Preferential posterior cerebellum defect in BETA2/NeuroD1 knockout mice is the result of differential expression of BETA2/NeuroD1 along anterior–posterior axis

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Abstract

BETA2/NeuroD1 has been shown to play a major role in terminal differentiation of the pancreatic and enteroendocrine cells, as well as for the survival of photoreceptors. Here, we report that the loss of BETA2/NeuroD1 affected the cerebellar development with a major reduction of granule cell number. However, there is a differential reduction of granule cells along the anterior and posterior axis of the cerebellum; while the reduction of granule cells in the anterior lobes is substantial, there is an almost complete loss of granule cells in the posterior compartment. To understand the mechanism for this anterior–posterior difference, we carried out detailed analyses. We found that both BETA2/NeuroD1 and its direct target TrkC, expression commence earlier in the posterior part than those in the anterior part during cerebellum development. Consequently, loss of BETA2/NeuroD1 enhances granule cell death in the posterior 2 days earlier than the anterior. Furthermore, the higher rate of cell death in the posterior of the cerebellum is concomitant with the reduction of TrkC expression in knockout mice. Thus, our data indicate that preferential expression of BETA2/NeuroD1 and TrkC in posterior lobes explains the earlier start of cell apoptosis and preferential loss of granule cells in the posterior lobes.

Keywords: BETA2; NeuroD1; bHLH; TrkC; A/P axis; Cerebellum

Introduction

The cerebellum is an excellent system for studying neuronal development. In the adult mouse, it is a relatively simple laminated structure consisting of three layers: the molecular layer, the Purkinje cell layer, and the granular layer. This part of the brain is essential for fine motor control movement and posture of the body (Kandel et al., 1991). The cerebellum is originally derived from the dorsal alar plate of neural tube and is part of the metencephalon. All cells in the cerebellum arise from a germinal matrix. The germinal matrix in the primordial cerebellum is bounded by the isthmus anteriorly and the choroid plexus posteriorly and forms the rhombic lip caudally (Goldowitz and Hamre, 1998). The cerebellar granule precursor cells are derived from the posterior edge of the cerebellar anlage close to the rhombic lip in mice as early as E10.5 (Alderb et al., 1996) and migrate to the pial surface of the developing cerebellum forming the external granular layer (EGL) (Altman and Bayer, 1978). During postnatal cerebellar development, granule precursor cells that have exited the cell cycle migrate radially along the Bergmann glial fibers to form the internal granular layer (IGL) (Fujita et al., 1966). Finally, the postnatal development of the cerebellum is completed by P21 (Altman and Bayer, 1985).

At the gross anatomical level, the adult cerebellum can be divided into several regions extending along the mediolateral axis from the vermis outward to two hemispheres and along the anterior–posterior axis which includes ten lobules in the vermis and eight lobules in each hemisphere. The vermis is also divided into the anterior compartment (lobule I to V or VI) and the posterior compartment (lobule V or VI to X) (Herrup and...
Kuemerle, 1997; Kuemerle et al., 1997; Eisenman, 2000). The existence of this morphological boundary for the anterior and posterior cerebellum is supported by the observations of structural abnormalities that are restricted primarily to the anterior or posterior cerebellar lobule in naturally occurring mutant mice and gene targeted mutant mice (Ackerman et al., 1997; Eisenman, 2000). For example, mice lacking Engrailed-2 (En-2), a homolog of Drosophila engrailed gene, exhibit abnormal anterior–posterior patterning and foliation of the cerebellum (Millen et al., 1994; Kuemerle et al., 1997). In addition, many transcription factors have been identified, characterized, and shown to play an important role in cerebellar granule cell development during cerebellar morphogenesis (Millen et al., 1994; Kuemerle et al., 1997; Ben-Arie et al., 1997; Miyata et al., 1999). Mice lacking Math1 fail to form an EGL (Ben-Arie et al., 1997), and loss of BETA2/NeuroD1 has been shown to result in a severe reduction in the granule cell population of the cerebellum during early postnatal stages (Miyata et al., 1999).

BETA2/NeuroD1 is a member of the basic helix–loop–helix (bHLH) transcription factor family and together with the Math and Neurogenin subfamilies, they belong to the Drosophila proneural Ath group. It is expressed in a subset of postmitotic neuronal precursor cells in the CNS and PNS as early as E8.5, and its expression persists through adulthood (Cho and Tsai, 2004). Initial studies show that mice lacking BETA2/NeuroD1 die within 5 days due to severe perinatal diabetes and have high ketone body levels in the urine as result of severe reduction of pancreatic β-cells, as well as some enteroendocrine cells (Mutoh et al., 1997; Naya et al., 1997). However, either changing genetic backgrounds (Liu et al., 2000a) or tissue-specific expression of an insulin transgene in pancreas (Miyata et al., 1999) rescues diabetic phenotypes in knockout mice. In both cases, surviving knockout mice display similar phenotypic behaviors, including impaired balance, ataxic gait, hyperactivity, circling, and swaying head movement (Miyata et al., 1999; Liu et al., 2000a,b). In addition, BETA2/NeuroD1 mutant mice have abnormal hearing and vision as a result of severe sensory neuronal defects in inner ear and neural retina, respectively (Morrow et al., 1999; Liu et al., 2000b; Kim et al., 2001; Pensesi et al., 2003). Histological analysis reveals that loss of BETA2/NeuroD1 leads to severe defects in granule cells in the dentate gyrus, as well as in a subset of EGL granule neuron of the developing cerebellum (Miyata et al., 1999; Liu et al., 2000a). This morphological abnormality in the cerebellum of BETA2/NeuroD1 knockout mice is consistent with the phenotypic behaviors. However, little is known about the role BETA2/NeuroD1 plays in the survival of granule cells. We have used the knockout mouse model to further examine the role of BETA2/NeuroD1 in cerebellar granule cell development.

In this study, we determined that BETA2/NeuroD1 is mainly expressed in the posterior part of the cerebellum, in the premigrating zone of the EGL at the early stages, and its expression expands to the anterior part of the EGL around birth. The cerebellum of BETA2/NeuroD1 knockout mice is smaller than wild-type littermates and shows severe granule cell reduction in the posterior cerebellum and almost a complete lack of foliation. Furthermore, EGL granule precursors lacking BETA2/NeuroD1 fail to express an appropriate level of neurotrophin receptor, TrkC, and undergo apoptosis. Therefore, BETA2/NeuroD1 is a putative upstream regulator of TrkC, and the differential expression of BETA2/NeuroD1 and TrkC in the anterior–posterior axis, during cerebellar development, likely accounts for the differences of anterior versus posterior defects of BETA2/NeuroD1 knockout mice.

Materials and methods

Animals

BETA2/NeuroD1 knockout mice were generated in the 129SvJ background as described previously (Liu et al., 2000a). The stage of the mouse embryos was determined as embryonic day 0.5 (E0.5) in the morning when the copulation plug was shown. All genotypes described were confirmed by polymerase chain reaction (Naya et al., 1997). All animals were handled in accordance with the policies on the treatment of laboratory animals of Baylor College of Medicine.

Immunohistochemistry

12-μm cryostat sections were obtained from neutral-buffered formalin (NBF) fixed brain samples from E17.5 to postnatal day 7 (P7). Endogenous peroxidase activities and nonspecific antibody binding were blocked with 3% hydrogen peroxide and 3% normal serum, respectively. Affinity purified polyclonal rabbit anti-TrkC and goat anti-NeuroD (N-19) (Santa Cruz biotec) antibodies were used at 1:3000 and 1:500 dilutions, respectively. Monoclonal anti-Calbindin-D-28K antibody (Sigma) and polyclonal anti-cleaved capase-3 (Asp175) antibody (Cell Signaling) were used at 1:200 dilutions. Antibodies were diluted in 3% normal serum in PBS (pH 7.2) containing 3% bovine serum albumin (BSA) and incubated overnight at 4°C. Immunoreactivities were visualized using ABC kit (Vector Laboratories) for colorimetric visualization. For the immunofluorescence staining, donkey cy3-conjugated anti-rabbit and donkey FITC-conjugated anti-goat antibodies were used at 1:400 dilutions (Molecular Probes). To test the possibility of the compensation or functional redundancy by BETA2/NeuroD1 homologues, NeuroD2 and Nexitl, we performed immunohistochemistry using anti-NeuroD2 antibody (Sigma, 1:200 dilution) and anti-Nexitl antibody (Chemicon, 1:200 dilution) along with anti-NeuroD antibody (Santa Cruz) on E17.5 to P5 stages of wild-type and KO mice.

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside activity staining (X-gal staining)

Embryonic (E17.5, E18.5), postnatal (P1-P2), and adult brains (4 months old) were dissected and fixed in 2% PFA for 2 h, rinsed three times with PBS, and incubated in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) solution (2 mM MgCl2, 0.01% deoxycholate, 0.02% NP-40, 100 mM PBS, pH 7.4, 5 mM potassium ferrocyanide (K3Fe(CN)6), and 5 mM potassium ferricyanide (K4Fe(CN)6), and 1 mg/ml X-gal) at room temperature for 8–16 h. X-gal-stained brains were postfixed with 2% PFA for 8–16 h and embedded in paraffin. Sections (7 μm) were deparaffinized, rehydrated, and counterstained with nuclear fast red.

Bromodeoxyuridine (BrdU) incorporation assay

Embryonic (E17.5, E18.5), postnatal (P1-P2), and adult brains (4 months old) were dissected and fixed in 2% PFA for 2 h, rinsed three times with PBS, and incubated in BrdU solution (2 mM MgCl2, 0.01% deoxycholate, 0.02% NP-40, 100 mM PBS, pH 7.4, 5 mM potassium ferrocyanide (K3Fe(CN)6), 5 mM potassium ferricyanide (K4Fe(CN)6), and 1 mg/ml X-gal) at room temperature for 8–16 h. X-gal-stained brains were postfixed with 2% PFA for 8–16 h and embedded in paraffin. Sections (7 μm) were deparaffinized, rehydrated, and counterstained with nuclear fast red.
BrdU positive cells per section was calculated for each animal, and the ratio of positive cells was analyzed. Mean ± SEM was evaluated between wild-type and BETA2/NeuroD1 knockout mice, and significance was determined by Student’s t test. To determined EGL area, same sections were traced and calculated in NIH Image J (version 1.31), and an average was determined.

Detection of apoptotic cells

Apoptotic cell was detected by the terminal deoxynucleotidylttransferase-mediated biotinylated UTP nick end labeling (TUNEL) assay (Gavrieli et al., 1992), with the same modifications. Briefly, the brains were dissected from embryos (E16.5 to E18.5) and postnatal (P0 to P7) mice. Samples were fixed in 10% neutral-buffered formalin (NBF) at 4°C for 16 h. NBF-fixed brains were embedded in paraffin and sectioned at 7 μm. Sections were deparaffinized and rehydrated in a series of graded ethanol solutions and incubated in PBS for 30 min. Sections were permeabilized with 20 μg/ml protease K in 10 mM Tris, pH 8.0, for 20 min at room temperature and were incubated in 3% H2O2 for 10 min at room temperature. After a brief rinse, slides were placed in the TUNEL buffer (incubated for 10 min). TUNEL buffer containing 1 nmol of biotin-16-deoxyUTP (Roche) and 50 U/ml of terminal transferase (Invitrogen) at 37°C for 1 h. Enzyme reactions were terminated by high-salt buffer (300 mM NaCl and 30 mM sodium citrate). Biotin-labeled cells were visualized by the ABC detection system (Vector Laboratories) and counterstained with methyl green. The TUNEL-positive cells in both the anterior and posterior part of EGL were counted from 6 sections (7 μm in thickness) selected from a total of 20 to 50 mid-vernix sections obtained from E17.5 to P7 of 3 independent embryos and animals of each age. The average number of TUNEL positive cells per 6 sections was calculated for each animal. Mean ± SEM was evaluated between wild-type and BETA2/NeuroD1 knockout mouse groups, and significance was determined by Student’s t test. To determined EGL area, same sections were traced and calculated in NIH Image J (version 1.31), and an average was determined. The anterior–posterior boundaries in each stage were determined by in situ hybridization using 35S-labeled Otx-2 probe (data not shown).

Western blot analysis

Cerebellum and cerebral cortex were dissected and extracted using sample buffer (0.5% sodium dodecyl sulfate (SDS), 8 M urea, and 50 mM 2-mercaptoethanol). Proteins were separated by 10% SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane electrophoretically. The membrane was blocked with 5% nonfat dried milk in Tris-buffered saline/0.1% Tween-20 (TBS-T) and incubated with anti-TrkC antibody (1:200 dilutions) in TBS-T. Visualization of the immunological reaction was done by peroxidase-conjugated anti-rabbit IgG in conjunction with enhanced chemiluminescence (ECL, Amersham). The density of the autoradiogram was measured and quantified in ImageJ software (Version 1.31, NIH), and the measurements were normalized by β-actin expression level. Mean ± SEM was evaluated between wild-type and BETA2/NeuroD1 knockout mouse groups, and significance was determined by Student’s t test.

Chromatin immunoprecipitation assay (ChiP)

P0–P1 stages of wild-type animal were sacrificed and granule cells were dissociated using Collagenase/Dispase mixture (Roche). ChiP assays were then performed as described in Yoon et al. (2003). The ChiP assays were performed with anti-NeuroD antibody (N-19). For PCR, 1 μl from 50 μl DNA extraction, and 30–35 cycles of amplification were used. The primers used for PCR were as follows: TrkC-5′, 5′-AGGCTTTCTACGAGAATAGC-3′; TrkC-3′, 5′-CCCCTCTGCCGTAGGCTC-3′.

Photography

Micrographs were photographed with a digital camera (Axiovision 3.1) mounted on Axiostkop2 microscope (Carl Zeiss). Photographs were assembled in Adobe Photoshop (version 7.0), with adjustments to contrast and brightness and color balance to obtain optimum photographs.

Results

To address the in vivo function of BETA2/NeuroD1 gene, we established a BETA2/NeuroD1 null and heterozygous mice showing no statistically significant difference from their wild-type littermates in their body weight by 1 month of age (data not shown).

Severe defects of cerebellar granule cells in the adult BETA2/NeuroD1 knockout mice

Morphological and histological observations reveal that the overall size of BETA2/NeuroD1 knockout mouse cerebellum is decreased to 60% in volume (Figs. 1A, B) and up to 75% in the vermis from those of wild-type littersmates by 4 months of age (Fig. 1D). Interestingly, the lobules VIIb to IX of the posterior compartment of the BETA2/NeuroD1 knockout mouse cerebellum are undistinguishable (Figs. 1A–D). In addition, the crucial (CIII) and paramedial lobes (P) in the lateral hemispheres are missing (Figs. 1A, B). Furthermore, mutants have a lesser degree of foliation throughout entire cerebellum (Figs. 1C–H). Most importantly, a drastic reduction of granule cells was found in the knockout cerebellum, and both granule and molecular cell layers were thinner in knockout mice (Figs. 1C–H). Interestingly, the loss of granule cells in the anterior lobes was partial but substantial; whereas the granule cells were almost completely ablated in the posterior lobes. This gross size reduction in BETA2/NeuroD1 knockout cerebellum was observed as early as the first week after birth with impaired righting reflex responses. Mid-sagittal view of X-gal stained cerebellum revealed that the prominent expression of BETA2/NeuroD is in the granular layer (GL) of adult cerebellum (Figs. 1E, F). Consistent with the reduction of granule cells as shown in Fig. 1D, X-gal-positive granule cells in knockout mice have a major reduction in numbers in the anterior compartment and are almost completely depleted in the posterior compartment of cerebellum (Fig. 1F). These results suggest that BETA2/NeuroD1 is important for the cerebellar development, and they also suggest that it plays a more critical role in the posterior compartment.

To determine whether the effect of ablation of BETA2/NeuroD1 is specific to granule cells, we examined Purkinje cells using an antibody against calbindin (Figs. 1G, H). In the
BETA2/NeuroD1 knockout mice, Purkinje cells appear to have differentiated normally in both anterior and posterior lobes (Fig. 1H), but their arrangement is significantly disturbed in the posterior compartment (Fig. 1H). The total population of the Purkinje cells in the BETA2/NeuroD1 knockout mice shows no statistically significant differences in number by 2 months of age (data not shown) but shows a 30% decrease by 4 months of age (BETA2+/+: 338 ± 30.7; BETA2−/−: 223.3 ± 24.5, P < 0.01). These results suggest that BETA2/NeuroD1 is important for the cerebellar development and even more important for the compartmentalization of the posterior lobe.

**Fig. 1. Cerebellar granule cell defect in the adult BETA2/NeuroD1 knockout mice.** (A, B) Dorsal view of wild-type (BETA2+/+) and BETA2/NeuroD1 knockout (BETA2−/−) mouse cerebellum at 4 months old. (C, D) Cresyl-violet stained mid-sagittal view of BETA2+/+ and BETA2−/− mouse cerebellum. (E, F) X-gal stained mid-sagittal view of BETA2/NeuroD1 heterozygote (BETA2+/−) and BETA2−/− mouse cerebellum. (G, H) Immunoreactivities of anti-Calbindin antibody on BETA2+/+ and BETA2−/− mouse cerebellum with methyl green counterstain. Anterior is to the top of photographs (A and B) and to the left of photographs (C to H). Cerebellar vermis, hemisphere, and fissures are labeled by roman numerals and given names as follows: S, simplex; CI, crus I; CII, crus II; P, paramegal lobe; 1, primary fissure; sp, superior posterior fissure; i, intercrural fissure; py, prepyramidal fissure; 2, secondary fissure; ce, precentral fissure; cu, preculminate fissure; po, posteriolateral fissure; GL, granule cell layer; ML, molecular layer; t, tectum. Scale bars = (A, B), 2 mm; (C –H), 0.5 mm.

Differential anterior–posterior expression of BETA2/NeuroD1 during early development of the cerebellum

To address the cause of differential depletion of granule cells in the anterior–posterior compartment of BETA2/NeuroD1 knockout mice, we asked whether BETA2/NeuroD1 is
differentially expressed along the anterior–posterior axis. Thus, we performed immunostaining on developing and postnatal cerebellum of wild-type mice using anti-NeuroD antibody (Fig. 2). As we suspected, BETA2/NeuroD1 is first detected in the posterior part of cerebellum anlagen around E14.5 (data not shown). It is not surprising to observe BETA2/NeuroD1 expression only in the posterior, since granule cells are only detected in the posterior compartment at this stage. BETA2/NeuroD1 expression later expands to the anterior region of cerebellum at E16.5 (data not shown), which correlates with granule cell development (Goldowitz and Hamre, 1998). Major BETA2/NeuroD1 immunoreactivity was observed in the inner half of EGL where postmitotic differentiating premigratory neuronal precursor cells are localized (Figs. 2A′–B″). In addition, a lower level of BETA2/NeuroD1 expression is also detected in the migratory or postmigratory neuronal cells in the IGL of the cerebellum (Fig. 2, arrows). At E17.5, BETA2/NeuroD1 is more intensively expressed in the posterior half than the anterior half of the EGL (Figs. 2A, A′, A″). However, the difference in BETA2/NeuroD1 expression between these two halves is almost undistinguishable at birth (P0, data not shown) and completely disappears by P1 (Figs. 2B, B′, B″). These results suggest that the different degree of defects between the anterior and posterior compartments of BETA2/NeuroD1 knockout mouse cerebellum can be attributed to differential BETA2/NeuroD1 expression in these two compartments.

Abnormal vermis foliation in perinatal BETA2/NeuroD1 knockout mice

Based on the morphology of the adult BETA2/NeuroD1 knockout mice, it was possible to identify which lobules were affected by BETA2/NeuroD1 null mutation during early cerebellar development. We analyzed the developmental profiles of foliation during early development of cerebellum. To aid our investigation of cerebellum foliation defects, we marked BETA2/NeuroD1 expression cells in the cerebellum. Since the BETA2/NeuroD1 gene of our mutant mice is replaced with β-galactosidase gene by a homologous recombination.
(Naya et al., 1997), it was possible for us to use X-gal staining for this purpose. X-gal stained brains were sectioned sagittally, and sections taken from the region of the cerebellar vermis close to mid-sagittal plane were photographed (Fig. 3). As shown in Fig. 3, BETA2/NeuroD1 is highly expressed in the developing EGL through all ages examined, and it allowed us to trace the dynamic changes of developing EGL very clearly. At E17.5, the initial cerebellar size and foliation patterning of the BETA2/NeuroD1 knockout cerebellum does not appear to be disrupted by the null mutation (Figs. 3A, B). At E18.5, the posterior fissure in the knockout is more shallow (Figs. 3C, D), but in contrast, the two developing anterior fissures, referred to as preculminate (cu) and primary (1) fissures, are visible in the knockout cerebellum as seen in the wild-type littermate (Figs. 3C, D). At P1, major lobulation patterning defect in the null mutant can be clearly observed (Figs. 3E, F). The precentral fissure (ce) has developed in the anterior part of wild-type cerebellum, but no precentral fissure-like formation is observed in the BETA2/NeuroD1 knockout cerebellum. In addition, the other two fissures, preculminate (cu) and primary fissures (1), in the anterior part of BETA2/NeuroD1 knockout cerebellum are much less developed than those of wild-type control mice. Furthermore, the pyramidal fissure (py) in the posterior part is not clearly developed in the

![Fig. 3. Abnormal lobulation/foliation of developing cerebellum of BETA2/NeuroD1 knockout mice.](image-url)

A: X-gal stained mid-sagittal sections from heterozygote (BETA2+/−) mice with nuclear fast red counterstain at E17.5, E18.5, P1, and P2, respectively. (B, D, F, H) BETA2/NeuroD1 knockout mice (BETA2−/−) mice at the same time points were shown. Developing cerebellar vermis and lobules are labeled by roman numerals and given names as follows; 1, primary fissure; ce, precentral fissure; cu, preculminate fissure; py, pyramidal fissure. ant, anterior; post, posterior. Scale bar = 200 μm.
**BETA2/NeuroD1** knockout. These differences persist at P2 (Figs. 3G, H).

**Cell proliferating in BETA2/NeuroD1 knockout mice**

Cerebellar foliation requires the proliferation of granule cells in the EGL (Mares et al., 1970). To address whether the abnormal foliation and reduction of granule cells numbers in the **BETA2/NeuroD1** knockout cerebellum are due to the lack of proliferation in EGL area, we examined the proliferating activities in the embryonic and neonatal cerebellum using bromodeoxyuridine (BrdU) incorporation assay (Fig. 4). No significant differences in EGL cell proliferation were detected between wild-type and knockout cerebellum before birth (Figs. 4A, B). At P0, both the thickness and the length of the layer of BrdU positive granule cells in the anterior EGL of knockout cerebellum (160.8 ± 10.6 cells/section with an average area of 58.3 ± 2.4 μm²/section) were quite similar to littermate controls (185.1 ± 16.7 cells/section with an average area of 66.7 ± 2.9 μm²/section) (P > 0.05) (Figs. 4C, D). However, the posterior EGL of **BETA2/NeuroD1** knockout exhibited a slight but significant difference (P < 0.05) in BrdU-positive cell numbers between the two genotypes (**BETA2**+/+ : 149.2 ± 16.7 cells/section with an average area of 29.0 ± 0.6 μm²/section; **BETA2**−/− : 117.3 ± 7.4 cells/section with an average area of 29.7 ± 0.9 μm²/section) (Figs. 4C, D). By P1, the differences in BrdU incorporation were even more prominent at the posterior part of knockout animals (**BETA2**+/+ : 201.2 ± 17.3 cells/section with an average area of 43.3 ± 1.2 μm²/section; **BETA2**−/− : 92.3 ± 4.9 cells/section with an average area of 30.0 ± 2.1 μm²/section), while the anterior part of the knockout cerebellum did not show any statistically significant difference (**BETA2**+/+ : 220.5 ± 18.3 cells/section with an average area of 78.7 ± 1.2 μm²/section; **BETA2**−/− : 201.2 ± 14.3 cells/section with an average area of 79.0 ± 2.1 μm²/section; P > 0.05) (data not shown). This proliferation defect in knockout mice is even clearer after P2, especially in the posterior part of the cerebellum (Figs. 2E, F) (**BETA2**+/+ : 293.5 ± 15.1 cells/section with an average area of 38.7 ± 2.1 μm²/section; **BETA2**−/− : 136.7 ± 16.2 cells/section with an average area of 31.7 ± 0.9 μm²/section).

Fig. 4. Reduction of proliferation in the cerebellum of **BETA2/NeuroD1** knockout mice. (A, C, E) BrdU incorporation assay from E17.5, P0, and P2 in wild-type (**BETA2**+/+), and (B, D, F) **BETA2/NeuroD1** knockout (**BETA2**−/−) mice with methyl green counterstain was shown. t, tectum. ant, anterior; post, posterior. Scale bar = 400 μm.
Differential anterior–posterior apoptosis in the developing EGL of BETA2/NeuroD1 knockout mice

Although the morphological differences between wild-type and knockout mice are not prominent before birth, the differences become apparent in the posterior part of knockout mice as early as P1 (Figs. 3E, F and 5T), and the overall size reduction is observed as early as P2 (Figs. 3G, H and 5S, T). Since proliferation likely plays little role in this defect, as indicated earlier, it is possible that apoptosis might be the reason for the observed defects of BETA2/NeuroD1 knockout mice during early cerebellum development, especially in the differentiating zone of the EGL where BETA2/NeuroD1 is highly expressed (Figs. 2A–B'). To address this possibility, we performed TUNEL assay on E17.5 all the way to P7 cerebella (Figs. 5E–P). TUNEL labeling of cerebellar tissue reveals an increased number of apoptotic cells in all postnatal BETA2/NeuroD1 null mice, and most of the apoptotic cells are restricted to the innermost layer of the EGL where postmitotic, differentiating, premigrating granule cells reside. However, we did not detect a statistically significant difference in the number of TUNEL-positive cells before birth in any part of the developing cerebellum (data not shown; Figs. 5Q, R). Using an anti-cleaved caspase-3 antibody (Figs. 5A–D), an earlier maker for cell death, we found that there are caspase-3-positive cells only in the posterior cerebellum of BETA2/NeuroD1 knockout mice (Fig. 5D, arrows). At P0, increased numbers of apoptotic cells are detected in the posterior part within the innermost layer of knockout EGL where BETA2/NeuroD1 is expressed (Figs. 5E–H, R). However, very few or normal levels of apoptotic cells are found in the anterior cerebellum of both genotypes (Figs. 5E, F, Q). The cell death in the posterior part of knockout mice peaked at P2 (Figs. 5L, R) and began to subside by P5 (Figs. 5P, R). In contrast, a majority of apoptosis in the anterior cerebellum occurred after P2 (Figs. 5J, N, Q). Total EGL areas were also measured for both wild and mutant cerebella (Figs. 5S and T). Although there is a difference in the vermis size after P0, this difference cannot account for the early increased apoptosis in the posterior cerebellum. Therefore, a more than 2-day head start in apoptosis explains the more severe defect of the posterior cerebellum. This result also suggests that BETA2/NeuroD1, a differentiating factor for neuronal cells (Liu et al., 2000a,b), is required for the terminal differentiation of granule cell precursors in both anterior and posterior lobes and, without it, cells go through apoptosis. Finally, it also indicates that the earlier requirement for the expression of BETA2/NeuroD1 in the posterior cerebellum results in a more severe defect when the BETA2/NeuroD1 gene is deleted.

Lack of neurotrophic receptor TrkC in BETA2/NeuroD1 knockout mice

One of the remaining questions is: what causes the granule cell to die during early cerebellar development? It was shown earlier that the neurotrophin receptors, TrkB and TrkC, which are necessary for survival of neurons (Minichiello and Klein, 1996), are putative downstream targets of BETA2/NeuroD1 (Liu et al., 2000b; Kim et al., 2001). Thus, we asked whether expression of Trks and their ligands is affected in the BETA2/NeuroD1 knockout cerebellum (Fig. 6). The anti-NeuroD antibody (N-19) from Santa Cruz Biotechnologies is designed to detect a short peptide from the initial N-terminal part of BETA2/NeuroD1 protein, for which the coding region is not deleted in the BETA2/NeuroD1 knockout mouse (Naya et al., 1997). Thus, this anti-NeuroD antibody allowed us to perform the immunohistochemistry even in the BETA2/NeuroD1 knockout mice where nonfunctional truncated BETA2/NeuroD1 is expressed. At E17.5, BETA2/NeuroD1 immunoreactivity is observed in posterior to anterior gradient manner in the inner half EGL of both genotypes (Figs. 2A, B; Figs. 6A, a, b). In the wild-type, anti-TrkC antibody immunoreactivity is highly co-localized with that of anti-NeuroD in the innermost layer of posterior part of the EGL (Figs. 6A, a, a'', arrows). Interestingly, some
BETA2/NeuroD-positive cells that reside in the more peripheral part of the EGL do not express TrkC (Fig. 6A, a’). TrkC is also found in the anterior half of the EGL at this stage, although at a much lower level (Figs. 6A, a, a’). In contrast to wild-type littermates, BETA2/NeuroD1 knockout mice exhibit a much reduced TrkC level in the posterior part (Figs. 6A, b, b’’) and almost completely absent in the anterior part (Figs. 6A, b, b’). To investigate this further, we examined the TrkC levels on BETA2/NeuroD1 knockout mice throughout the cerebellum development to correlate TrkC with the level of cell death in the knockout mice (Fig. 6B). In the wild-type
cerebellum, TrkC immunoreactivity was first detected in the developing posterior part of the cerebellar anlagen at E16.5 (data not shown). As shown in Fig. 6B, a high level of TrkC was detected in the posterior part of wild-type animals throughout development (Figs. 6B, c, g, k, o); whereas it remained at a moderate level in the anterior part (Fig. 6B, a, e, i, m). In comparison to wild-type animals, BETA2/NeuroD1 knockout EGL exhibited a much reduced level of TrkC in the posterior as well as anterior parts of the EGL (Figs. 6B, d, h, l, p). Consistent with our immunohistochemistry data, cerebellar extracts from BETA2/NeuroD1 knockout mice showed statistically significant decreases in all age examined (Figs. 6C, a, c). The effect of TrkC levels is specific to the cerebellum because the cerebral extracts obtained from both genotypes did not show any differences in TrkC levels (Fig. 6C, b). Interestingly, BETA2/NeuroD1 knockout mice did not show any significant differences in immunoreactivities for other neurotrophin factors and their receptors, such as brain-derived neurotrophin factor (BDNF), neurotrophin-3 (NT-3), and TrkB (data not shown). This result indicates that TrkC is the major player in mediating the role of BETA2/NeuroD1 in controlling the survival of cerebellar granule cells. The fact that preferential expression of TrkC was observed in the posterior cerebellum could explain the anterior–posterior difference in the cerebellum defect.

A ChIP assay was performed to determine if TrkC is a direct target of BETA2/NeuroD1 (Fig. 7). P0–P1 mice cerebellum was used because TrkC is expressed at high level in the inner half of the EGL at this stage (Fig. 6). The ChIP assay was performed with anti-NeuroD antibody (N-19). Specific PCR amplification was obtained in the NeuroD antibody precipitated chromatin (Fig. 7, lane 1) but not detected when preimmune goat IgG was used (Fig. 7, lane 2). In addition, amplified band was also detected in immunoprecipitated DNA from the cerebellum with anti-NeuroD antibody, but the level was lower than that of the cerebellum (Fig. 7, lane 3). This result suggested that BETA2/NeuroD1 binds to the promoter region of TrkC and directly regulates the expression of TrkC in the differentiation zone of the EGL.

Discussion

Null mutation of BETA2/NeuroD1 exhibits severe neurological symptoms in the nervous system with abnormal behaviors, as we reported previously (Liu et al., 2000a, b; Pennesi et al., 2003). Among those symptoms, abnormal ataxic gait and imbalance are most prominent behavior problems that result from cerebellar malformation and seen as early as the second postnatal week in BETA2/NeuroD1 knockout mice. We observed that the cerebellum of BETA2/NeuroD1 knockout mice is reduced in size starting at P1 (Figs. 3E, F; Fig. 5T), and the foliation patterning defect becomes evident during postnatal stages (Figs. 3E–H), affecting all the posterior fissures while the anterior fissures appear to be less affected. Furthermore, the anterior–posterior differences in granular cell layer formation in adult knockout mice were observed clearly (Fig. 3).

Anterior–posterior axis in the cerebellum

Transgenic expression analysis of meandertail cerebellar mutant provides evidence that the developing cerebellum can be divided into anterior and posterior developmental domains, with the compartment boundary in the lobule VI (Hallonet et al., 1990; Ross et al., 1990; Logan et al., 1992). Significantly, this is the same lobule that divides the differential anterior–posterior defect we observed in BETA2/NeuroD1 knockout mice (Fig. 1). The anterior–posterior boundary is marked by two homeodomain genes, Otx-1 and Otx-2, two murine homologues of the Drosophila orthodenticle (otd) gene. The functional attributes of the anterior–posterior compartments have been studied during embryonic and postnatal development (Frantz et al., 1994). Interestingly, the anterior and posterior compartments of the cerebellum receive signals from the different organs of the body, thereby, defining the function of each cerebellum compartment. For example, the anterior compartment receives a major input from the spinal cord, while the posterior compartment receives signals from the spinal cord and the auditory system (Brodal, 1981; Carpenter, 1985). In contrast to the expression of Otx2, which is restricted to the posterior compartment of cerebellum (Frantz et al., 1994), BETA2/NeuroD1 knockout mice (b) with DAPI (blue) counterstain. High-magnifications of wild-type (a, VV, aV) and BETA2/NeuroD1 knockout mice (V, b, VV, b) were shown. Arrows, anti-NeuroD and TrkC double-positive cells. (B) Anti-TrkC antibody immunoreactivities from E17.5, P0, P2, and P5 in the wild-type mice anterior (a, e, i, m) and posterior part (c, g, k, o) of the EGL, and BETA2/NeuroD1 knockout mice anterior (b, f, j, n), and posterior part (d, h, l, p) of the EGL were shown. (C) Western blot analysis of TrkC level from cerebellum (E17.5 to P2) (a), and cerebrum (P0 and P2) (b). Quantification of the TrkC level from immunoblot analysis (c). Data represent the mean ± SEM from three independent experiments (*P < 0.05; **P < 0.01, Student’s t test) EGL, external granular cell layer; IGL, internal granular layer. Scale bars = (A), 200 μm; (a', a", b', b"), 25 μm.
NeuroD1 is expressed in both compartments in the postnatal brain (Fig. 1E). However, during early cerebellar development, BETAV/NeuroD1 is mainly expressed in the posterior compartment of the cerebellum (Figs. 2A, A’). This preferential expression of BETAV/NeuroD1 and its target TrkC to the posterior compartment, early in the development, may explain the more severe granule cell defects than the anterior compartment of adult cerebellum. Our results, thus, provide further support to our hypothesis that the developing cerebellum is compartmentalized in an anterior–posterior fashion and provides a candidate gene for maintaining such a functional boundary in the cerebellum.

It is possible that differential defect along the anterior–posterior axis can also be contributed by the differential compensation of BETAV/NeuroD1 related genes, NeuroD2 and Next1. To rule out this possibility, we examined the expression of these two related genes and found that there is no apparent compensation in the cerebellum (data not shown). Thus, it is unlikely that differential compensation is the reason of the differential anterior–posterior defect in BETAV/NeuroD1 mutant.

Foliation defect in BETAV/NeuroD1 knockout mice

Morphological analysis shows that the cerebellum of BETAV/NeuroD1 knockout mice is small and has an abnormal pattern of foliation. The foliation defect is much more evident in the vermis, especially in the posterior part of the cerebellum (Figs. 1, 3). Previous studies have shown a correlation between cerebellar foliation and changes in granular cell proliferation and/or cell viability (Mares et al., 1970; Aruga et al., 1998; Olson et al., 2001). For instance, mice lacking Zip1, a zinc finger protein, display a severe foliation defect in the anterior part of the cerebellum due to the abnormal proliferation of cerebellar granule cells (Aruga et al., 1998); whereas enhanced apoptosis of granule neurons in NeuroD2 mutant mice results in foliation defects similar to our mice lacking BETAV/NeuroD1 (Olson et al., 2001). In addition, we previously found that BETAV/NeuroD1 knockout mice have a defect in the granule cell population in the dentate gyrus of hippocampal formation, which shares many developmental, morphological, and cellular characteristics with the granule cells in the cerebellum (Vicario-Abejon et al., 1995; Yang et al., 1996), due to increased apoptosis and decreased proliferation (Liu et al., 2000a). Therefore, we asked whether granule cell proliferation was altered in the BETAV/NeuroD1 knockout mice cerebellum. However, we found that the major expression of BETAV/NeuroD1 is restricted in the inner half of the EGL (Fig. 2) where postmitotic neuronal precursor cells are localized (Lee et al., 1995), and no expression was observed in the outer half of the EGL where proliferating cells are distributed (Fig. 2; Miyata et al., 1999). Thus, the reduced proliferation in the posterior part of the developing cerebellum after birth and in the anterior compartment at P2 is likely secondary to the massive reduction in the number of granule cells due to apoptosis in the BETAV/NeuroD1 knockout EGL. Taken together, our data suggest the cerebellar foliation defect in BETAV/NeuroD1 knockout mice cerebellum is the result of an excessive number of apoptoses rather than on defect of proliferation in the EGL.

Cell death and neurotrophic factors

Cerebellar development involves massive proliferation, cell death, and migration during early postnatal stage. In particular, molecules that protect cerebellar neurons from cell death are just beginning to be identified. The survival of neurons depends upon the neurotrophic factors and the formation of functional contact with neighboring cells. It has been demonstrated that the neurotrophins NT-3 and BDNF and their receptors, TrkB and TrkC, are both essential for the survival of granule neuron in vivo (Schwartz et al., 1997; Bates et al., 1999; Minichiello and Klein, 1996). These factors are expressed in the cerebellar granule neurons throughout the cerebellar development (Klein et al., 1990, Lamballe et al., 1993).

Single mutation of either of TrkB or TrkC gene shows a statistically significant increase in apoptosis in the EGL, and the double mutation of neurotrophin receptors TrkB and TrkC results in a more significant increase in cell death in the EGL, than a single mutation at the second week of postnatal stages (Minichiello and Klein, 1996). Thus, both TrkB and TrkC neurotrophin receptors are required for cerebellar granule cell survival during differentiation processes. This leads us to believe that the spatiotemporal expression pattern of neurotrophin receptor of either TrkB or TrkC would be important for the cerebellar granule cell survival during critical points of granule cell development in the BETAV/NeuroD1 knockout mice.

TrkC mRNA has been shown by in situ hybridization to be expressed in the embryonic, early postnatal stages, and adult cerebellum (Lamballe et al., 1993) and together with its ligand, NT-3, promotes the differentiation of postmitotic, premigratory granule cell precursor, accelerating cell exit from the EGL (Doughty et al., 1998). Null mutation of BDNF, a ligand of TrkB, shows an impaired EGL granule cell migration with no differentiation defect in the EGL (Borghesani et al., 2002), indicating that BDNF and its receptor, TrkB, are more important for regulating granule cell migration to the IGL than granule cell differentiation in the EGL. This agrees with our data showing a reduction of TrkC, a direct target of BETAV/NeuroD1, but not TrkB expression, in the EGL of BETAV/NeuroD1 knockout mice and suggests that a reduction in TrkC expression, as a consequence of BETAV/NeuroD1 loss, results in the improper differentiation and survival of granule neurons.

In conclusion, we have demonstrated that BETAV/NeuroD1, by inducing its target, TrkC, is necessary for the proper differentiation and survival of cerebellar neurons. Therefore, null mutation of BETAV/NeuroD1 results in the increased granule cell apoptosis and, consequently, the reduction of the cerebellar size and fissure formation. In addition, the spatiotemporal expression patterns of BETAV/NeuroD1 and TrkC correlate well with a 2-day lead in the start of apoptosis and...
more severe defects in the posterior lobes. Taken together, our results suggest that the differential requirement in these two compartments is necessary for their differentiation and survival. In addition, our results demonstrate the importance of BETA2/NeuroD1 in cerebellar development and in anterior–posterior axis establishment.

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