NeuroD2 Is Necessary for Development and Survival of Central Nervous System Neurons

James M. Olson,*†,1,2 Atsushi Asakura,*1,3 Lauren Snider,* Richard Hawkes,‡ Andrew Strand,* Jennifer Stoeck,* Andrew Hallahan,*† Joel Pritchard,* and Stephen J. Tapscott*2

*Clinical Research and Human Biology Divisions and Program in Developmental Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109; †Division of Pediatric Hematology and Oncology, Children’s Hospital and Medical Center, Seattle, Washington 98105; and ‡Department of Cell Biology and Genes & Development Research Group, University of Calgary, Calgary, Alberta, Canada T2N 4N1

NeuroD2 is sufficient to induce cell cycle arrest and neurogenic differentiation in nonneuronal cells. To determine whether this bHLH transcription factor was necessary for normal brain development, we used homologous recombination to replace the neuroD2 coding region with a β-galactosidase reporter gene. The neuroD2 gene expressed the reporter in a subset of neurons in the central nervous system, including in neurons of the neocortex and hippocampus and cerebellum. NeuroD2−/− mice showed normal development until about day P14, when they began exhibiting ataxia and failure to thrive. Brain areas that expressed neuroD2 were smaller than normal and showed higher rates of apoptosis. Cerebella of neuroD2-null mice expressed reduced levels of genes encoding proteins that support cerebellar granule cell survival, including brain-derived neurotrophic factor (BDNF). Decreased levels of BDNF and higher rates of apoptosis in cerebellar granule cells of neuroD2−/− mice indicate that neuroD2 is necessary for the survival of specific populations of central nervous system neurons in addition to its known effects on cell cycle regulation and neuronal differentiation. © 2001 Academic Press

Key Words: neurogenic bHLH transcription factors; cerebellum; BDNF; neuronal apoptosis; microarray analysis.

INTRODUCTION

Neurogenic bHLH transcription factors contribute to the development of a rich assortment of neurons in the brain and peripheral nervous system (Jan and Jan, 1993; Guillemot, 1995; Lee, 1997). The neurogenin members of this family act as determination factors, inducing transcription of genes that confer a neuronal fate on multipotent progenitors (Ma et al., 1996; McCormick et al., 1996; Fode et al., 2000). Neurogenin1-null mice fail to develop proximal sensory cranial nerve ganglia and exhibit abnormal dorsal telencephalon development (Ma et al., 1998; Fode et al., 2000). Neurogenin2-null mice lack distal cranial nerve ganglia (Fode et al., 1998). Because neurogenins are not expressed in appreciable levels in postmitotic neurons, no role in long-term neuronal survival has been ascribed to these family members.

The members of the neuroD subset of neurogenic bHLH transcription factors are characterized as neuronal differentiation genes because they induce cell cycle arrest in neural precursors and induce transcription of genes that contribute to mature neuronal phenotype (Lee et al., 1995; Naya et al., 1995; McCormick et al., 1996; Farah et al., 2000). These transcription factors are expressed in postmitotic neurons throughout adulthood. The deletion of NeuroD in mouse brain revealed a previously unappreciated role for NeuroD Delta-like 1, and neural differentiation genes such as neuroD and neuroD2 (J. Olson, unpublished data, and Ma et al., 1997). Neurogenin1-null mice fail to develop proximal sensory cranial nerve ganglia and exhibit abnormal dorsal telencephalon development (Ma et al., 1998; Fode et al., 2000). Neurogenin2-null mice lack distal cranial nerve ganglia (Fode et al., 1998). Because neurogenins are not expressed in appreciable levels in postmitotic neurons, no role in long-term neuronal survival has been ascribed to these family members.

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in neuronal survival (Naya et al., 1997; Miyata et al., 1999). The cerebellar granule cells in these mice underwent apoptosis during the first weeks of life, resulting in premature death of >90% of this cell population (Miyata et al., 1999). Hippocampal granule cells also failed to form in the absence of NeuroD, contributing to an epileptogenic phenotype (Miyata et al., 1999; Liu et al., 2000).

Like neuroD, neuroD2 is expressed in postmitotic cerebellar neurons as well as other neuronal populations in the adult brain (McCormick et al., 1996; Yasunami et al., 1996; Kume et al., 1998; Schwab et al., 1998; Miyata et al., 1999). Although the capacity of NeuroD2 to induce differentiation in mammalian neural precursors has been established (Kume et al., 1998), its role in differentiation and survival of neurons in developing brain remained to be elucidated. Toward this goal, we deleted the neuroD2 gene by homologous recombination. The resulting mice experienced progressive neurologic deterioration and early death. Neuronal populations that normally express neuroD2 were reduced in neuroD2–/– mice and exhibited increased TUNEL staining. Cerebella of neuroD2-null mice expressed reduced levels of genes encoding proteins that support cerebellar granule cell survival, suggesting a mechanism by which neuroD2 promotes neuronal maintenance in addition to its known effects on cell cycle regulation and neuronal differentiation.

**MATERIALS AND METHODS**

**Targeted Disruption of the neuroD2 Gene**

The pPGKneobAlox2PGKDTA vector containing the coding region of the lacZ gene, the neomycin-resistance gene flanked by lox sites, and the negative selection marker diphtheria toxin A was used to generate recombinants (gift from Phil Soriano). The lacZ and neomycin genes were flanked by a 2-kb HindIII–SalI fragment from the 5′ upstream region and a 4-kb Xhol–HindIII fragment from the 3′ region of the neuroD2 gene. G418-resistant colonies were transfected with a plasmid encoding Cre recombinase to eliminate the pgk–N neo cassette, leaving the lacZ gene in place of the entire neuroD2 coding region. Chimeras generated from two independent targeted ES clones were maintained on a 129/SV background or backcrossed onto C57/BL/6 background (seizure analyses). Littermate controls were used for all experiments. Genotyping was performed using AX2R primer (ACAGTCTGACAAGGTAA G-GCTGTTCCCACG) and ND21F primer (GTCTTACGATA TG-CACCTTCACCACGATCG) to detect wild-type and mutant PCR products from which sufficient tissue was available. Ten micrograms of total RNA per lane was electrophoresed, transferred onto nitrocellulose membranes, and probed with random-primed probes (generated from least cross-reactive region of neuroD1 and neuroD2 coding sequences). RNA loading was confirmed by 18S probe. Gene expression analyses were performed as previously described (Mancini et al., 1998). Eight parasagittal sections (every sixth section beginning at midline) were stained for apoptosis using the TUNEL method. TUNEL-positive cells were counted separately in the entire molecular and granule cell layer by an investigator that was blinded to the genotype. For quality control, the first 100 sections were counted by two independent investigators. Interinvestigator variance was less than 5% in all cases.

**Histology and Protein Analysis**

Mice were anesthetized with Avertin, then transcardially perfused with saline followed by 4% paraformaldehyde. X-gal staining was performed as previously described (Miyata et al., 1999). The antibodies used for immunocytochemistry were mouse monoclonal anti-calbindin-D 28K (1/1000; Sigma, St. Louis, MO), mouse monoclonal anti-zebrin II (1/200; Brochu et al., 1990), mouse monoclonal anti-parvalbumin (1/1000; Sigma), mouse monoclonal anti-NF200 (Dako), mouse monoclonal anti-glial fibrillary acidic protein (anti-GFAP; 1/100; Dako), mouse monoclonal anti-human natural killer cell antigen 1 (anti-HNK-1; Eisenman and Hawkes, 1993), mouse monoclonal anti-neurofilament-M (1/1000; Chemicon), and rabbit polyclonal anti-BDNF (1/200; Santa Cruz) at the dilutions shown in parentheses. Western analyses used the same anti-NF-M (1/500) and anti-BDNF (1/150) or anti-calbindin (1/7500; Chemicon) for primary staining and horseradish peroxidase-conjugated secondary antibodies.

**Gene Expression Analyses**

RNA was extracted using TRIZOL Reagent (Gibco) according to the manufacturer's recommended protocol. mRNA pellets were resuspended in nuclease-free water and quantitated spectrophotometrically. Northern analysis was performed on a subset of specimens from which sufficient tissue was available. Ten micrograms of total RNA from each sample was electrophoresed, transferred onto Schleicher and Schuell Nyttran membrane, hybridized with 10 × 106 cpm random-primed probe (generated from least cross-reactive region of neuroD1 and neuroD2 coding sequences) overnight in F1 buffer (1.5× SSPE, 10% PEG 8000, 7% SDS), washed 3 × 20 min at 52–57°C in 0.1× SSC/0.1% SDS, and then exposed to Kodak film for 18–42 h. RNA loading was confirmed by 18S probe. NeuroD1 probe was a 350-bp Psrl fragment from the mouse cDNA that encompasses the region spanning amino acids 187–304; NeuroD2 probe was a 635-bp mouse cDNA fragment encompassing amino acids 210 through the 3′ nontranslated region. Probes were used for immunocytochemistry were mouse monoclonal anti-glial fibrillary acidic protein (anti-GFAP; 1/100; Dako), mouse monoclonal anti-human natural killer cell antigen 1 (anti-HNK-1; Eisenman and Hawkes, 1993), mouse monoclonal anti-neurofilament-M (1/1000; Chemicon), and rabbit polyclonal anti-BDNF (1/200; Santa Cruz) at the dilutions shown in parentheses. Western analyses used the same anti-NF-M (1/500) and anti-BDNF (1/150) or anti-calbindin (1/7500; Chemicon) for primary staining and horseradish peroxidase-conjugated secondary antibodies.

**Neurologic Assessment**

Gait analysis was performed by measuring inked paw prints from mice walking through a 30 × 4-in. tunnel, then dividing width by stride. Motor analysis was performed on a Rota-Rod that accelerated to a speed of 8–10 rpm. Kainic acid-induced seizure threshold analysis was performed as previously described in C57/BL/6 mice (Hu et al., 1998). Seizure analysis was conducted on F4 C57/BL/6 backcrossed mice. Briefly, litters were injected intraperitoneally with 30 mg/kg kainic acid, marked with colored ink, then observed for 1 h by an investigator that was blinded to the genotype. An “X” was recorded each time an animal exhibited seizure activity (e.g., staring, circus movements). To avoid recording the same seizure multiple times, a maximum of one “X” per event was assigned within each 5-min period. Animals were sacrificed upon entering status epilepticus or at the completion of the 60-min observation period.

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cloning into Topo-TA vectors using the following primers and wild-type mouse cerebellum: BDNF sense (CGCCCATGAAAGAATGAAC), BDNF antisense (CAGTGACTATACACAGGAA), c-fos sense (AGAGGCAGACATGCGCA), c-fos antisense (TTGCTGACTCTTGACTG), c-kit sense (ATCGAGATTTGCAATTACG), and c-kit antisense (AGCGGTCGCTGAGAAGA). Resulting plasmids were sequenced to verify the insert. All Northern analyses revealed a single primary band (approximately 2.6 kb for NeuroD, NeuroD2, Math1, and Math2 and approximately 3 kb for c-kit and c-jun) with the exception of that for BDNF, which yielded multiple bands, ranging from 1.5 to approximately 4.2 kb, representing multiple splice variants consistent with previous reports.

Preparation of labeled cRNA. RNA from the cerebellum of three neuroD2-/- or three age-matched wild-type animals at each age was combined to comprise each sample, consisting of approximately 150 μg of total RNA (two independent pools were generated for each genotype). Poly(A) RNA was isolated from the samples using Oligotex mRNA isolation kits (Qiagen, Chatsworth, CA). Biotinylated cRNAs were prepared per Affymetrix (Santa Clara, CA) protocol. Labeled cRNA (32 μg) was fragmented in 40 μl of 40 mM Tris-acetate, pH 8.0, 100 mM KOAc, 30 mM MgOAc for 35 min at 95°C. The fragmented cRNA was brought to a final volume of 300 μl in hybridization buffer containing 100 mM M es, 20 mM EDTA, 0.01% Tween 20 (all from Sigma Chemical), 1 M NaCl (Ambion, Austin, TX), 0.5 mg/ml acetylated BSA (Gibco BRL, Gaithersburg, MD), 0.1 mg/ml herring sperm DNA (Promega, Madison, WI), and biotinylated controls B2, BioB, BioC, BioD, and Cre (Affymetrix) at 50, 1.5, 5, 25, and 100 pM, respectively.

Array hybridization and analysis. Mu6500 A, B, C, and D oligonucleotide arrays were prehybridized, hybridized, washed, and stained as recommended by the manufacturer (Affymetrix) using a GeneChip Fluidics Station 400 (Affymetrix). The arrays were hybridized sequentially over the course of 4 days using 200 μl of cRNA (0.1 μg/ml) per hybridization. After hybridization of one array, the aliquot was recovered from the array, recombined with the unused portion of the sample, and frozen. This process was repeated with each sample until the sample had been hybridized to the entire set of four arrays. Immediately after being washed and stained, the probed arrays were scanned with a Hewlett-Packard GeneArray scanner. The scanned images were analyzed and compared using GeneChip 3.1 software (Affymetrix; Lipshutz et al., 1999). Images were globally scaled to compensate for minor variations in fluorescence and bring the mean average difference for all of the genes on each array to 2500 intensity units. Difference calls (increased or decreased) were based on the default proprietary GeneChip 3.1 algorithm. No secondary thresholds (e.g., minimum fold change) were applied.

RESULTS

Growth Arrest, Motor Deficits, and Seizure Threshold in NeuroD2 Mutant Mice

NeuroD2-null and heterozygous mice were derived by homologous recombination using a strategy that replaced the entire coding region of the neuroD2 gene with the lacZ reporter gene and removed the pgk-Neo selection cassette (Fig. 1A). Two independent lines of mice, in which the neuroD2 gene was successfully eliminated as determined by Northern and Southern analysis (Fig. 1B and data not shown), yielded identical results. Genotyping of 198 live pups from NeuroD2-/- intercrosses in a mixed C57/129SV background demonstrated a normal Mendelian distribution of the mutant locus: 52 +/- (26%), 95 +/- (48%), 51 +/- (26%). Both heterozygous and homozygous pups developed normally until approximately postnatal day 14 (P14), but...
the neuroD2−/− mice died between P14 and P35. Following P14, the neuroD2−/− mice developed a wide-based ataxic gait (data not shown), demonstrated growth arrest (Fig. 2A), and had impaired motor performance on a Rota-Rod test (Fig. 2B), a test of gross motor coordination. In contrast to neuroD-null mice, glucose levels were within normal limits at all ages tested (data not shown). Although the mutant mice had decreased growth, they were observed to eat and drink, and autopsy revealed food remnants in the stomach and intestines. Remarkably, the heterozygous mice also showed decreased weight gain and a significantly worse performance on the Rota-Rod test compared to wild-type littermates (Figs. 2A and 2B). In addition, the adult heterozygous mutants had a low incidence of unexplained death, and occasional spontaneous seizure activity was observed.

To evaluate seizure threshold, we injected wild-type and neuroD2-null mice with kainic acid, which induces seizures by activating excitatory amino acid neurotransmitter receptors (Hu et al., 1998). In three independent experiments, neuroD2-null mice uniformly developed status epilepticus within seconds to several minutes following kainic acid (20–30 mg/kg kainic acid, data not shown). In contrast when given 30 mg/kg kainic acid, wild-type mice exhibited no effect, or only staring, during a 1-h observation period (Fig. 2C). At the same dose, the neuroD2+/− mice typically exhibited circling, excessive grooming, other stereotypic behavior, posturing, and tonic clonic episodes (Fig. 2C).

Expression of NeuroD2-Driven lacZ Gene in Cerebellum and Cortex

In an attempt to understand the reason for the early death and neurologic impairment of the neuroD2 mutant mice, we examined the expression pattern of the lacZ gene that had been used to replace the neuroD2 coding sequence. Consistent with the previous observation that neuroD2 mRNA was first detected at embryonic day 12.5 (E12.5) by Northern analysis (McCormick et al., 1996), neuroD2+/− embryos exhibited β-galactosidase activity in the neocortex and spinal cord beginning at E12 (Fig. 3A). At E19, β-galactosidase activity persisted in these structures and was also observed in optic tectum, brain stem, cerebellar vermis, and deep cerebellar nuclei and external germinal

FIG. 2. NeuroD2-null and -heterozygous mice differ from wild type mice. (A) Growth curves for wild-type (triangle, n = 7), heterozygote (square, n = 8), and NeuroD2-null (diamond, n = 6) offspring of F4 × F4 mice crossed onto C57 black/6 background. Null mice were significantly smaller than wild type at day 21 (P < 0.01 by paired t test). (B) Motor performance analysis in P21 mice. Rota-Rod testing demonstrated that wild-type mice typically remained on the turning cylinder for >18 min, neuroD2−/− mice fell off within 10 s and the performance of neuroD2 heterozygotes was intermediate. (C) 21-day-old neuroD2 heterozygotes (gray arrows) exhibited circling, tremor, excessive grooming, posturing, and tonic clonic seizures at doses that were subthreshold for wild-type littermates (black arrows). An “X” is shown for each time the animal exhibited the behavior. Observations were made by an investigator that was blinded to mouse genotype. To avoid multiple recordings of the same seizure, each behavior was recorded only once per 5-min period. Also, staring was recorded only once per animal, though most animals developed staring within the first 5 min and continued to stare throughout the observation period. Primary data from two experiments are shown.
layer of cerebellum (Figs. 3B–3D). Mature basket, stellate, and granule cells all stained with β-galactosidase in neuroD2-heterozygous and -null mice as did deep cerebellar nuclei neurons (Figs. 3E–3G and data not shown). Purkinje cells were β-galactosidase negative and in situ hybridization using a probe that does not cross-react with other known neurogenic bHLH genes revealed absence of staining in Purkinje cells (Figs. 3G and 3H and data not shown). Purkinje cells were β-galactosidase negative and in situ hybridization using a probe that does not cross-react with other known neurogenic bHLH genes revealed absence of staining in Purkinje cells (Figs. 3G and 3H and data not shown). (Although earlier studies using in situ hybridization reported expression of neuroD2 in the Purkinje cells of the cerebellum (McCormick et al., 1996), it is possible that this represented cross-hybridization since we do not detect any expression of the neuroD2 gene in our current study.) In other regions of the postnatal brain, β-galactosidase staining was present in all layers of cerebral cortex and hippocampus and in a subset of thalamic and hypothalamic nuclei (detailed expression data to be presented elsewhere) (Figs. 3E–3G). At all ages examined, expression was restricted to the central nervous system, and no staining was observed in pancreas, retina, nasal epithelium, or peripheral nerves.

**Decreased Survival of NeuroD2-Expressing Neurons in NeuroD2 Mutant Mice**

In a comparison of the gross histology of wild-type, neuroD2+/−, and neuroD2−/− brains, all major populations of neurons that expressed NeuroD2 were present, although regions that had large populations of NeuroD2-expressing cells appeared smaller. Specifically, it was evident that the cerebella of null mice had the appropriate number of folia, but were smaller and had a reduced fissure depth (Fig. 4). The area of the granule cell layer was measured on midsagittal sections using an MCID image analysis system. Com-
pared to wild-type littermate controls, the cerebellar granule cell layer area in neuroD2-null mice was 75 ± 10% of control, whereas the cerebellar molecular layer was no different between the two genotypes (Fig. 4 and data not shown).

To determine whether the diminished cerebellar size in neuroD2-null mice was due to loss of a specific subpopulation of neurons, immunohistochemical staining for each type of cerebellar cell was performed. Purkinje cells, basket cells, granule cells, stellate cells, Golgi cells, fibrous astrocytes, and Bergman glia were all present and morphologically normal (Figs. 5A and 5B).

Since all neuronal populations were present, but there was a reduction in the area of the granule cell layer, we conducted bromodeoxyuridine (BUDR) incorporation studies and TUNEL assays to determine whether deficiency of NeuroD2 might alter granule cell birth or death, respectively. Wild-type and mutant mice had similar numbers of cells in the external germinal layer (the population that generates the granule cells) that incorporated BUDR (data not shown), indicating that granule cell generation was not altered by NeuroD2 deficiency. Although we detected no increase in apoptosis by TUNEL staining prior to P14, increased apoptosis was observed in neuroD2−/− mouse cerebella at P14, P19, P21, and P22. Despite the cerebella being smaller in null mice than wild type, there were significantly more TUNEL-positive cells in neuroD2-null cerebella than wild type at all of these ages (Figs. 6A–6C).

**FIG. 4.** Influence of neuroD2 deletion on cerebellum. Hematoxylin and eosin staining of cerebella from P21 wild-type (A, B) and neuroD2-null (C, D) mice. Lobulation differences are minor. NeuroD2-null mouse granule cell layers are thinner than wild type, demonstrated in high-power images (B, D) of the primary fissure base. In contrast to neuroD-null mice, no axial differences were apparent. Roman numerals I–X mark the 10 cerebellar lobules in sagittal view.
FIG. 5. Immunocytochemical analysis of neurons and glia in neuroD2-null (left columns) and wild-type (right columns) mice. Cellular constituents of cerebella are all present in P21 neuroD2-null mouse cerebella. (A and B) Purkinje cells stained with anti-calbindin. (C and D) Parasagittal compartmentation is intact as shown by anti-zebrin II/aldolase C. (E and F) Molecular layer interneurons (stellate cells) are present (arrowheads, anti-parvalbumin). (G and H) Granule cell layer, stained with anti-10B5, is present but thinner (see Fig. 4). (I and J) Basket cell interneurons stained with anti-neurofilament 200. (K and L) Golgi cells stained with anti-HNK-1. (M and N) Fibrous astrocytes in the granular layer and Bergman glia in the molecular layer stained with anti-glial fibrillary acidic protein. Scale bar, 250 μm.
FIG. 5—Continued
This was observed in both the granule cell layer and in the molecular layer. Based on position within the molecular layer, it appeared that both stellate and basket cells underwent increased cell death. Migrating granule cells are no longer present in the molecular layer after P14. Apoptosis was not quantitated in deep cerebellar neurons, the other cerebellar neuronal population that normally expresses neuroD2, because concordant sections could not be reliably generated between mice. Similarly, reliable quantitation was not possible in hippocampus. It is worth noting, however, that TUNEL-positive cells were rarely seen in the granule cell layer of the hippocampus of wild-type mice, but were present in most sections from neuroD2−/− mice (Figs. 6D–6F).

Altered Gene Expression in NeuroD2 Mutants

To assess the influence of the NeuroD2 deletion on a subset of neurogenic bHLH transcripts expressed in the cerebellum, Northern analysis was performed for NeuroD, Math1, and Math2/Nex in day 14 and day 21 cerebellum and cerebral cortex. There were no dramatic differences in the expression of these bHLH genes in neuroD2−/− mice, although NeuroD was slightly increased at day 14 and decreased at day 21 (Fig. 1B).

Expression array analysis was used to identify transcripts with altered abundance in the NeuroD2 cerebellum at P21. Of approximately 6000 mRNAs that were evaluated, only 27 were diminished in neuroD2-null mice compared to controls using standard Affymetrix GeneChip software criteria (Table 1). Notable among these were brain-derived neurotrophic factor (BDNF), tyro3/sky, ras-p21, 3CH134 (mitogen-activated protein kinase [MAPK] phosphatase), c-fos, and c-jun, all of which may play a role in cerebellar neuron survival (Stitt et al., 1995; Courtney et al., 1997; Crosier and Crosier, 1997; Schwartz et al., 1997; Skaper et al., 1998). Northern analysis for a subset of mRNAs confirmed the microarray data and extended the findings to a second time point (14 days) and to cerebral cortex (Fig. 7A).

To determine whether the gene expression changes from the array data and the Northern analysis reflected alteration of protein levels, Western blots and immunocytochemistry for BDNF and neurofilament-m (NF-M) were performed. Western analysis of protein from cerebellum showed that both BDNF and NF-M were decreased in neuroD2-null mice compared to wild-type littermates (Fig. 7B). Immunocytochemistry showed BDNF in both the Purkinje cells and the granule neurons in wild-type cerebellum, consistent with prior reports (Fig. 6C; Schwartz et al., 1997). In the neuroD2−/− mice, the BDNF staining was significantly reduced in the granule cell layer but relatively maintained in the Purkinje cells (Fig. 7D). Immunocytochemistry for NF-M demonstrated decreased staining in basket cell projections surrounding Purkinje cells and reduced staining of the fiber tracts between the folia and the deep cerebellar nuclei (Figs. 7G and 7H). It is notable that basket cells are present and make heavy (NF-H, neurofilament 200), but not medium, neurofilament in the absence of NeuroD2 (compare Figs. 5C and 5D with 7G and 7H). NF-M was also decreased in the processes surrounding the cell bodies of the pyramidal neurons of the hippocampus, possibly reflecting decreased NF-M expression in the basket cell population of the hippocampus (Figs. 7E–7J). In addition, NF-M staining was reduced in some fiber tracts, such as the corpus callosum, but was unchanged in some areas of the brain, such as the fiber tracts in the brain stem (data not shown). Therefore, expression of some neural genes, such as BDNF and NF-M, was decreased only in selective subsets of neurons, whereas these same genes were expressed at apparently normal levels in other neuronal populations in the brain.

DISCUSSION

NeuroD2−/− mice exhibited small stature, postnatal premature death, ataxia, motor deficits, reduced seizure threshold, and excessive neuronal cell death possibly related to diminished expression of survival factors. Compared to the other neurogenic bHLH transcription factor-null mice that have been reported, neuroD2-null mice are most like neuroD−/− mice in which diabetes-mediated neonatal lethality has been rescued by transgenic expression of NeuroD under the regulation of the insulin promoter; Miyata et al., 1999). This similarity might be expected since NeuroD and NeuroD2 share a high degree of homology and their expression patterns are broadly overlapping (McCormick et al., 1996). Even so, the cerebellar phenotype of the neuroD−/− and neuroD2−/− mice differed substantially. NeuroD−/− mice exhibited massive granule cell loss in an axial gradient early in postnatal cerebellar development. In contrast, in neuroD2−/− mice the apoptosis of cerebellar neurons occurred later postnatally, was less extensive, and did not display an axial gradient. The apoptosis of the granule cells of the hippocampus was also less extensive in the neuroD2−/− mice, whereas in the neuroD−/− mice the entire population of dentate gyrus neurons was eliminated (Miyata et al., 1999; Liu et al., 2000). Since both NeuroD and NeuroD2 are expressed in the granule cells of the cerebellum and dentate gyrus, the extensive death of these cells in the neuroD−/− mice indicates that the high degree of homology does not result in complete functional redundancy.

Indeed, there is a complex expression pattern of bHLH proteins in the developing cerebellum. The cerebellum forms from two alar plate germinal matrices adjacent to the fourth ventricle. The cerebellar primordium forms the rhombic lip which gives rise to the neurons and glia of the cerebellar folia and the precerebellar primordium gives rise to the deep cerebellar nuclei. During cerebellar development, Math1 is expressed at the midbrain/hindbrain junction at E9.5 and is later expressed in the rhombic lip and the...
external granule cell layer (Akazawa et al., 1995). Deletion of Math1 results in the absence of cerebellar granule cells (Ben-Arie et al., 1997). In contrast to the death of differentiated granule cells in the NeuroD and NeuroD2 mutant mice, the absence of granule cells in the Math1 mutant is due to diminished generation of granule cell precursors (Ben-Arie et al., 1997). Therefore, the role of Math1 is consistent with a determination gene, whereas NeuroD and NeuroD2 appear to function in differentiation and survival.

The role of other bHLH genes in the developing cerebellum has yet to be determined. By E12, neurogenin and neurogenin2 are also expressed in rhombic lip progenitors (Sommer et al., 1996; Ma et al., 1998). Reports on ngn1-, ngn2-null mice have focused on other brain areas and have not yet reported a cerebellar phenotype, but the absence of specific neuronal populations in these studies suggests that they also have a role in neuronal determination. Subsequent to the expression of these genes implicated in neuronal determination, NeuroD and NeuroD2 are expressed in the germinl trigone (a population of cells in the rhombic lip that give rise to the external granule cell layer (EGL)), deeper cells in the rhombic lip, and deep cerebellar nuclei progenitors. These transcription factors continue to be expressed in cerebellar granule cells and deep cerebellar nuclei during terminal differentiation and through adulthood. By E18, the EGL spreads over the cerebellar surface and continues to proliferate until it is approximately 10 cell layers thick by gestation. The deeper EGL cells, which express NSCL1 and NSCL2, stop proliferating and migrate through the molecular and Purkinje cell layers to form the internal granule cell layer (IGL; Duncan et al., 1997; Haire et al., 1996). Nex/Math2 is not expressed in external granule cells, but is expressed along with NeuroD and NeuroD2 in postmigratory IGL. N ex1/Math2-deficient mice had apparently normal cerebella and NSCL1 and NSCL2 knock-out mice have not yet been reported (Schwab et al., 1998).

It is informative that in both the neuroD 

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**FIG. 6.** Reduced granule cell number is due to apoptosis in neuroD2-null mice. Eight parasagittal sections (every sixth section beginning at midline) were stained for apoptosis using the TUNEL method. No significant differences were observed in animals younger than P14. (A) In the granule cell layer, TUNEL staining was significantly increased in neuroD2-null mice compared to wild type at P14, P19, P21, and P22 (*P < 0.05, **P < 0.01). NeuroD2 heterozygotes showed increased apoptosis at P19. (B) In the molecular layer, TUNEL staining was significantly increased in neuroD2-null mice compared to wild type at P14, P19, P21, and P24. No significant differences were seen in the molecular layer of heterozygous mice. (C and D) Images of TUNEL-positive cells in cerebellum and hippocampus (arrowheads). (E and F) TUNEL staining in hippocampal dentate gyrus from neuroD2-null (E) and wild-type (F) mice. Images shown are representative of both TUNEL staining and dentate gyrus thickness (granule cell number) observed in multiple litters.
TABLE 1
mRNAs That Differ in NeuroD2-Null Cerebella

<table>
<thead>
<tr>
<th>Fold Δ</th>
<th>GenBank</th>
<th>Entrez definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decreased mRNAs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−9.3</td>
<td>U58471</td>
<td>NeuroD2</td>
</tr>
<tr>
<td>−2.8</td>
<td>X55573</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>−2.4</td>
<td>U18343</td>
<td>Growth factor receptor tyrosine kinase tyro3)</td>
</tr>
<tr>
<td>−2.5</td>
<td>Y00864</td>
<td>c-kit</td>
</tr>
<tr>
<td>−2.4</td>
<td>V00727</td>
<td>c-fos</td>
</tr>
<tr>
<td>−1.1</td>
<td>J04115</td>
<td>c-jun</td>
</tr>
<tr>
<td>−1.1</td>
<td>AA027387</td>
<td>*RAS-related protein RAB-4</td>
</tr>
<tr>
<td>−1.7</td>
<td>X61940</td>
<td>Protein tyrosine phosphatase type 16 (3CH134)</td>
</tr>
<tr>
<td>−1.9</td>
<td>AA138791</td>
<td>*GTPase-activating protein (ras-p21)</td>
</tr>
<tr>
<td>−2</td>
<td>W10081</td>
<td>*Guanine nucleotide regulatory protein</td>
</tr>
<tr>
<td>−1.7</td>
<td>X05640</td>
<td>NF-M middle-molecular-mass neurofilament protein</td>
</tr>
<tr>
<td>−2.7</td>
<td>M15525</td>
<td>Laminin B1</td>
</tr>
<tr>
<td>−3.7</td>
<td>U08372</td>
<td>Asialglycoprotein receptor (MHL-1)</td>
</tr>
<tr>
<td>−1.6</td>
<td>M73741</td>
<td>α-B-crystallin</td>
</tr>
<tr>
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<td>M13227</td>
<td>Enkephalin</td>
</tr>
<tr>
<td>−2.7</td>
<td>AA011789</td>
<td>*Chlorine channel protein P64</td>
</tr>
<tr>
<td>−4.4</td>
<td>X54149</td>
<td>MyoD118, myeloid differentiation primary response gene</td>
</tr>
<tr>
<td>−1.7</td>
<td>AA168903</td>
<td>*SPOB transcriptional regulator</td>
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<td>AA016424</td>
<td>*X box binding protein</td>
</tr>
<tr>
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<td>U37091</td>
<td>Carbonic anhydrase IV</td>
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<tr>
<td>−1.4</td>
<td>W85270</td>
<td>*Inorganic phosphatase</td>
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<td>W97576</td>
<td>*Dihydrolipoamide acetyltransferase</td>
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<tr>
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<td>Phosphatidylinositol 4-phosphate 5-kinase type I-α</td>
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<td>L40406</td>
<td>Heat shock protein (hsp-E71)</td>
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<td>M21332</td>
<td>MHC class III RD (H2-d and H2-Sk haplotypes)</td>
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<td>−2.2</td>
<td>X58438</td>
<td>MP4 gene for a proline-rich protein</td>
</tr>
<tr>
<td>−1.7</td>
<td>W77226</td>
<td>*ADP-ribosylation factor-like protein 3</td>
</tr>
</tbody>
</table>

| Increased mRNAs                                     |
| 2.5     | W13136     | *Angiotensinogen precursor         |
| 2.3     | W17473     | *Angiotensinogen precursor         |
| 2.3     | AA031158   | *Extensin precursor               |
| 2.5     | U51805     | Liver arginase mRNA               |
| 2.2     | W81960     | *Aryl sulfotransferase            |
| 4.2     | L04961     | Nuclear-localized inactive X-specific transcript (Xist) |
| 1.7     | U17259     | p19                                |
| 2.5     | D78135     | Glycine-rich RNA binding protein CIRP |
| *EST similar to listed gene                        |

neuroD2−/− mice, the generation and differentiation of neurons can occur, but specific populations of neurons have decreased survival. Therefore, NeuroD and NeuroD2 appear to have a critical role in mediating the survival of determined neurons during critical periods of differentiation. Particularly in the nervous system, survival is an important component of differentiation, since large numbers of neurons are eliminated if appropriate synaptic contacts are not achieved. The alterations of gene expression in the neuroD2−/− cerebellum give some insight into the role of these transcription factors in neuronal survival. It is remarkable that, of the approximately 6000 genes assessed on the expression array, the small number reduced in neuroD2−/− cerebella included BDNF, tyro3, c-fos, c-jun, ras-p21, 3CH134 (MAPK phosphatase), and MyD118. In the normally developing brain, neurotrophins such as BDNF are soluble factors that support neuron survival by activating Trk tyrosine kinase receptors. BDNF-mediated neuronal survival is mediated by the phosphatidylinositol-3 kinase and the ras/MAPK signaling pathway in turn phosphorylates BAD, a proapoptotic protein, which suppresses BAD-mediated cell death (Bonni et al., 1999).

Mice deficient in BDNF exhibit ataxia, cerebellar hypoplasia, and increased apoptosis of cerebellar neurons (Schwartz et al., 1997), similar to neuroD2−/− mice, raising the possibility that reduced BDNF in neuroD2−/− mice is partially responsible for the cerebellar phenotype. The cerebella of neuroD2−/− and BDNF−/− mice are not identical, however, particularly with regard to Purkinje cells. In neuroD2−/− mice, BDNF protein expression is preserved in Purkinje cells, which do not express neuroD2, and their dendritic trees develop normally. In BDNF−/− mice, Purkinje cell dendrite formation is delayed, stunted, and incomplete. In contrast to the BDNF−/− mice, in the neuroD2−/− mice BDNF is decreased only in the granule cell population and is preserved in the Purkinje cells. Also, the Northern analysis suggests that the decrease of BDNF expression by the cerebellar granule cells might occur only following P14. In the neuroD2−/− mice, the early expression of BDNF and continued expression by the Purkinje cells might support the survival of Purkinje cells and some of the granule cells.

It is interesting that select populations of neuroD2-expressing cells showed decreased expression of a subset of neural-specific genes. The granule cells of the cerebellum had decreased expression of BDNF, whereas BDNF expression appeared preserved in the Purkinje cells and in the cerebral cortex. Similarly, some neuronal populations had preserved expression of Nf-M, whereas Nf-M expression was decreased in processes surrounding the Purkinje cells and pyramidal neurons of the cerebellum and hippocampus, respectively. Although we have not unambiguously identified the neurons with decreased Nf-M expression, in both regions a population of basket cell neurons is a likely candidate. The fact that NeuroD2 would be necessary for BDNF and Nf-M expression in some neurons, but not in...
FIG. 7. Confirmation of a subset of microarray findings by Northern analysis, Western analysis, and immunocytochemistry. (A) Representative mRNAs that were called decreased in neuroD2-null cerebella at P21 were also decreased by Northern analysis. The same genes were measured in cerebral cortex and in P14 brains. A representative loading control probed with 28S is shown. (B) Western analysis of brain-derived neurotrophic factor (BDNF) and neurofilament-m (NF-M) shows decreased protein level for both in cerebella of P21 neuroD2-null mice compared to wild type. The same blots were reprobed for calbindin, which did not change on microarray analysis. Anti-calbindin binding was equal in cerebella from all genotypes, demonstrating that the lanes were evenly loaded. (C and D) BDNF immunocytochemistry in cerebella of wild-type (C) and neuroD2−/− (D) mice at P21. Cerebellar granule cell BDNF staining (g) was reduced in P21 neuroD−/− mice compared to wild type, whereas Purkinje cell staining (arrow) was similar in both genotypes. Staining along fiber tracts was equal in both genotypes (not shown). (E-J) Neurofilament-m staining. Staining was diminished in fiber tracts of cerebellum and in processes that appear to be basket cell neurites in neuroD2-null mice (F) compared to wild type (E). NF-M staining in cerebellar basket cells is shown in closer view in G and H (compare to Figs. 5C and 5D). Similarly, NF-M staining in processes of the hippocampal dentate gyrus was markedly reduced in neuroD2-null mice (J) compared to controls (I). The reference NF-M staining in H is in cerebellar fiber tracts similar to those shown in F and reference staining in I in fiber tracts ventral to the dentate gyrus. NF-M staining was similar in brain stem and multiple other brain regions of neuroD2-null mice. Shown are representative sections. Staining patterns were consistent among multiple litters of animals.
others, argues that the family of neurogenic bHLH proteins might act in a partly redundant manner. Our findings are consistent with a model in which other neurogenic bHLH proteins might regulate BDNF in early cerebellar development in a manner independent of neuroD2, as evidenced by the relatively normal levels of BDNF RNA at P14 in the cerebellum, but later in development the granule cells become dependent on the expression of neuroD2 for continued expression of normal levels of BDNF. If this model is correct, then much more severe phenotypes would be expected in mice with disruption of additional neurogenic bHLH genes.

The growth arrest and early postnatal death of the neuroD2−/− mice remain unexplained, though we have ruled out diabetes, gastrointestinal malformations, and anorexia as primary causes of death. The failure of neuroD2-null animals to gain weight after day 14 and depleted fat stores are reminiscent of diencephalic syndrome, which develops in children that develop gliomas that compress hypothalamic nuclei and resolves after decompression (Gropman et al., 1998). Corresponding nuclei in mice express neuroD2 and are grossly normal in neuroD2−/− mice, but we have not ascertained whether pathways involving these nuclei were intact.

The fact that neuroD2-heterozygote mice demonstrated diminished motor performance, impaired growth, and reduced seizure threshold compared to wild-type controls suggests that both copies of neuroD2 are necessary for normal brain development and function. This raises the possibility that polymorphisms in the human neuroD2 locus could contribute to variations in motor or cognitive development or predispose patients to epilepsy or other neuropsychiatric diseases. Precedent for functionally relevant polymorphisms in bHLH transcription factor genes have been established for neurod/beta2, which is associated with diabetes predisposition in humans, and for the myogenin locus, which influences porcine skeletal muscle mass and growth rate (Iwata et al., 1999; Malecki et al., 1999). The human neuroD2 gene is located on 17q12 but has not yet been implicated in human variation and disease.

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REFERENCES


Brain Development in NeuroD2−/− Mice


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