

## Regulation of neuroD2 expression in mouse brain

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### Abstract

The basic helix–loop–helix (bHLH) transcription factor, neuroD2, induces neuronal differentiation and promotes neuronal survival. Reduced levels of neuroD2 were previously shown to cause motor deficits, ataxia, and seizure propensity. Because neuroD2 levels may be critical for brain function, we studied the regulation of neuroD2 gene in cell culture and transgenic mouse models. In transgenic mice, a 10-kb fragment of the neuroD2 promoter fully recapitulated the endogenous neuroD2 staining pattern. A 1-kb fragment of the neuroD2 promoter drove reporter gene expression in most, but not all neuroD2-positive neuronal populations. Mutation of two critical E-boxes, E4 and E5 (E4 and E5 situated 149 and 305 bp upstream of the transcriptional start site) eliminated gene expression. NeuroD2 expression was diminished in mice lacking neurogenin1 demonstrating that neurogenin1 regulates neuroD2 during murine brain development. These studies demonstrate that neuroD2 expression is highly dependent on bHLH-responsive E-boxes in the proximal promoter region, that additional distal regulatory elements are important for neuroD2 expression in a subset of cortical neurons, and that neurogenin1 regulates neuroD2 expression during mouse brain development.

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### Introduction

NeuroD2/NDRF/KW8 (hereafter neuroD2) plays a critical role in neuronal differentiation and survival. Like the related neurogenic basic helix–loop–helix (bHLH) transcription factor neuroD1/beta2, neuroD2 is sufficient to convert non-neuronal cells into neurons in a *Xenopus* model. NeuroD2 also induces neuronal differentiation in mammalian P19 embryonal carcinoma cells, to a greater degree than other neurogenic bHLH transcription factors tested (Farah et al., 2000). Mice deficient for neuroD2 experience excessive apoptosis in central nervous system

populations that normally express neuroD2. These mice exhibit small brains, ataxia, reduced seizure threshold, growth failure, and early death (Olson et al., 2001). Mice that were heterozygous for neuroD2 exhibited the same deficits as neuroD2-null mice, though not as severe. This haploinsufficiency phenotype in mice raised the possibility that neuroD2 expression levels may influence human brain development and function. Since neuroD2 participates in genesis of neurons involved in learning, memory, coordination, and cranial nerve function, it is important to understand how the gene is regulated in the central nervous system.

In the developing mouse brain, neuronal determination proteins such as neurogenin1, neurogenin2, and MASH1 commit multipotent progenitors to a neuronal rather than astroglial fate (Nieto et al., 2001; Sun et al., 2001). In *Xenopus*, chick, and zebrafish, neurogenins induce expression of neuroD (Blader et al., 1997; Koyano-Nakagawa et al., 1999; Perez et al., 1999). Regulation of neuroD family

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members by neurogenins in mammalian brain is implied by the absence of neuroD1 expression in precursors of cranial sensory nerves of neurogenin-null mice (Fode et al., 1998; Ma et al., 1998). In telencephalon, neurogenin expression begins at E8.5, peaks at E11–14, and wanes by E16 in mouse brain (Ma et al., 1998). Neurogenin2 peaks in a similar fashion and is maintained into late embryonic and early postnatal stages. Concurrent with peak expression of neurogenin1 and neurogenin2, neuroD1 and neuroD2 mRNAs become detectable (E10.5 and E11, respectively). Neurogenins bind to E-box promoter elements (CANNTG) and have been shown to induce transcription driven by neuroD1 and neuroD2 promoter fragments in cell lines (Farah et al., 2000; Huang et al., 2000; Oda et al., 2000). In neuroendocrine cells, neurogenin3 positively regulates the expression of neuroD1 promoter-driven reporter constructs through two E-box sequences (CAGATG and CAT-ATG) that are located within 400 bp upstream of the transcriptional start site. Similarly, neurogenin1 activation of a neuroD2 promoter-reporter construct is reduced by mutation of a CAGATG E-box (E4, located 149 bp upstream of exon 1) in P19 embryonal carcinoma cells (Oda et al., 2000).

In this study, we confirm that E4 is necessary for neurogenin1 regulation of neuroD2. We also show that a nearby E-box, E5, is necessary for full neurogenin1 activity. Toward the goal of understanding neuroD regulation in mammalian brain, we report for the first time a detailed analysis of the brain regions in which the endogenous neuroD2 promoter is active. This expression pattern is fully recapitulated in mice that transgenically express the *lacZ* gene driven by a 10-kb fragment of the neuroD2 promoter (10 kb:*lacZ* mice) and largely recapitulated by a 1-kb fragment (1 kb:*lacZ* mice). Mutation of E4 and E5 in the 1 kb:*lacZ* mice completely abrogates transcriptional activity in mouse brain demonstrating that the neurogenin- and neuroD-responsive elements are critical for induction and maintenance of neuroD2 expression. Finally, we show that the neuroD2 promoter is inactive in certain populations of developing neurons that lack neurogenin, providing direct evidence that this neuronal determination factor is necessary for activation of the neuroD2 promoter in certain mammalian neuronal populations.

## Materials and methods

### Plasmid construction

A 10-kb fragment of DNA immediately upstream of the neuroD2 transcription start site was isolated during the initial cloning of neuroD2 (McCormick et al., 1996). The pPD46.21 plasmid was used as the backbone for  $\beta$ -galactosidase reporter constructs and the pGL3 basic plasmid was used as the backbone for luciferase reporter constructs. The 1-kb fragment and deletion mutants of the 1-kb fragment

were generated using standard cloning techniques. Point mutations were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with PCR primers containing the specified mutation (original E4 sequence: TAG AGA GTG **ACA GAT GGC GGC GGG TCC CGG**; mutant E4: TAG AGA GTG **AAA GCT TGC GGC GGG TCC CGG**; original E5 sequence: CCA TTG TTC **CCA TGT GGG GGG TTC TAT ATC**; mutant E5: CCA TTG TTC **CCT GCA GGG GGG TTC TAT ATC**). All constructs were sequenced to verify the absence of unintended mutations and the presence of intended mutations. In particular, the E4/E5 mutant constructs were shown to have an intact transcription start site and no mutations other than the intended E-box mutations.

### *In vitro* transfection

P19 embryonal carcinoma cells were maintained in subconfluent monolayers in DMEM with 10% fetal bovine serum and 5% bovine calf serum (FBS and BCS; Hyclone, Logan, UT). NSH neuroblastoma cells were maintained in DME with 10% BCS. Cells in 35-mm dishes were transfected with 2.1  $\mu$ g of DNA (total) using FuGENE 6 transfection reagent according to manufacturer's directions (P19 cells) or 4.1  $\mu$ g of DNA using Superfect reagent (NSH cells) (FuGENE: Roche; Superfect: Qiagen).  $\beta$ -galactosidase and luciferase assays were conducted 48 h after transfection unless otherwise noted using previously described techniques (Olson et al., 2001).

### EMSA methods

NeuroD1, neuroD2, neurogenin1, neurogenin2, E12 proteins (and empty CS2) vector were prepared using TNT coupled wheat germ *in vitro* transcription/translation extract system (Promega). For gel mobility shift assays, 5 ml samples of each protein were first incubated together at 37°C for 20 min. Each protein mix was subsequently incubated with approximately 50,000 cpm of  $^{32}$ P-labeled double-stranded oligonucleotide probe at room temperature for 15 min in the binding buffer: 20 mM Hepes (pH 7.6), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 1  $\mu$ g double-strand poly dI-dC. The DNA–protein complex was resolved on 5% polyacrylamide gel at room temperature. The following probes were used: E4 (AGAGTGACAGATGCGGGCGG), E5 (TTGTTCCCATGTGGGGGGTT), mutE4 (AGAGTGAAAGCTTGCGGGCGG), and mutE5 (TTGTTCCCTGCAGGGGGTT).

### Generation and analysis of transgenic mice

Transgenic mice were generated through the University of Washington Department of Comparative Medicine Transgenic Core and maintained in the Fred Hutchinson Cancer Research Center Animal Health Resource facility in accordance with federal and institutional regulations. Fragments

containing the appropriate fragment of neuroD2 promoter, the  $\beta$ -galactosidase gene, and a polyA sequence were isolated by restriction digest (1 kb digested with *EagI*, 10 kb digested with *PstI*–*EagI*), purified using Schleicher and Schuell columns according to the manufacturer's directions, and provided to the transgenic core for zygote (E0.5 fertilized eggs) injection. Transgene presence was confirmed on genomic DNA derived from tail or toe using PCR with the following primers AATCTCTGCTTTTCCTGCGTTGGG (forward) and GGGCGATCGGTGCGGGCCTCTTCGC (reverse). Southern analysis was performed using genomic DNA cut with *BamHI* and *NdeI* for 10 kb:*lacZ* mice and with *EcoRI* for 1 kb:*lacZ* and mutE4E5:*lacZ* mice, using the 1 kb promoter fragment as probe. We analyzed at least two independent lines from the 10 kb:*lacZ* and 1 kb:*lacZ* transgenic mice totaling the following numbers: 10 kb:*lacZ* mice: 6 embryos and 8 adults; 1 kb:*lacZ* mice: 7 embryos and 32 adults. We also analyzed four independent lines of mutE4E5:*lacZ* transgenic mice totaling six embryos and eight adults. There were no consistent differences between lines generated from the same construct.

#### *X-gal staining of embryos*

Timed-pregnant females were anesthetized with avertin and euthanized by cervical dislocation. Embryos were removed, rinsed briefly in ice-cold PBS, and then incubated for 2 h at 4°C in fixative solution (2% formaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3) with gentle agitation. The fixed embryos were washed three times with rinse solution (0.01% sodium deoxycholate, 0.02% NP-40, 2 mM MgCl<sub>2</sub> in 0.1 M phosphate buffer, pH 7.3) and then soaked in X-gal solution containing 2 mM MgCl<sub>2</sub>, 20 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 20 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 0.01% sodium deoxycholate, 0.02% NP-40, and 10 mg/ml X-gal in 0.1 M phosphate buffer. Stained embryos were rinsed with PBS, paraffin embedded, and then cut into 12- $\mu$ m sections. Following deparaffinization, sections were counterstained with Fast Red (Vector Laboratories, Burlingame, CA).

#### *X-gal staining and anti- $\beta$ -galactosidase antibody immunostaining for postnatal mice*

Postnatal and adult mice were anesthetized with avertin and perfused with PBS and then 4% paraformaldehyde. Both 50- and 500- $\mu$ m sections were cut by vibratome in cold phosphate buffer and stained with X-gal solution as described previously. Subsequently, after X-gal staining, the 500- $\mu$ m sections were paraffin embedded, cut into 12- $\mu$ m sections, and then counterstained with Fast Red. For anti- $\beta$ -galactosidase antibody immunostaining, 50- $\mu$ m sections were cut by vibratome in cold phosphate buffer, blocked with 10% normal goat serum, 2% BSA, 0.1% Triton X-100 in PBS, incubated with 1:500 dilution of monoclonal anti- $\beta$ -

galactosidase antibody (Promega), and then incubated with rhodamine- or FITC-conjugated secondary antibody.

#### *Analysis of double mutants of neurogenin1:neuroD2 and neurogenin2:neuroD2*

Heterozygous neurogenin1 (*ngn1*) and neuroD2 (*nd2*) males and females were bred to get *ngn1*<sup>-/-</sup>:*nd2*<sup>+/-</sup> and *ngn1*<sup>+/-</sup>:*nd2*<sup>+/-</sup>. Two time points at E13.5 and P0.5 were processed and analyzed by X-gal staining as described previously. The same breeding strategy and analysis was performed on *ngn2*:*nd2* double mutants. Analyses were performed on six *ngn1*:*nd2* mice, six *ngn2*:*nd2* mice and six of each control.

## Results

### *Identification of necessary proximal promoter elements*

Approximately 10- and 1-kb fragments immediately upstream of the neuroD2 transcription start site were cloned into reporter constructs. Preliminary experiments indicated that the 10- and 1-kb fragments were both responsive to endogenous transcription factors in subsets of P19 embryonal carcinoma and NSH neuroblastoma cell lines (not shown). We first focused on the 1-kb fragment (Fig. 1A). This fragment contains nine E-boxes (labeled E2–E10 from proximal to distal according to the convention of Oda et al., 2000).

A series of deletion and point mutants were generated from this fragment and cloned into luciferase reporter plasmids. In NSH neuroblastoma cells, neurogenin1 increased the expression of the nonmutated 1 kb:*luc* reporter 79.7  $\pm$  9.1-fold. Neurogenin1 activity was retained in the absence of E6 or E10 and most neurogenin activity was retained in the absence of E7 and E8. Neurogenin1 failed to induce all constructs that lacked E4 and E5 (Fig. 1B). NeuroD2 increased the expression of the nonmutated 1 kb:*luc* reporter by 9.4  $\pm$  0.6-fold. Like neurogenin1, neuroD2 had minimal activity on constructs that lacked E4 and E5, but showed baseline activity on other mutants (Fig. 1B).

To further explore the role of E4 and E5, these sites were altered by site-directed mutagenesis, cloned into luciferase reporter constructs, and co-transfected with neurogenin, neuroD2, or vector control DNA into NSH neuroblastoma cells (Fig. 1C). Compared to control, neurogenin1 increased activity of the parent vector ( $\Delta$  –1003 to –506, refer to Fig. 1B), 62  $\pm$  46.2-fold but did not activate the vector with mutated E4 (1.1  $\pm$  0.3-fold). The activity on the construct with mutated E5 was diminished to a lesser extent (11.3  $\pm$  5.6-fold). Induction of the nonmutated vector was less for neuroD (10.6  $\pm$  4.8) and neuroD2 (17.7  $\pm$  11.6) than neurogenin. The activity of neuroD and neuroD2 were diminished by mutation of E4



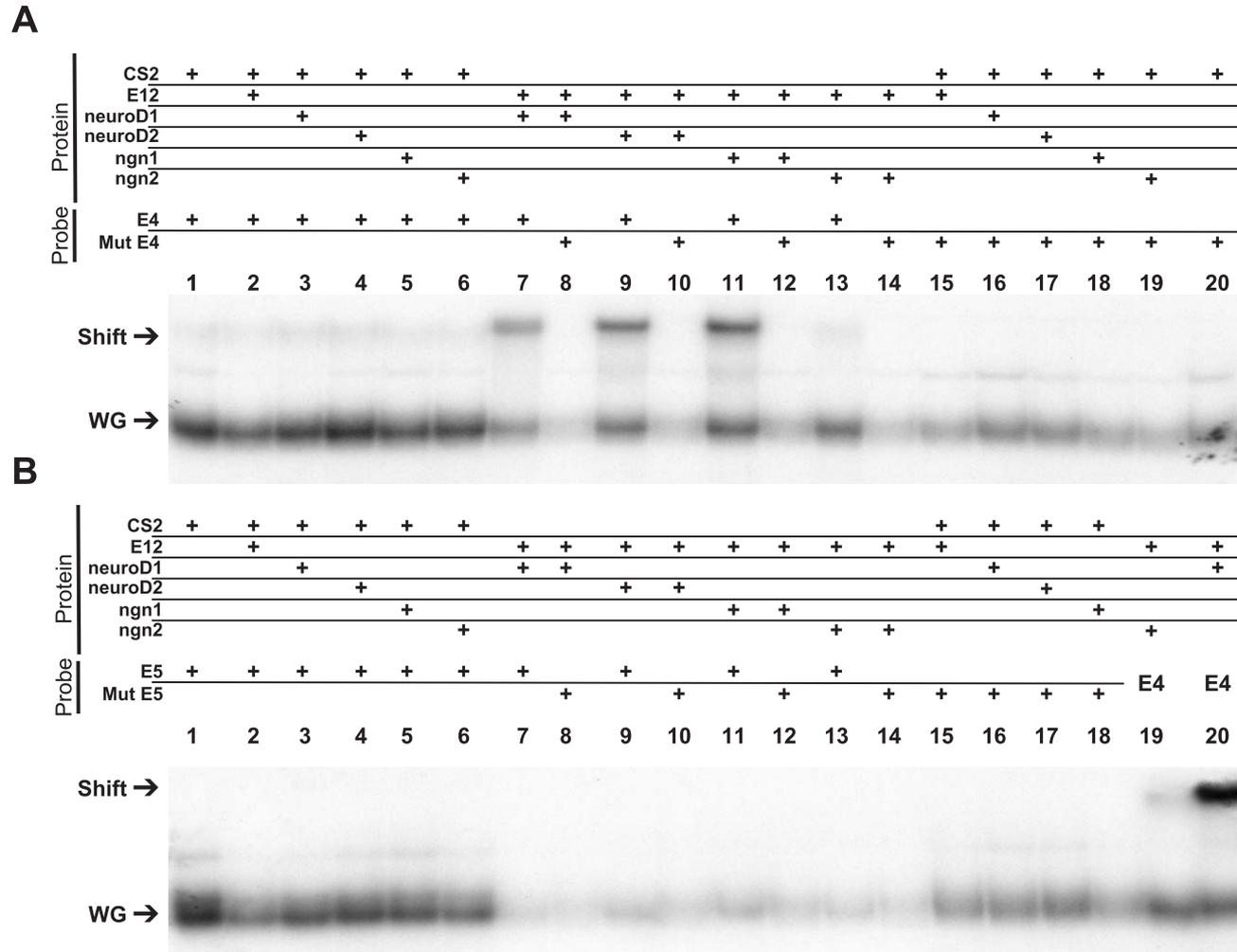


Fig. 2. Electrophoretic mobility shift assay of neuroD1, neuroD2, neurogenin1 (ngn1), and neurogenin2 (ngn2) complexed with E12, using wild type and mutant E4 and E5 promoter elements. Panel A demonstrates the binding of neuroD1, neuroD2, ngn1, and ngn2 to E4 and mutant E4 (mutE4) elements. Lanes 1–6 and 15–20 are controls for nonspecific (CS2) and monomeric (E12, neuroD1, neuroD2, ngn1, ngn2) binding to wild type and mutE4 probes. Lanes 7, 9, and 11 show neuroD1, neuroD2, and ngn1 complexed with E12 binding to the E4 promoter element. Lane 13 shows ngn2/E12 weakly binding the E4 element. Lanes 8, 10, 12, and 14 show neuroD1, neuroD2, ngn1, ngn2 not binding the mutE4 probe. Panel B demonstrates the binding of neuroD1, neuroD2, ngn1, and ngn2 to E5 and mutant E5 (mutE5) promoter elements. Lanes 1–6 and 15–18 are controls for nonspecific (CS2) and monomeric (E12, neuroD1, neuroD2, ngn1, ngn2) binding to wild type and mutE5 probes. Lanes 7–14 show neuroD1, neuroD2, ngn1, and ngn2 complexed with E12 binding neither the E5 nor the mutE5 promoter elements. Lanes 19 and 20 provide positive controls of varying intensity (ngn2/E12 + E4, neuroD1/E12 + E4). WG, wheat germ.

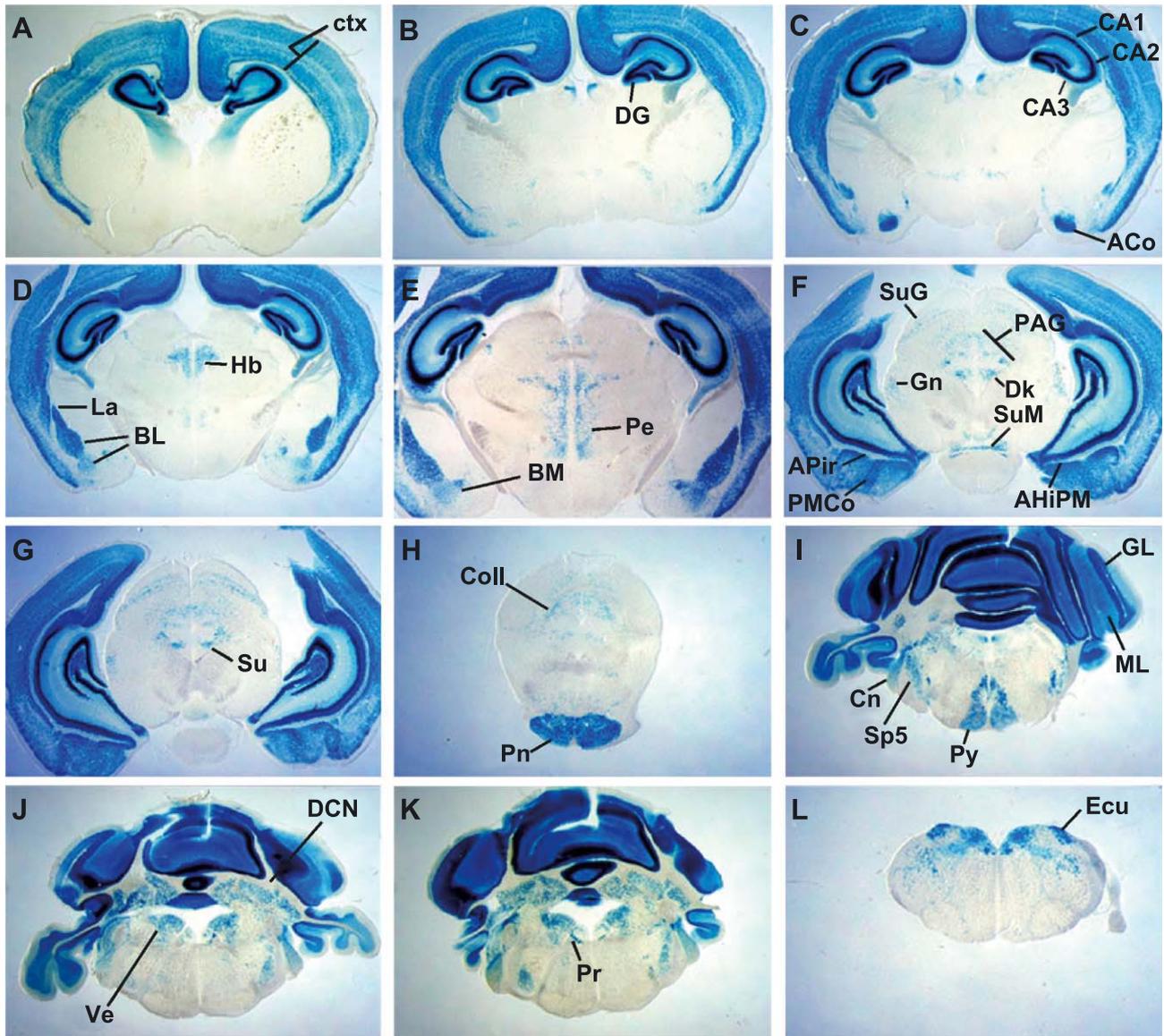


Fig. 3. Expression pattern of a reporter driven by the endogenous *neuroD2* promoter. Coronal sections (100  $\mu$ m) from a *neuroD2*<sup>+/-</sup> mouse brain stained for  $\beta$ -galactosidase activity. Sections shown are unevenly spaced from anterior to posterior, selected to demonstrate the following structures that express *neuroD2* (panels referred to in parentheses): ACo, anterior cortical amygdaloid nuclei (C); AHiPM, amygdalohippocampal posteromedial nucleus (F); APir, amygdalopiriform transition area (F); BL, basolateral amygdala nuclei (D); BM, basomedial amygdala nucleus (E); Cn, cochlear nuclei (I); Coll, colliculus (H); ctx, cerebral cortex (A); DCN, deep cerebellar nuclei (J); DG, dentate gyrus (B); Dk, nucleus of Darkschewitsch (F); Ecu, external cuneate nuclei (L); Gn, geniculate nucleus (F); GL, cerebellar granule cell layer (I); Hb, habenular nuclei (D); La, lateral amygdaloid nucleus (D); ML, cerebellar molecular layer (I); PAG, periaqueductal gray (F); PMCo, posteromedial cortical amygdala nuclei (F); Pe, paraventricular hypothalamic nuclei (E); Pn, pontine nuclei (H); Pr, prepositus hypoglossal nucleus (K); Py, pyramidal nucleus (I); Sp5, spinal trigeminal nucleus (I); Su, supraocular nuclei (G); SuG, superficial grey layer (F); SuM, supramammillary nuclei (F); Ve, vestibular nuclei (J). The olfactory bulb is not shown. Fiber tracts are not labeled.

E5 that were used for the reporter construct studies described above or the transgenic mouse studies described below.

From *in vitro* studies, we conclude that 1- and 0.5-kb fragments of the *neuroD2* promoter are induced by *neurogenin1* and *neuroD2*, that this activity is abolished by mutation of both E4 and E5 bHLH response elements, and that E4 is responsive to both *neurogenin1* and *neuroD* family members. In addition, E5 appears necessary for the activation by *neurogenin1*, although the gel shift assay did

not show binding, perhaps indicating that other factors are necessary for *neurogenin1* to bind to E5.

#### *Endogenous neuroD2 expression pattern*

To gain insight into *in vivo* regulation of the *neuroD2* gene, we utilized mice that express the *lacZ* gene in place of the *neuroD2* coding sequence to evaluate the populations of neurons in which the *neuroD2* promoter is active (Olson et al., 2001). In *neuroD2*<sup>+/-</sup> mice, *lacZ* staining

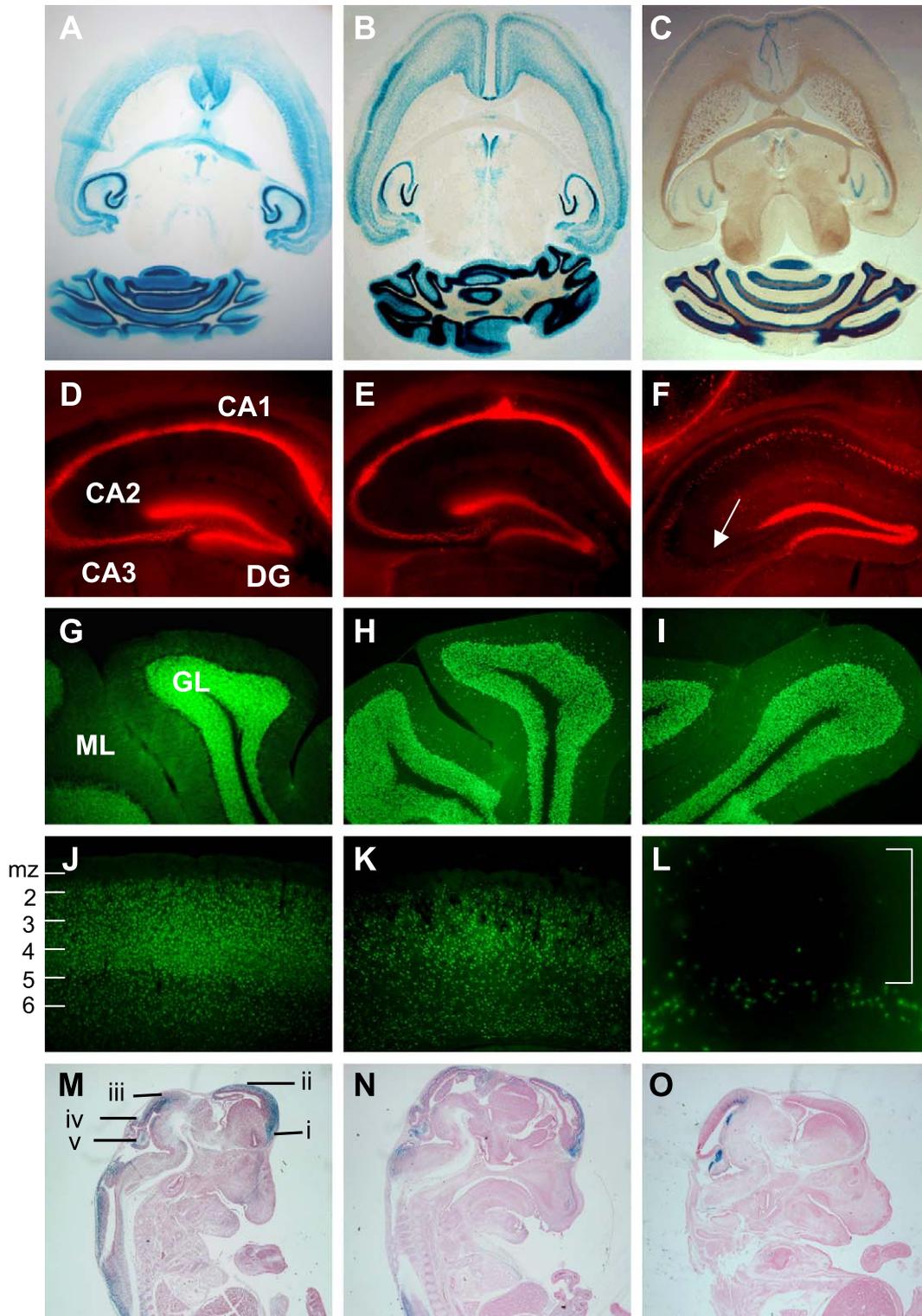


Fig. 4. Expression pattern of reporter driven by 10 and 1 kb neuroD2 promoter fragments and neuroD2<sup>+/-</sup>. (A–C) Horizontal sections of 21-day-old neuroD2<sup>+/-</sup>, 10 kb:lacZ, and 1 kb:lacZ mice stained for β-galactosidase activity (left to right). Note that the 10 kb:lacZ and 1 kb:lacZ mice have a nuclear localization signal (NLS) on the β-galactosidase gene, accounting for the difference in gross appearance between neuroD2<sup>+/-</sup> mice, which lack the NLS. (D–F) Hippocampus of adult neuroD2<sup>+/-</sup>, 10 kb:lacZ, and 1 kb:lacZ mice (left to right) shows consistent staining in dentate gyrus of all three lines. Arrow in F shows absence of staining in CA3. Staining was weaker in CA1 and CA2 of 1 kb:lacZ mice as well. (G–I) Cerebellum of neuroD2<sup>+/-</sup>, 10 kb:lacZ, and 1 kb:lacZ mice (left to right) shows no consistent differences between lines. (J–L) Cerebral cortex of neuroD2<sup>+/-</sup>, 10 kb:lacZ, and 1 kb:lacZ mice (left to right). Cortex layers indicated by text on left of panel J; mz: marginal zone. The bracket in L shows absence of staining in most cortical layers of 1 kb:lacZ mice. Panels D–L stained with an antibody that recognizes β-galactosidase. (M–O) Embryos at E13.5 with neuroD2<sup>+/-</sup>, 10 kb:lacZ, and 1 kb:lacZ genotype (left to right) stained for β-galactosidase activity, then sectioned sagittally. i, Olfactory lobe; ii, neopallial cortex; iii, ventral midbrain; iv, intraventricular cerebellar primordium; v, dorsal medulla oblongata. Again, the NLS in transgenic embryos causes more discrete staining than in neuroD2<sup>+/-</sup> embryos. Other abbreviations as in Fig. 3.

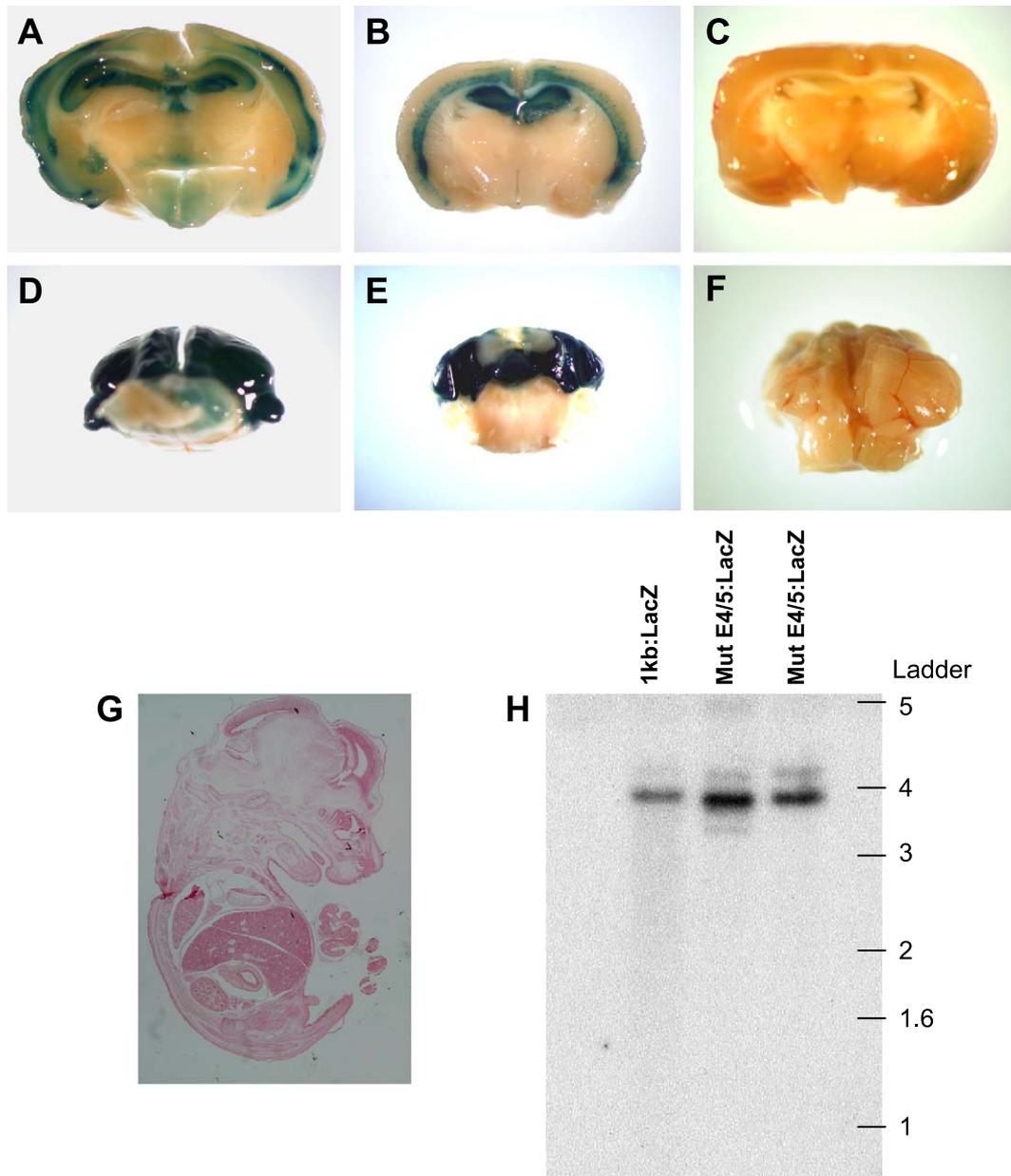


Fig. 5. Effect of E4 and E5 mutation on neuroD2 promoter activity in mice. (A–F) Thick cut coronal sections (500  $\mu$ m) through forebrain (top) and cerebellum (bottom) of 10 kb:*lacZ* (A, D), 1 kb:*lacZ* (B, E), and mutE4E5:*lacZ* (C, F) mice stained for  $\beta$ -galactosidase activity. (G) Cross section of an E13.5 mutE4E5:*lacZ* embryo stained for  $\beta$ -galactosidase activity at the same time as embryos shown in Fig. 4. (H) Representative genomic Southern blot of two of the four mutE4E5:*lacZ* transgenic lines with 1 kb:*lacZ* line as positive control.

revealed an active promoter in all layers of the cerebral cortex (Fig. 3A and Table 1), CA1–3 and dentate gyrus of the hippocampus (Figs. 3B and C), habenular thalamic nuclei, paraventricular hypothalamic nuclei, lateral, basolateral and basomedial amygdala nuclei (Figs. 3D and E), periaqueductal gray, geniculate nuclei, supramammillary nucleus, nucleus of Darkschewitsch, amygdalopiriform transition area, posteromedial cortical amygdala nuclei, amygdalohippocampal posteromedial nucleus (Fig. 3F), supraocular nuclei (Fig. 3G), neurons in the colliculus, pontine nuclei (Fig. 3H), and the pyramidal nucleus (Fig. 3I). Notably, the remaining regions of thalamus and

hypothalamus were negative as were the entire basal ganglia (Figs. 3A–D). In the cerebellum, granule cells, molecular layer neurons, and deep cerebella nuclei were positive and Purkinje cells were negative as previously described (Figs. 3I–J). Brainstem neurons in the external cuneate nucleus and central gray were positive and the Raphe nucleus was negative (Fig. 3L). The neuroD2 promoter was active in many cranial nerve nuclei including cochlear, spinal trigeminal (Fig. 3I), prepositus hypoglossal (Fig. 3K), medial vestibular, and vestibular nuclei (Fig. 3J). Sites of  $\beta$ -galactosidase expression are shown in Fig. 3 and Table 1.

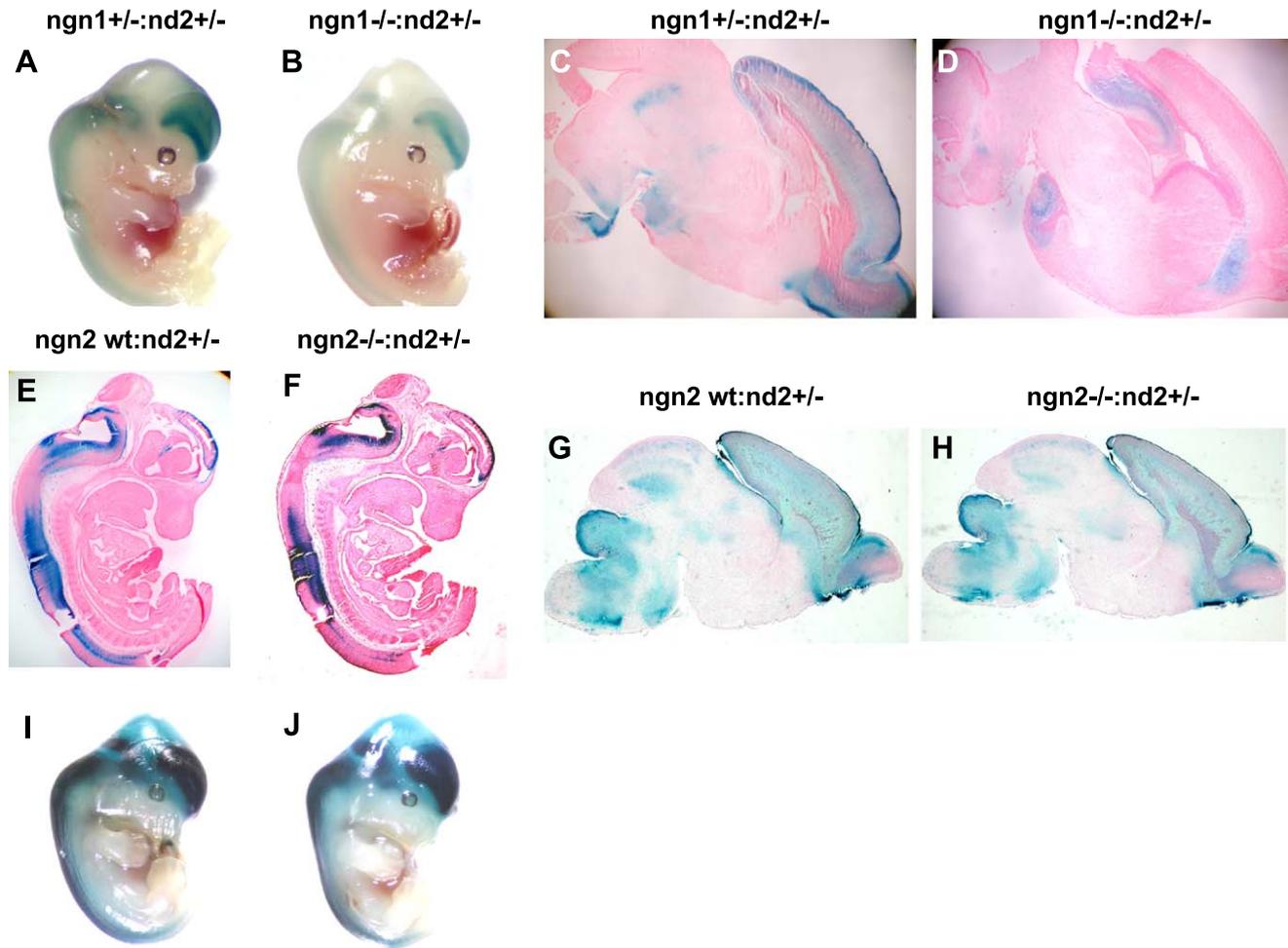


Fig. 6. Role of neurogenin1 and neurogenin2 in mouse brain neuroD2. E13.5 *ngn1*<sup>+/-</sup>, *neuroD2*<sup>+/-</sup> embryos (A) show more  $\beta$ -galactosidase activity than *ngn1*<sup>-/-</sup>, *neuroD2*<sup>+/-</sup> embryos (B). The difference is more pronounced at postnatal day P0.5 (C–D). E13.5 and P0.5 *ngn2* wt, *neuroD2*<sup>+/-</sup> embryos (E, G, I) show similar  $\beta$ -galactosidase activity as *ngn2*<sup>-/-</sup>, *neuroD2*<sup>+/-</sup> embryos (F, H, J). Three mice were evaluated for each condition, representative images are shown.

Table 1  
*LacZ* expression pattern in mouse models

Region	NeuroD2 <sup>+/-</sup> and 10 kb: <i>lacZ</i>	1 kb: <i>lacZ</i>	Negative in all mice
Cerebral cortex layers	All	Faint layer 5, 6 subplate Ventricular zone NOT layers 2, 3	
Hippocampus	CA1, 2, 3 Dentate gyrus	Variable in CA1, 2, 3 Between mouse lines Dentate gyrus	
Thalamus	Habenular nuclei	Same	Remainder of thalamus
Hypothalamus	Paraventricular hypothalamic nuclei	Same	Remainder of hypothalamus
Amygdala	Amygdalopiriform transition area Cortical amygdaloid nuclei Lateral n. basolateral nuclei Basomedial nuclei	Same	Central and medial nuclei
Basal ganglia	None	None	Caudate, putamen, globus pallidus
Cranial nerve related	Supraocular nuclei Cochlear nuclei Prepositus hypoglossal Vestibular nuclei Spinal trigeminal nuclei	Same	Trigeminal
Colliculus	Subset of neurons Superficial gray	Same	
Cerebellum	Granule cells Molecular layer neurons Deep cerebellar nuclei	Granule cells Molecular layer neurons NOT deep cerebellar nuclei	
Brainstem	Ext. cuneate nucleus Central gray Pontine nuclei Pyramidal nuclei	Ext. cuneate nucleus NOT central gray	Raphe nucleus
Miscellaneous	Nucleus of Darkschewitsch Periaqueductal gray Geniculate nuclei Supramammillary nuclei Superficial gray Olfactory bulb	Same	Mammillary bodies

#### *Transgenic mice distinguish proximal and distal neuroD2 promoter activity*

We developed transgenic mice that utilized either a 10- or 1-kb fragment of the *neuroD2* promoter to drive the  $\beta$ -galactosidase gene (10 kb:*lacZ* and 1 kb:*lacZ* mice, respectively, Fig. 4). The 10 kb:*lacZ* mice express  $\beta$ -galactosidase in the same neuronal populations as the endogenous *neuroD2* promoter indicating that all of the promoter elements that are necessary for *neuroD2* expression are contained within this region (Fig. 4 and Table 1). In contrast, 1 kb:*lacZ* mice failed to express  $\beta$ -galactosidase in several layers of the cerebral cortex (Figs. 4C and L), in CA3 of the hippocampus (Figs. 4C and F, arrow), deep cerebellar nuclei, and brainstem central gray matter (not shown). The staining pattern in 1 kb:*lacZ* mice otherwise recapitulated *neuroD2*<sup>+/-</sup> and 10 kb:*lacZ* mice (Fig. 4 and Table 1). Note that the 10 kb:*lacZ* and 1 kb:*lacZ* mice have a nuclear localization signal (NLS) on the  $\beta$ -galactosidase gene, accounting for the difference in gross appearance between *neuroD2*<sup>+/-</sup> mice which do not have an NLS.

Like the endogenous promoter, the 1- and 10-kb-driven reporters were active by embryonic day E13.5. At this

early stage, the differences between the two promoters were more pronounced than in adult mice.  $\beta$ -galactosidase staining was present in the olfactory lobe, neopallial cortex, the ventral region of the midbrain, the cerebellar primordium, and dorsal and ventral medulla oblongata of E13 10 kb:*lacZ* embryos, but was restricted to the ventral part of the midbrain, cerebellar primordium, and dorsal and ventral medulla oblongata in 1 kb:*lacZ* embryos (Figs. 4M–O).

To establish the importance of E4 and E5 in the context of mammalian brain neurons, we developed additional transgenic mice that utilized the 1 kb:*lacZ* construct with mutations of E4 and E5 (mutE4E5:*lacZ*). The brains of these mice were completely devoid of  $\beta$ -galactosidase activity at all ages (Figs. 5C, F, and G). To ensure that this was not due to an unexpected sequence error in the transgenic mice, we performed transgene sequencing and genomic southern analyses on four distinct mouse lines and found no sequence errors and expected transgene components, respectively (data not shown and Fig. 5H). Therefore, the absence of  $\beta$ -galactosidase activity in these mice clearly demonstrates that E4 and E5 are critical components of the proximal *neuroD2* promoter.

### Promoter activity in the absence of neurogenin1 or neurogenin2

To determine the influence of neurogenin1 and neurogenin2 on neuroD2 expression, we obtained neurogenin1<sup>+/-</sup> and neurogenin2<sup>+/-</sup> mice and bred them with neuroD2<sup>+/-</sup> mice. Through multiple rounds of breeding, we generated embryos that contained the  $\beta$ -galactosidase gene in place of the neuroD2 coding sequence and were null for neurogenin1 (ngn1<sup>-/-</sup>:nd2<sup>+/-</sup>) or neurogenin2 (ngn2<sup>-/-</sup>:nd2<sup>+/-</sup>). Because neurogenin1<sup>-/-</sup> and neurogenin2<sup>-/-</sup> mice die shortly after birth, *lacZ* staining was done only at E13 and P0.5. For E13.5 embryos in the absence of neurogenin1, the endogenous neuroD2 promoter was less active in the neopallial cortex compared to controls (Figs. 6A–D). By P0.5, in the absence of neurogenin1, neuroD2 promoter activity was restricted to the intermediate zone of the cerebral cortex, and was missing from the cortical plate, including the superficial layers where it is normally expressed in the vast majority of neurons. The number and density of neurons in these structures were unchanged in neurogenin1-null mice compared to controls, suggesting that the cell types that normally express neuroD2 are not specifically missing. NeuroD2-*lacZ* expression was also missing from the mitral cell layer in the neurogenin1 mutant olfactory bulb, where it is normally present at high level (Figs. 6C–D). We have not observed a consistent difference in neuroD2 expression between wild type and neurogenin2 mutant brain (Fig. 6).

### Discussion

In the developing mammalian brain, a small number of bHLH and other neurogenic transcription factors act in a combinatorial fashion to influence the fate of neuronal cells. The spatial and temporal expression patterns of each transcription factor serve as templates for the rich diversity of neuronal subtypes.

In this study, we evaluated neuroD2 gene regulation in mouse brain. We presented an analysis of neuronal populations in which the endogenous neuroD2 promoter is active and demonstrated by generating transgenic mice that all of the necessary regulatory elements appear to be present in a 10-kb fragment of DNA immediately upstream of the transcriptional start site. A 1-kb fragment was sufficient to recapitulate endogenous neuroD2 expression patterns in most neuronal populations, but apparently lacked critical response elements that are necessary for expression in subsets of cortical neurons. Although we have not identified these regulatory elements, comparison of human and mouse sequences shows two regions of significant homology. These regions correspond with the murine promoter region (base pairs -1 to -2583 relative to the translation start codon) and a region about 5-kb upstream of the promoter (-4454 to -6635). The latter may be a suitable starting

point for future studies that map the distal regulatory elements.

In the proximal regulatory region, E4 is recognized by neurogenin and neuroD family members and is particularly responsive to neurogenin1. The importance of this E-box in mammalian brain is demonstrated by the absence of neuroD2 1-kb promoter activity following mutation of E4 and E5. Mutation of E5 reduced the response of a 0.5-kb reporter construct to neurogenin1 in NSH cells. Since neurogenin and neuroD family members failed to bind E5 in electrophoretic mobility shift assays, we conclude that neurogenin does not directly regulate neuroD2 via E5. This notion is supported by the fact that E4 but not E5 is conserved between mice and humans. Deletion of the promoter region that contains E6 and E7 caused approximately 30% decline in neurogenin1-induced reporter activity. This region does not contain elements that are sufficient to promote gene expression *in vivo*, however, since transgene expression was eliminated by mutation of E4 and E5 in mice. An alternative explanation is that promoter elements other than E-boxes play a modulatory role in neuroD2 expression only after the promoter is activated by transcription factors that utilize E4.

Complete absence of neuroD2 promoter activity in superficial layers of the neocortex of P0.5 mice that lack neurogenin1 underscore the importance of neurogenin1 in the regulation of neuroD2 in mammalian brain. NeuroD2 promoter activity was similar in the presence and absence of neurogenin2 at both ages. Coupled with consistently weak binding of neurogenin2 to E4 in gel shift assays, these data suggest that neurogenin2 may have little influence on neuroD2 expression. It is possible that neurogenin family members differentially regulate neuroD family members based on affinity for particular response elements in the neuroD family promoters. Additionally, the extent of neurogenin1 or neurogenin2 regulation of neuroD2 may be underestimated in these studies due to functional redundancy between neurogenin family members that share homology and overlapping expression patterns (Fode et al., 1998; Ma et al., 1996, 1998).

Because neurogenin family members are only transiently expressed during development, neurogenin1 cannot account for long-term expression of neuroD2 postnatally. Furthermore, while neuroD2 expression, like several other bHLH proteins, has been shown to be autoregulatory and the neuroD2 promoter is transactivated by neuroD2, autoregulation cannot account for the majority of neuroD2 promoter activity in adulthood as evidenced by persistent expression of the *lacZ* gene (driven by the endogenous neuroD2 promoter) in neuroD2-null mice. The most likely alternative is that neuroD2 expression is largely regulated by neuroD1. In developing mouse brain and differentiating P19 cells, neuroD1 expression precedes neuroD2 expression and neuroD1 transactivated the neuroD2 promoter reporter constructs in both P19 and NSH cells. Comparison of neuroD1 and neuroD2 expression reveals broadly overlapping patterns in cerebral cortex, hippocampus, cerebellum, and cranial nerve ganglia.

In addition to providing insight into regulation of neuroD2 in neurons that are critical for memory, learning, balance, and cranial nerve function, this study provided information about neuroD2 promoter fragments that might be useful for generation of future transgenic mice. For example, the 1-kb promoter fragment is particularly useful for driving gene expression in cerebellar granule cells, granule cell precursors, and hippocampal dentate gyrus without expression in most cerebral cortex, thalamic or basal ganglia neurons. Crossing the neuroD2<sup>+/-</sup> or 1 kb:*lacZ* mice with mice that lack other neurodevelopmental factors offers a tool for understanding which transcription factors are necessary for production and migration of neuroD2-expressing neurons.

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