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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