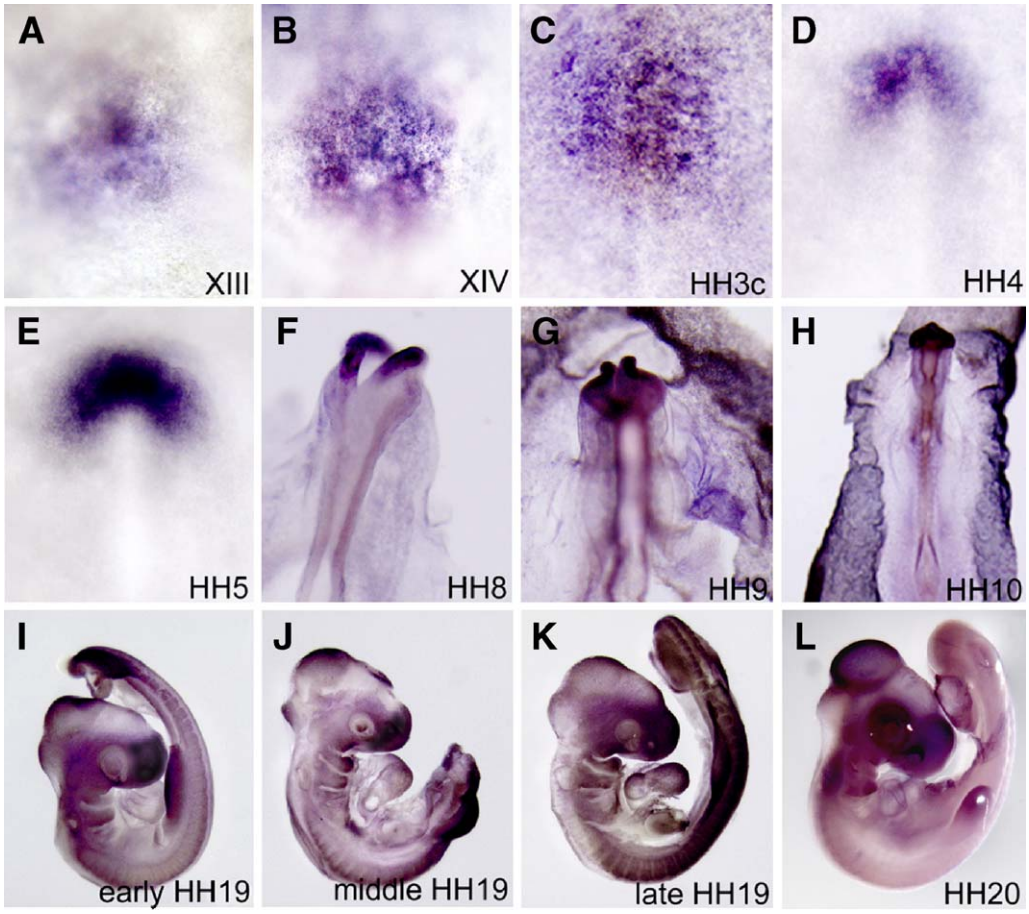

Fig. 1. Analysis of *CNBP* expression pattern in early chick embryo. *CNBP* expression is detected in epiblast and hypoblast cells of the unincubated embryo (stages XIII/XIV) (A and B). Hypoblast expression continues with the elongation of the streak (stage 3c) (C). At stage 4, expression of *CNBP* is detected in the neuroectoderm of the prospective forebrain, while expression within the streak itself is down-regulated (D). Strong expression of *CNBP* restricted to the most anterior portion of the embryos at stage H.H. stage 5 (E). A few hours later, at H.H. stage 8, *CNBP* expression is strongly expressed at anterior neural plate (F). *CNBP* is expressed strongly in the region of the prospective forebrain region at stage 9 and 10 (G and H). 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) ($N = 3$).

expressed at the anterior neural plate (Fig. 1F). *CNBP* is 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 (Fig. 1I). 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 (Fig. 1J). At late H.H. stage 19, *CNBP* transcripts began to assemble predominantly in the forebrain, with significant expression in the midbrain. More expression of *CNBP* was detected in the tail, heart and wing bud at this stage (Fig. 1K). 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 (Fig. 1L). Our data show that *CNBP* is expressed in the hypoblast, which is embryologically and functionally equivalent to the mouse AVE. The expression pattern of *CNBP* during organogenesis (H.H. stages 8 to 20) corresponds to results in mice (Chen et al., 2003). The highly conserved expression pattern of *CNBP* suggests that *CNBP* plays a pivotal role in rostral head formation in the chick embryo. We also found *CNBP* expressed in the heart and tail, where CNBP function remains unclear.

A CNBP knockdown by CNBP siRNAi 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′-GCAGCUGACCCUGAAGUUCAU-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

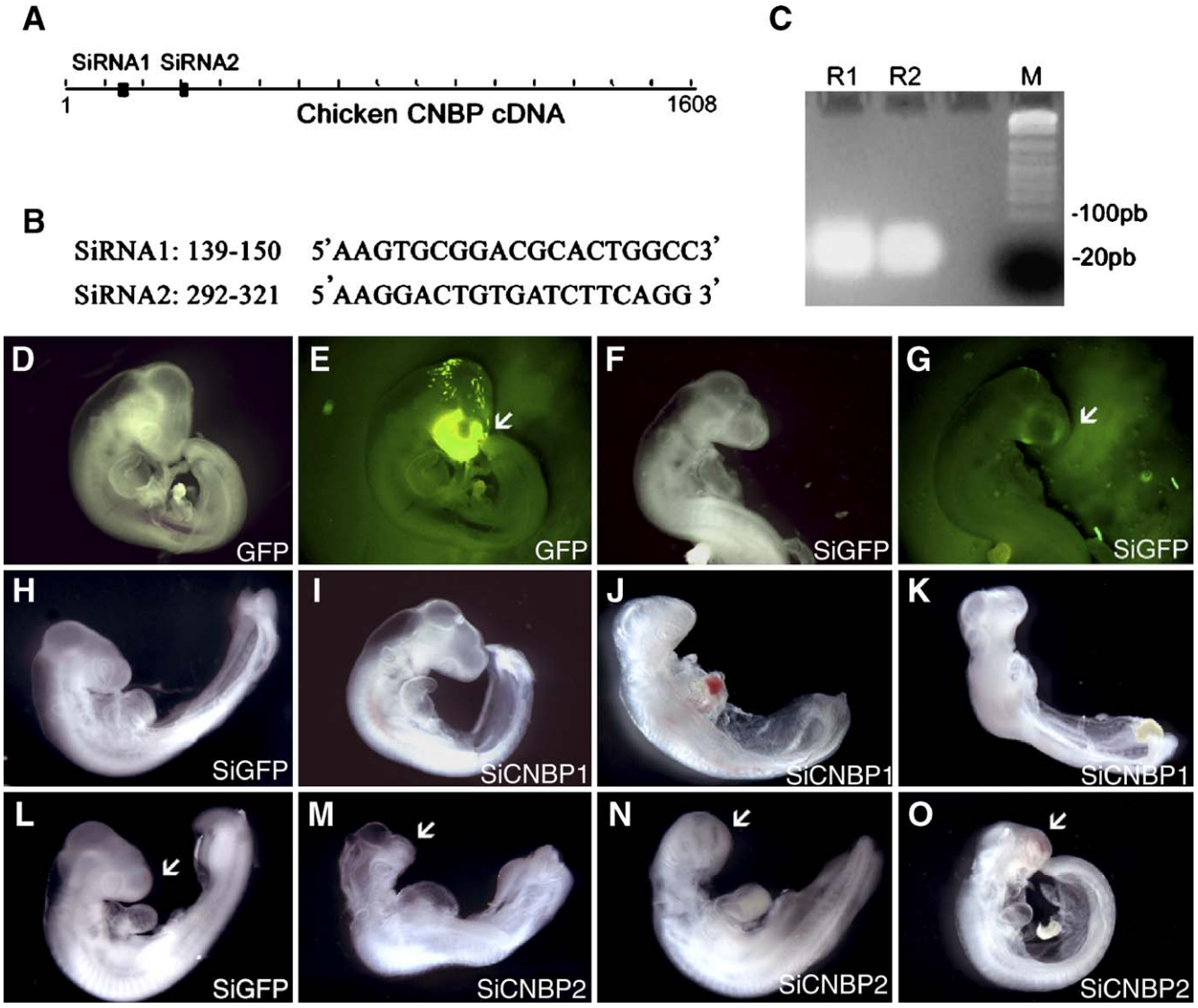

Fig. 2. siRNA in ovo electroporation approach in chicken system. Chick embryos in H.H. stage 9 were windowed. DNA (1.0 mg/ml) and siRNA (1.0 mg/ml) were pipetted into the embryos. (A, C) Generation of *CNBP* siRNAs. Two 21-bp *CNBP* siRNAs against chick *CNBP* (siCNBP1 and siCNBP2) were designed (C) 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 siCNBP2 (Figs. 2M–O) were more severe than those with SiCNBP1 (Figs. 2I–K), indicating that siCNBP2 may have higher silencing activity. The siCNBP2 was utilized in all the remaining silencing experiments. About 90% of the embryos 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.

Forebrain truncation phenotype restricted to electroporated region, and time course of siRNA effects on CNBP expression

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

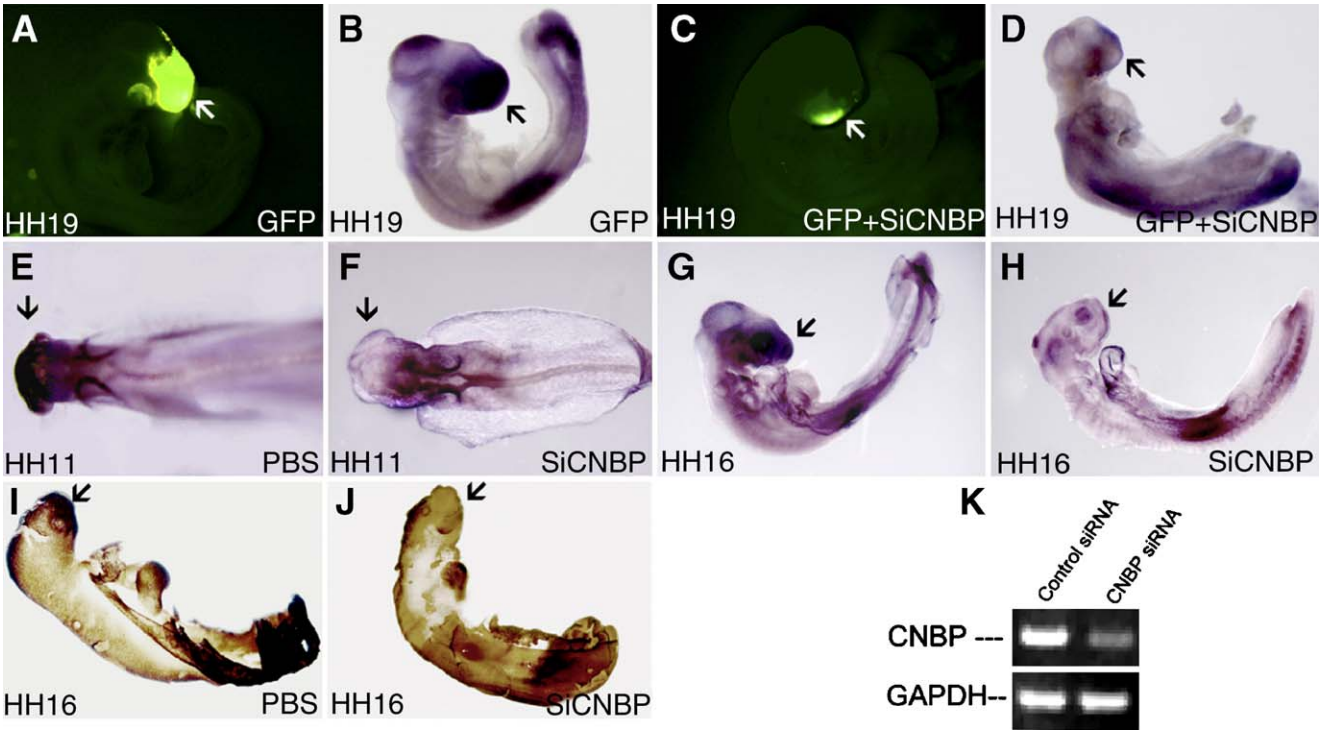

Fig. 3. Forebrain truncation phenotype restricted to electroporated region, and time course of siRNA effects on *CNBP* expression. The forebrain truncation phenotypes are restricted to the electroporated region using GFP expression as a control (A–D). The figure shows the dorsal view of an embryo 10 h (E, F) and 20 h (G, H, I, J) after electroporation. Examination of the *CNBP* expression level by in situ hybridization indicated a marked reduction of *CNBP* mRNA signal 10 h (H.H. stage 11) after electroporation with siCNBP (E, F, arrows). By 20 h after electroporation, *CNBP* mRNA was silenced in 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 (I, J, arrows). RT-PCR showed that *CNBP* siRNA strongly reduces *CNBP* mRNA but not *GAPDH* mRNA derived from the forebrain of a 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 d- e- v- e- l- o- p-

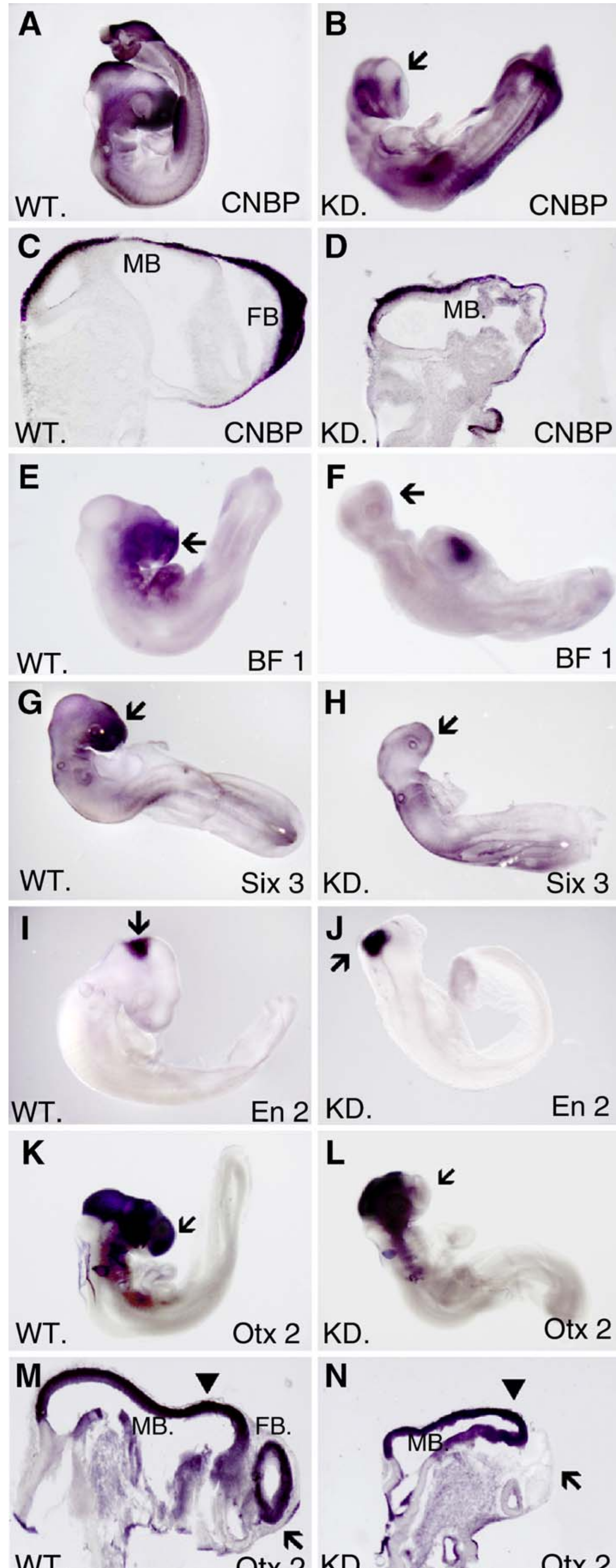

Fig. 4. Morphological and histological analyses of the rostral head phenotype in *CNBP* knockdown embryos with rostral head tissue-specific molecular markers. Whole-mount RNA in situ hybridization of wild-type (A, C, E, G, I, K, M), and *CNBP* knockdown embryos (B, D, F, H, J, L, N) at H.H. stage 19. *CNBP* is expressed in the forebrain, midbrain and tail in the wild type, but the forebrain is truncated. *CNBP* expression is undetectable in the rostral head of the *CNBP* knockdown chick embryos (A, B, arrows). Sagittal sections of wild-type whole-mount *CNBP* in situ hybridization (C) and *CNBP* knockdown embryos show an underdeveloped forebrain (D). Forebrain tissue expressing *CNBP* is completely absent in the *CNBP* knockdown embryos in H.H. stage 19 (D). Expression of the telencephalon forebrain marker *BF-1* is absent in *CNBP* knockdown embryos (F, arrow) compared with the wild-type embryos (E, arrow). Panel H shows the absence of diencephalon marker *Six3* in a *CNBP* mutant embryo (H, arrow) as compared to the wild-type embryo (G, arrow). *En2*, a marker for the midbrain and anterior hindbrain, is normally expressed in H.H. stage 19 in wild-type and *CNBP* knockdown embryos (I, J, arrows). *Otx2* is expressed in the midbrain and forebrain in wild-type embryos (K, arrow), but is absent only in the truncated forebrain in knockdown embryos (L, arrow). Sagittal sections of the whole-mount Otx2 cRNA in situ hybridization show that expression of Otx2 is normally restricted to the midbrain and forebrain regions of wild-type embryos (M, arrow) and is completely absent in the forebrain region of the CNBP knockdown mutant embryo (N, arrow) ($N = 5$).

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), *Hesx1* (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 *Hesx1* throughout the entire hindbrain of chick embryo (Figs. 6C–H). We had previously reported that *BF-1*, *Six3,* and *Hesx1* 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 *Hesx1* 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.

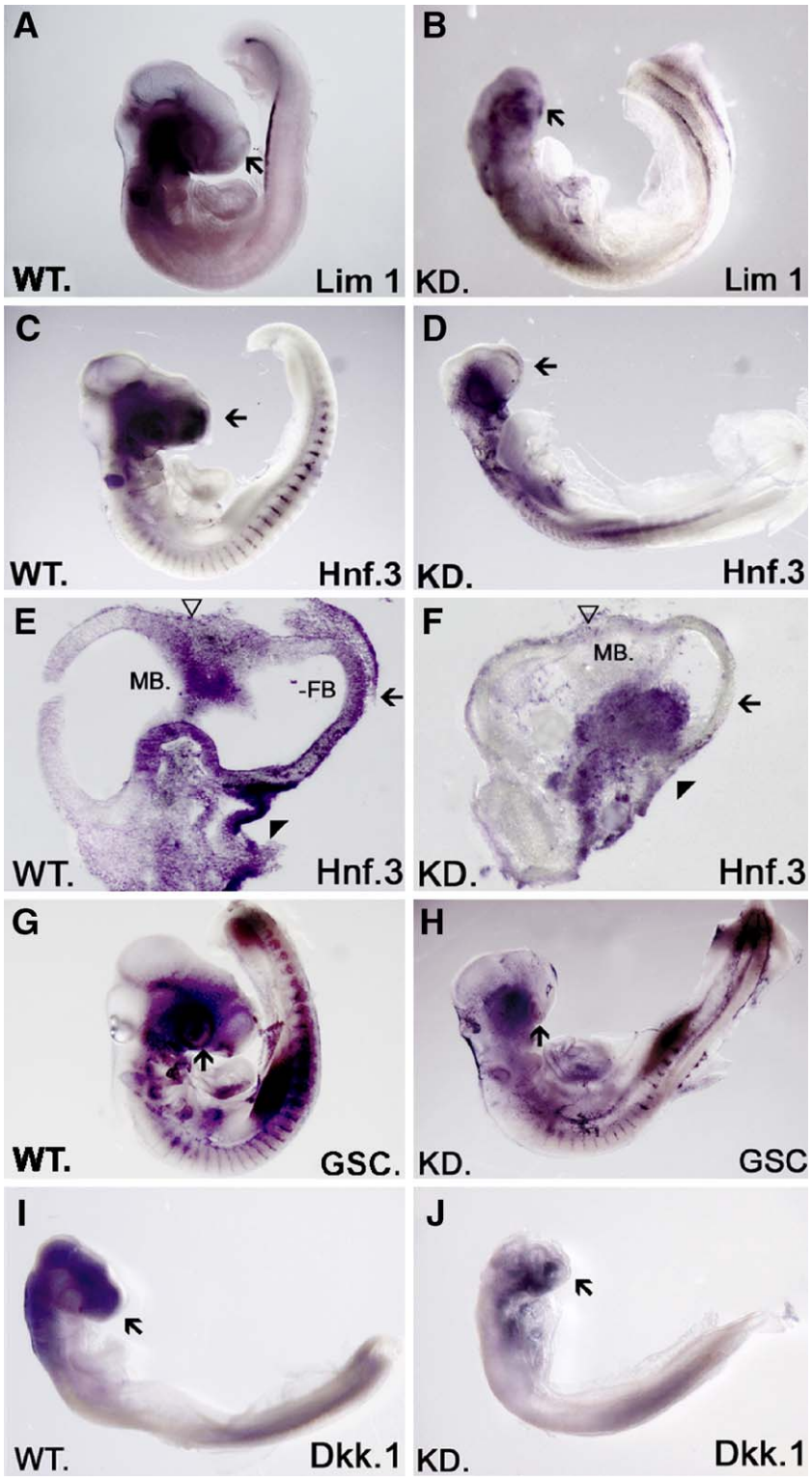

Fig. 5. Molecular analysis of anterior CNS markers in *CNBP* knockdown embryos by whole-mount RNA in situ hybridization. *Lim1* is expressed in the ingressing axial mesendoderm and notochord and can also be detected in the ventral midbrain and hindbrain of both wild-type and mutant embryos (A, B, arrows). 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) ($N = 5$).

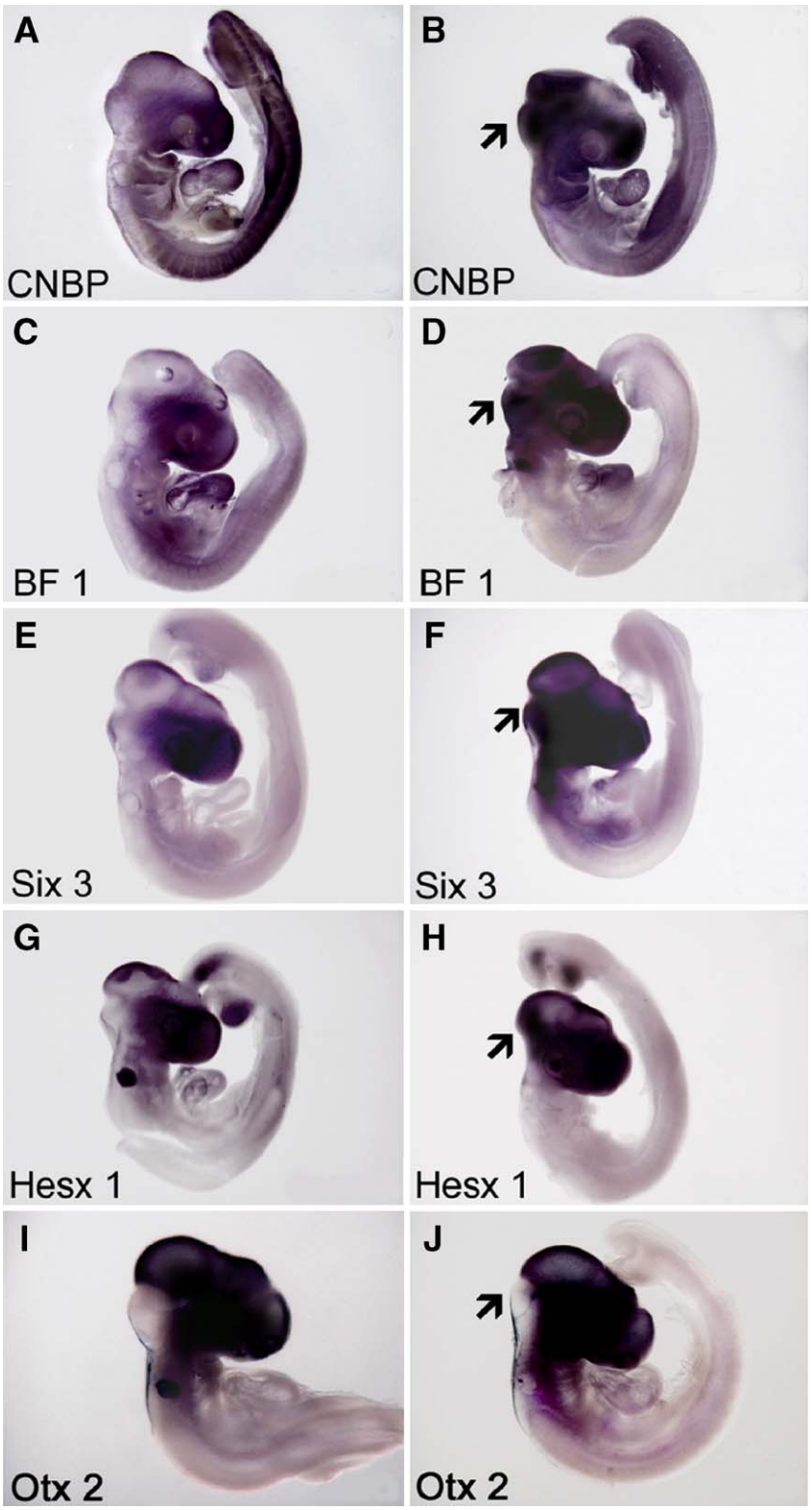

Fig. 6. Induction of ectopic expression of forebrain markers in hindbrain by *CNBP* misexpression. Chick embryos in H.H. stage 9 of development were injected with *CNBP* expression − RCASBP virus in the prospective hindbrain region (B, D, F, H). The contrasting embryos (A, C, E, G, I) were injected with 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, including severe anterior truncation

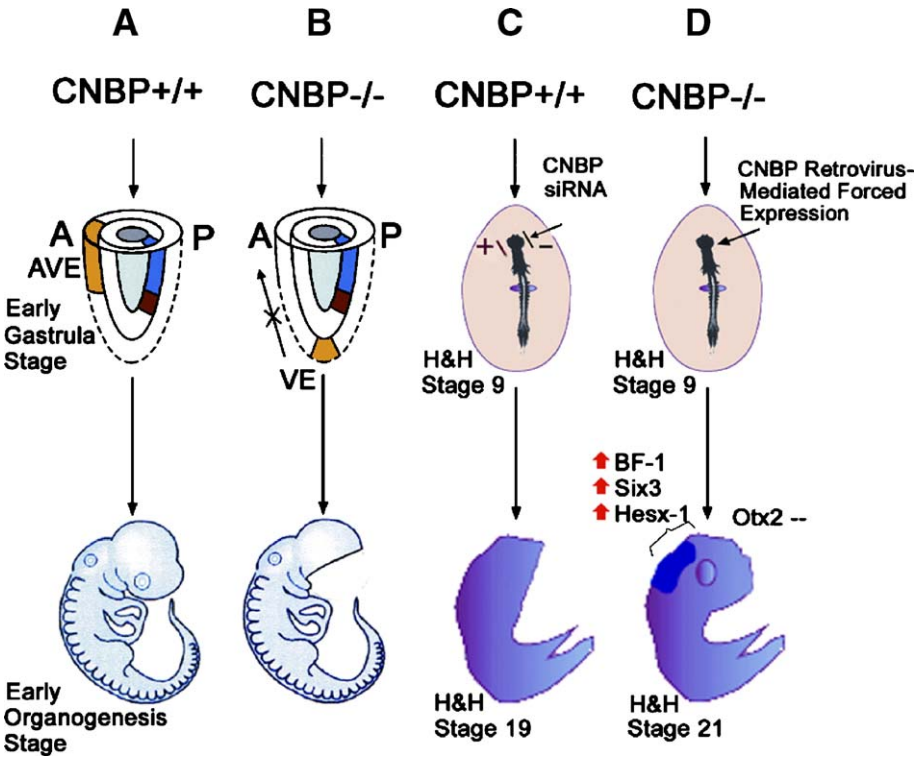

Fig. 7. Model for *CNBP* function in establishing the forebrain. Loss of *CNBP* function in different stages clearly showed the importance of multiple *CNBP* actions in the forebrain developmental process. In the mouse model (A), *CNBP* is normally expressed in the AVE and in the developing forebrain during early organogenesis. (B) In *CNBP*$^{-/-}$ mutant mouse embryos, defects in the anterior movement of the visceral endoderm and anterior definitive endoderm formation cause severe forebrain truncation. Using the *in ovo* electroporation in chicks (C) knockdown of *CNBP* by siRNA approach in organogenesis caused forebrain truncation, indicating the important role of *CNBP* during this stage. (D) Retrovirus-mediated misexpression of *CNBP* in the hindbrain of chicks induced the expression of *BF-1*, *Six3* and *Hesx-1* but not *Otx2* during organogenesis. This indicates that *BF-1*, *Six3* and *Hesx-1* are possibly *CNBP*'s downstream genes, while *Otx2* induces and maintains the anterior identity of the embryo in a parallel pathway to *CNBP*. A lack of *CNBP*'s downstream genes in the forebrain is possibly the cause of the anterior truncation seen in *CNBP* knockdown chick embryos. It is possible that *CNBP* is essential in forebrain formation during early organogenesis through the regulation *BF-1*, *Six3*, *Hesx-1* and *Hnf3β*.

recluded 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 identify 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 *Hesx1* is also required in the anterior neural ectoderm, and variable forebrain truncations have been observed in *Hesx1*-null embryos (Martinez-Barbera and Beddington, 2001; Martinez-Barbera et al., 2000). *Six3* and *Hesx1* 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 *Hesx1*. Our finding suggests that *CNBP* plays a role in the gene regulation of important rostral head transcription activators, including *BF-1*, *Six3* and *Hesx1* (Fig. 7), in forebrain formation during chick embryo organogenesis.

Misexpression of *CNBP* in chick embryos induced the expression of *BF-1*, *Six3* and *Hesx1*, 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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