Gene Expression of NeuroD/BETA2 during Development of the Mouse Central Nervous System

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Abstract

NeuroD/BETA2, a basic helix-loop-helix transcription factor, has been known to play a role in terminal differentiation during neurogenesis. To gain further insight into the function of NeuroD/BETA2 in the nervous system development, we examined the expression pattern of NeuroD/BETA2 during embryonic and postnatal development by *in situ* hybridization. Dynamic changes of NeuroD/BETA2 expression were observed in the developing nervous system. Gene expression of the NeuroD/BETA2 in developing cerebellum and hippocampus increased during the embryonic stages and persisted throughout postnatal development and remained at a stable level in the adult brain. NeuroD/BETA2 expression was detected in postmitotic cells in the subventricular zone of the cerebrum during embryogenesis. This observation confirms that NeuroD/BETA2 may have a role in terminal differentiation during neurogenesis.

Key words : NeuroD, BETA2, Brain, Mouse

INTRODUCTION

Cellular diversity of the nervous system is achieved by a progressive or stepwise expression of a certain set of genes. Basic helix-loop-helix (bHLH) factors have been shown to play an important role in this process from invertebrates to mammals. Tissue specific factors of the bHLH family heterodimerize with ubiquitous bHLH factors such as E12/E47 to control both the determination and differentiation of specific cell types in various tissues, including muscle and nerve (for review, Jan and Jan, 1993). NeuroD, a bHLH transcription factor, was cloned as a gene that could interact with the Daughterless gene product in *Drosophila*. Ectopic expression of NeuroD induces premature differentiation of neurons in *Xenopus* oocytes (Lee *et al*., 1995), whereas mutation of NeuroD causes an increase of glia (Morrow *et al*., 1999). In addition, overexpression of NeuroD promotes premature differentiation of late-born neurons in retina (Ahmad *et al*., 1999), suggesting that NeuroD may play a role in the terminal stage of neuronal differentiation.

NeuroD was also isolated from insulinoma cells as an important factor for transactivation of the insulin gene, referred as BETA2 (Naya *et al*., 1995). Knock out mice lacking BETA2 die within 5 days after birth due to severe diabetes mellitus resulted from the loss of insulin-producing β cells. BETA2 also regulates transcription of hormones in neuroendocrine cells, such as the secretin gene in enteroendocrine cells and the proopiomelanocortine gene in pituitary as well as a homeodomain protein, PDX-1 (Mutoh *et al*., 1997; Poulin *et al*., 1997; Sharma *et al*., 1997; Mutoh *et al*., 1998). The BETA2 mutant mice fail to develop secretin- and cholecystokinin-producing enteroendocrine cells with no obvious defects in the nervous system (Naya *et al*., 1997), suggesting an important role in development of neuroendocrine cells. Taken these data, it is suggested that NeuroD/BETA2 participates in successive stages of cell fate determination during development of nervous as well as neuroendocrine tissues.

This work was supported by the Academic Research Fund of Ministry of Education, Republic of Korea (Grant number: BM 97-107) to Haeyoung Suh-Kim and by Korean Ministry of Education through a Research Fund (Grant number: 1998-021-F00031) to both Haeyoung Suh-Kim and Yeon-Soo Kim.

In this study, we show that expression of NeuroD/BETA2 is regulated in a time- and region-dependent manner during development of the nervous system, especially in cerebellar development.

MATERIALS AND METHODS

1. Animals and Tissue Preparation

Prenatal mice (BALB/c) from embryonic day 13 (E13), postnatal mice from postnatal days 1 to 13 (P1～P13) and adult were used *in situ* hybridization. The occurrence of a vaginal plug was defined as E0.5 and the day of birth as P1. Embryos and brains were immediately removed without fixation, snap-frozen in isopentane cooled at −50°C, and stored at −80°C until sectioned. Serial sagittal sections (14 μm) were prepared on a cryostat at −20°C and thaw-mounted onto slides (ProbeOn Plus™, Fisher Scientific). For *in situ* hybridization, sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline, pH 7.4 (PBS) for 15 min at room temperature. After washing in PBS, sections were acetylated with 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 min and dehydrated.

2. *In Situ* Hybridization

To generate antisense probes, a plasmid pGEM-BETA2N986 containing N-terminal 986 bp of BETA2 (Genebank Accession No. U24679) was constructed. A 214 bp antisense probe corresponding from 773 bp to 986 bp of the BETA2 cDNA was generated by linealizing pGEM-BETA2N986 with NcoI and subsequent *in vitro* transcription by using T7 RNA polymerase and digoxigenin-UTP (Boehringer Mannheim, Germany) (Fig. 1). A sense probe covering the same 214 bp, from 773 to 986, was generated similarly using pGEM-BETA2△773 containing a deletion of N-terminal 773 bp of BETA2 cDNA.

Sections were hybridized with digoxigenin labeled probes in hybridization buffer (40% formamide, 4? SSC, 1? Denhardt's, 10% dextran sulfate, 10mM dithiothreitol, 1mg/ml yeast tRNA, and 1 mg/ml single strand DNA) in a humidified chamber at 52°C for 14～16 hours. After hybridization, sections were washed in 2 × SSC at room temperature, and incubated with 20 μg/ml ribonuclease A in 0.5 M NaCl, 10 mM Tris-Cl, pH 8.0 and 1 mM EDTA for 30 min at room temperature. Then the sections were washed in 2 × SSC for 20 min at room temperature and in 0.1 × SSC for 90 min at 60°C. After washing off the unbounded probes, sections were incubated for 16 hours with anti-digoxigenin antibody (Boehringer-Mannheim) that was preabsorbed with 12 mg/ml mouse brain powder in TBS (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 7.4). After removing all unbound antibodies, the alkaline phosphatase-conjugated antibody was visualized as blue precipitates using 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Sigma). The slides prepared from BrdU injected mice were subjected to subsequent BrdU assays.

3. Bromodeoxyuridine (BrdU) Incorporation Assay

To distinguish undifferentiated, proliferative cells from postmitotic, premigratory neuronal cells in the developing mouse brain, mice at E13 were injected intraperitonially with BrdU (1 mg/g body weight), and sacrificed 2 hours after injection. After performing *in situ* hybridization assay (see above), sections were incubated in 2 N HCl and neutralized in 0.1 M sodium borate at room temperature for 25 min each. Sections were incubated for 12 hours at 4°C with anti-BrdU antibody (1 : 1000 dilution; Sigma). After removal of unbound antibodies, sections were incubated for 1 hour with biotinylated anti-mouse antibody (1 : 200 dilution; Vector Laboratories), and then incubated with avidine-biotin horseradish peroxidase complex for 30 min (Vector Laboratories). The peroxidase activity was visualized by a color reaction using 0.04% diaminobenzidine (Sigma) and 0.02% hydrogen peroxide as a substrate. The sections were mounted with 50% glycerol in PBS.

RESULTS

1. Identification of NeuroD/BETA2 in the embryo

To investigate the expression of NeuroD/BETA2 in postmitotic cells during embryogenesis, we performed the non-radioactive *in situ* hybridization in E13 whole mouse. To avoid cross-reactivity with other members of bHLH transcription factors, anti-sense probe was generated for the region of 773 to 986 of NeuroD/BETA2 cDNA, which did not contain the bHLH domain (Fig. 1). NeuroD/BETA2 expression was dominant in developing brain. In particular, expression of NeuroD/ BETA2 appeared to be restricted in subventricular zone of developing cerebral cortex (Fig. 2A and 2B). To confirm this area contains postmitotic premigratory cells, we carried out bromodeoxyuridine incorporation assay. BrdU, a thymidine analogue, could be incorporated to DNA during replication. As shown in Fig. 2, immunoreactive region was localized in the ventricular zone of develop-ing brain. NeuroD/BETA2 expressing cells were not overlapped with BrdU positive regions.

2. Expression of NeuroD/BETA2 during postnatal development

At P1, antisense NeuroD/BETA2 transcripts were localized

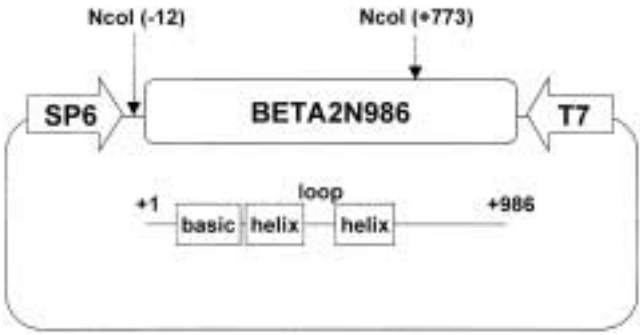

Fig. 1. A schematic model for synthesis of antisense RNA probes of NeuroD/BETA2. Antisense RNA probe of NeuroD/BETA2N 986 was synthesized by *in vitro* transcription using T7 RNA polymerase in the presence of dNTP mix including digoxigenin-UTP after linerization with NcoI restriction enzyme.

chiefly in specific regions of the brain, such as olfactory bulb, hippocampus and granule cells of cerebellum, and cerebral cortex (Fig. 3A and 3B). The expression in olfactory bulb was declined gradually during early postnatal period and specifically confined to the mitral cell layer at P7 (Fig. 3C and 3I). At postnatal stages, pyramidal cells in Ammon's horn (CA1 to CA3) and granule cells in dentate gyrus contained high level of NeuroD/BETA2 transcripts (Fig. 3J). Moderate level of expression was also detected in olfactory bulb and hippocampal formation throughout postnatal periods as tested in P13 (Fig. 3E). The expression in hippocampus and dentate gyrus remained at moderate level in the adult stage (Fig. 3G).

Expression of NeuroD/BETA2 was dramatically changed during postnatal cerebellum development. In postnatal cerebellum, several layers were clearly identified, including external granular layer (EGL), internal granular layer (IGL), Purkinje layer, and molecular layer. Expression of NeuroD/BETA2 was highly detectable in EGL at P7 (Fig. 3C and 3D). However, NeuroD/BETA2 transcripts were mainly found in IGL at P13 as most cells EGL had migrated to IGL (Fig. 3E and 3F). As Purkinje cell layer became evident at P13, NeuroD/BETA2 expression was also detectable in Purkinje cells (compare Fig. 3D to 3F). Strong expression of NeuroD/BETA2 in IGL and Purkinje cells was maintained through the adult stage (Fig. 3H).

DISCUSSION

In this study we showed that dynamic expression of NeuroD/BETA2 in specific regions of the early postnatal and adult brain. Strong expression of NeuroD/BETA2 was detected in the regions undergoing active differentiation, especially in olfactory bulb, hippocampus, and cerebellum.

In situ hybridization and BrdU incorporation assay with E13 embryos demonstrate that NeuroD/BETA2 is expressed in subventricular zone in developing brain where most cells just exit cell cycle and start to differentiate (Fig. 2A and 2B). This result is consistent with the previous notion that in *Xenopus* NeuroD is expressed in postmitotic differentiated neuron at late terminal differentiation stages (Lee *et al*., 1995).

After birth, rapid proliferation in the EGL in cerebellum expands the zone from a single cell layer to a multiple cell layer in thickness (Fugita *et al*., 1966). Precursor granule cells continue to proliferate within the EGL until P13, when the zone disappears due to the inward migration to form the IGL. Thus, in the EGL, proliferating precursor cells tend to reside in the region close to

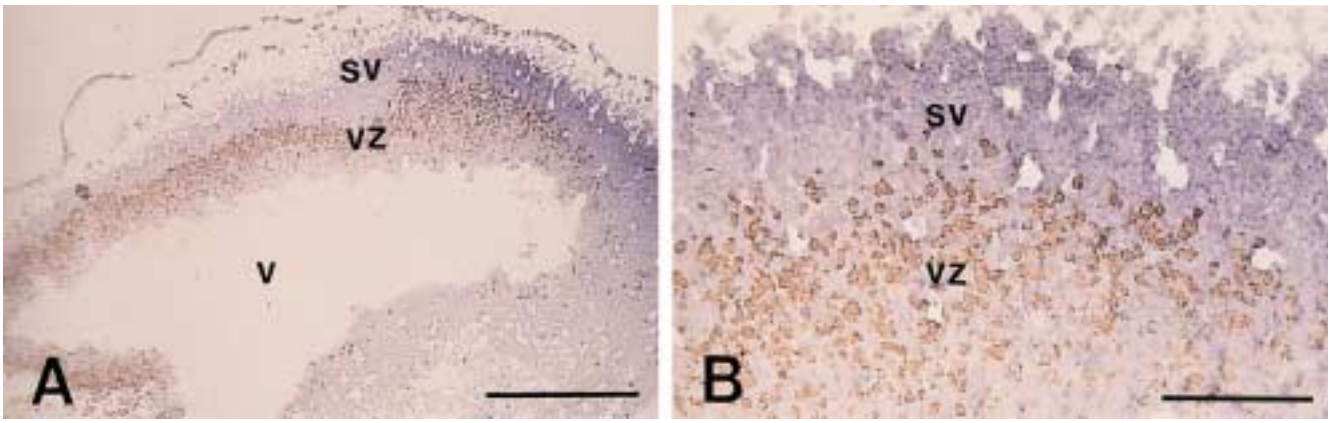

Fig. 2. Localization of NeuroD/BETA2 and BrdU in embryonic day 13 mouse brain. (A) NeuroD/BETA2 was expressed in the subventricular zone of the developing brain (violet). (B) BrdU immunoreactive proliferating cells were localized in the ventricular zone (brown). v, ventricule; vz, ventricular zone; sv, subventricular zone. Scale bars = A, 400 μm; B, 100 μm.

pial surface, whereas postmitotic, premigratory cells are mostly found in the inner zone adjacent to the nascent molecular layer. Interestingly, expression of NeuroD/BETA2 became noticeable in primordial cerebellum at the edge of rhombic lips at E14 (data not shown) where the EGL progenitor cells were beginning to expand (Fugita *et al*., 1966). Since then predominant expression of NeuroD/BETA2 was maintained in the entire region of the EGL as well as in the IGL throughout cerebellar development. This result is consistent with our previous report that the NeuroD /BETA2-immunoreactivity was localized in granular layer and Purkinje cells of adult rat cerebellum (Cho *et al*., 1997). Persistent expression of NeuroD/BETA2 in the adult brain suggests a role of NeuroD/BETA2 in maintenance of neuronal characteristics in mature neurons. Since during postnatal stage EGL contains both mitotic and postmitotic, premigrationg cells, it would be interesting to know whether NeuroD/BETA2 is also expressed in mitotic cells during cerebellum development. Further studies are under investigation.

Our data that strong expression of NeuroD/BETA2 in hippocampus and cerebellum is consistent with a recent report that NeuroD null mice are deficit of neuronal cell in the granular layer of cerebellum and hippocampus (Miyata *et al*., 1999) and a notion that the granular cells of cerebellum share many developmental, morphological, and cellular characteristics with granule cells within dentate gyrus (Altman, 1967; Vicario-Abejon *et al*., 1995).

In summary, expression of NeuroD/BETA2 in developing brain as well as in the mature adult brain suggests that NeuroD/ BETA2 is important not only for neuronal differentiation, but also for terminal differentiation and maintenance of neuronal cells in the adult brain.

ACKNOWLEDGEMENTS

This work was supported by the Academic Research Fund of Ministry of Education, Republic of Korea (Grant number : BM 97-107) to Haeyoung Suh-Kim and by Korean Ministry of Education through a Research Fund (Grant number : 1998-021-F00031) to both Haeyoung Suh-Kim and Yeon-Soo Kim.

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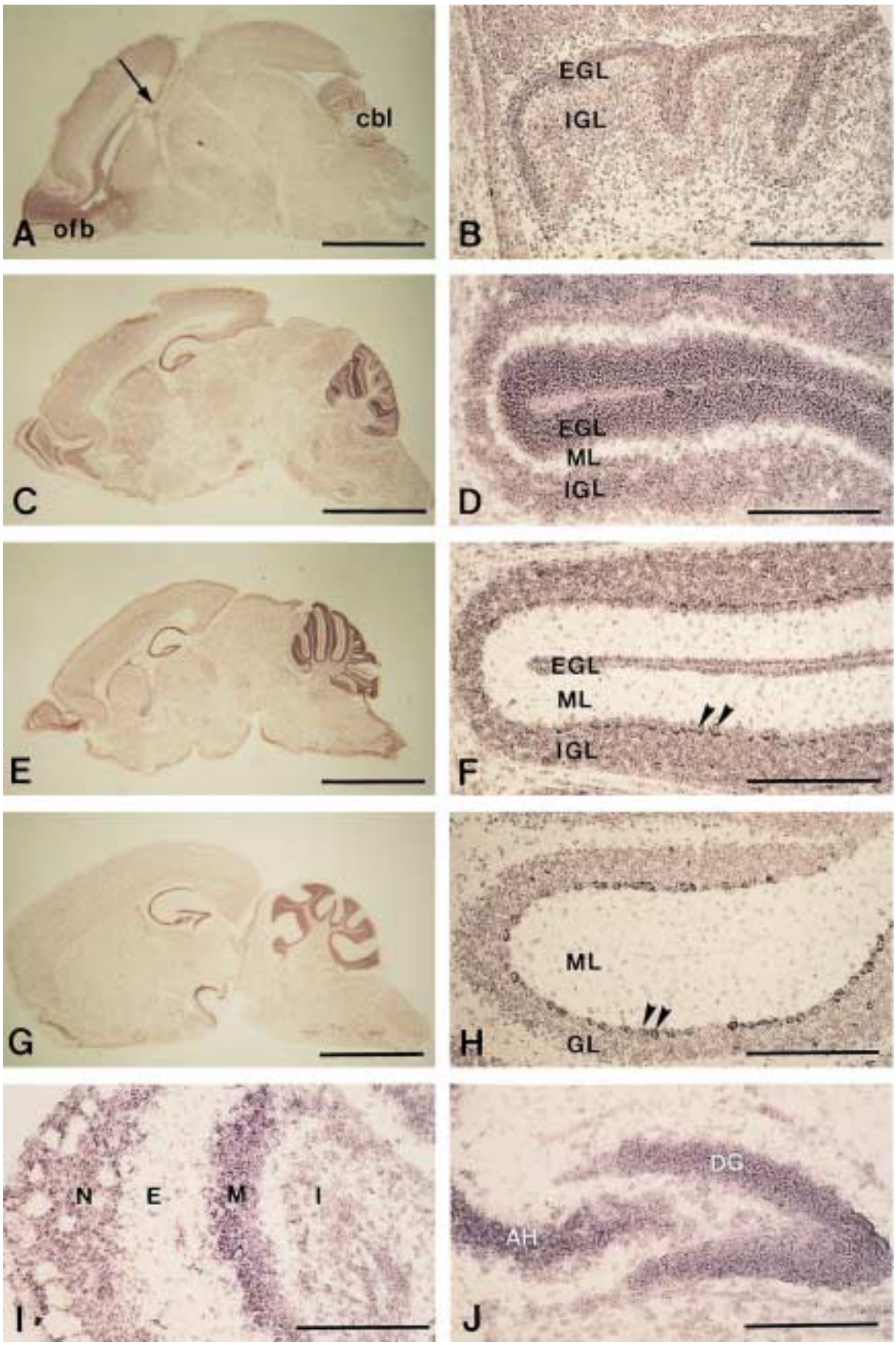
Legends for Figures

Fig. 3. Expression of NeuroD/BETA2 in postnatal mouse brain. Brains of postnatal day 1 (A, B), day 7 (C, D, I, J), day 13 (E, F), and adult (G, H) were hybridized with antisense probes. NeuroD/BETA2 was highly detectable in hippocampus, olfactory bulb, and developing cerebellum. Neither sense probes nor omission of antisense probes gave any significant signals (data not shown). arrow, developing hippocampus; ofb, olfactory bulb; cbl, cerebellum, EGL, external granular layer of cerebellum; IGL, internal granular layer of cerebellum; ML, molecular layer; GL, granular layer; arrowheads, Purkinje cell; N, olfactory nerve; E, external plexiform layer; M, mitral cell layer of olfactory bulb; I, internal granular layer of olfactory bulb; AH, Ammon's horn; DG, dentate gyrus. Scale bars = A, 2.0 mm; C, 3.0 mm; E, 3.2 mm; G, 3.0 mm; B, D, F, and H to J, 200 μm.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〈초 록〉

생쥐 중추신경계에서 NeuroD/BETA2 유전자의 발현

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basic helix-loop-helix 전사인자(transcription factor)에 속하는 NeuroD/BETA2 유전자는 신경형성과정 중 종말분화에 중요한 기능을 하는 것으로 알려져 왔다. 신경계의 발생 및 분화과정에서 NeuroD/BETA2 유전자의 기능을 알기 위해서 본 연구에서는 생쥐의 배자발생과정, 출생초기 및 성숙한 생쥐를 대상으로 *in situ* hybridization방법을 이용하여 유전자의 발현 양상을 조사하였다.

NeuroD/BETA2 유전자는 발생중인 생쥐의 신경계에서 매우 높게 발현되었다. 출생 초기에 NeuroD/BETA2 유전자는 소뇌와 해마에서 시간이 경과할수록 지속적으로 증가하는 발현양상을 보여주었고 성숙 후에도 비교적 안정한 상태로 발현되었다. 출생 전 NeuroD/BETA2 유전자는 배자발생과정 동안 뇌실밑층의 분열을 마친 세포에서 발현되었다. 이러한 결과는 NeuroD/BETA2 유전자가 신경형성과정 중 종말분화과정에 중요한 역할을 담당한다는 사실을 확인시켜 준다.

찾아보기 낱말 : NeuroD, BETA2, 뇌, 생쥐