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A Role for Nuclear Factor I in the Intrinsic Control of Cerebellar Granule Neuron Gene Expression*[S]

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Nervous system formation requires the elaboration of a complex series of differentiation events in both a spatially and maturation-regulated manner. A fundamental question is how neuronal subtype specification and developmental gene expression are controlled within maturing neurons. The α6 subunit of the γ-aminobutyric acid type A ($GABA_A$) receptor (GABRA6) is preferentially expressed in cerebellar granule neurons and is part of an intrinsic program directing their differentiation. We have employed a lentiviral approach to examine the transcriptional mechanisms controlling neuronal subtype-selective expression of this gene. These studies demonstrated that nuclear factor I (NFI) proteins are required for both transgenic GABRA6 promoter activity as well as endogenous expression of this gene in cerebellar granule neurons. Chromatin immunoprecipitation also showed that NFI proteins are bound to the GABRA6 promoter in these cells *in vivo*. Furthermore, analyses of gene knockout mice revealed that *Nfia* is specifically required for normal expression of the GABRA6 gene in cerebellar granule neurons. NFI expression and DNA binding activity are highly enriched in granule neurons, implicating this transcription factor family in the neuronal subtype-selective expression of the GABRA6 gene. These studies define a new role for NFI proteins as neuronal subtype-enriched transcriptional regulators that participate in an intrinsic transcriptional program directing the differentiation of cerebellar granule neurons.

Fundamental to nervous system development is the specification and maturation of diverse neuronal populations that ultimately assemble into a complex synaptic network. These events require the appropriate spatiotemporal expression of an array of different genes during development, and a critical question is the nature of the transcriptional program that controls their proper expression. Several neuron-enriched transcription factors have been directly implicated in the determination of different neuronal populations (1). However, much remains to be learned regarding the roles of individual trans-regulators and their interactions in directing the phenotypes of distinct neuronal subtypes at different phases of their maturation.

Cerebellar granule neurons (CGNs)[1] elaborate a well defined terminal differentiation program (2) and provide an excellent model of both cell-specific and developmental regulation of neuronal differentiation. Postnatally, newly born granule neurons migrate from the premigratory zone to form the internal granule cell layer, whereupon dendrite formation and synaptic connections with excitatory mossy fibers and inhibitory Golgi type II neurons ensue. Type A α-aminobutyric acid receptors ($GABA_AR$) are present in synaptic and extrasynaptic regions of CGNs associated with inhibitory inputs from Golgi type II cells. During CGN differentiation, $GABA_ARs$ undergo a maturation-dependent change from mainly benzodiazepine-sensitive to benzodiazepine-insensitive forms (3). This is associated with a switch in $GABA_AR$ subunit expression wherein the α2 and α3 subunits conferring benzodiazepine sensitivity are down-regulated while the α6 subunit is induced (3). In mature CGNs, $GABA_ARs$ containing the α6 subunit function mainly at extrasynaptic sites where they mediate tonic regulation of CGN excitability (4).

The $GABA_AR$ α6 subunit (GABRA6) is primarily expressed in granule neurons of the cerebellum (5). Furthermore, its expression in these cells is intrinsically determined (6, 7). Thus, the GABRA6 gene provides an excellent opportunity to investigate intrinsic transcriptional mechanisms controlling granule neuron subtype-specific gene regulation.

The nuclear factor I (NFI) family consists of four separate genes (*Nfia*, *b*, *c*, and *x*) that are implicated in the developmental regulation of gene transcription in many tissues (8). Multiple splice variants for each NFI gene have been identified, adding further complexity to their potential functions in diverse cell types. Gene inactivation studies have demonstrated the importance of individual NFI genes in distinct organ systems, including lung formation (*Nfib*) (9) and tooth root development (*Nfic*) (10). *Nfia*-null mice exhibit neurological defects, including agenesis of the corpus callosum, hydrocephalus, and disrupted development of midline glia (11, 12). However, specific roles for these factors in neuronal maturation and function, and the specific neuronal target genes they regulate *in vivo*, remain unclear. The present studies demonstrate that NFI proteins are enriched in CGNs and are required for the expression of the GABRA6 gene.

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[S] The on-line version of this article (available at http://www.jbc.org) contains Supplemental Table S1.

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[1] The abbreviations used are: CGN, cerebellar granule neuron; $GABA_A$, α-aminobutyric acid, type A; $GABA_AR$, $GABA_A$ receptor; GABRA6, $GABA_AR$ α6 subunit; mGABRA6, mouse GABRA6; NFI, nuclear factor I; CMV, cytomegalovirus; IRES, internal ribosomal entry site; RT, reverse transcription; ChIP, chromosomal immunoprecipitation; EMSA, electrophoretic mobility shift assay; DIV, days *in vitro*.

MATERIALS AND METHODS

Cell Culture—Mouse CGNs were prepared from 6-day-old CD1 pups according to previously described procedures (13). Briefly, dissociated cerebellar cells were prepared by trypsin-DNase digestion and mechanical trituration, and CGNs were enriched to >98% purity by using Percoll™ (Sigma) gradient centrifugation and pre-plating on poly-D-lysine-coated Petri dishes. Cells were cultured on plastic dishes or on glass coverslips coated with 100 μg/ml poly-D-lysine at a density of 2×10^6/ml in Neurobasal™ medium containing B27 serum-free supplements, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and 0.45% glucose at 37 °C/5% CO_2. Mouse cortical neurons were prepared from E18 CD1 pups. Dissected cortices were digested with 0.05% trypsin at room temperature for 2 min. After mechanical trituration, the cells were plated on plastic dishes coated with 100 μg/ml poly-D-lysine at a density of 2×10^6/ml in the same Neurobasal™/B27 medium used for CGNs.

Human embryonic kidney 293T cells and human choriocarcinoma JEG3 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium (Sigma) containing 10% heat-inactivated fetal bovine serum (Invitrogen).

Protein Extraction—Nuclei were isolated from cell cultures and tissues and extracted using protocols outlined in earlier studies (14). Whole-cell protein extraction was performed as previously described (15). Protein concentrations were assayed with Bio-Rad protein assay reagent (Bio-Rad) or Micro BCA™ protein assay reagent kit (Pierce).

Electrophoretic Mobility Shift Assays—The A region probe was generated by PCR using primers containing EcoRI sites: GGAATTCAAATGCTGAGCCCATTG (sense); GGAATTCTGGAGAGTCAGAGCAATG (antisense). The PCR product was digested with EcoRI, and the resulting fragment was radiolabeled with [α-^{32}P]dATP using Klenow fragment. Seven overlapping competitors covering the A region (see Fig. 1 for sequences) were generated by annealing and Klenow fill-in of oligonucleotide pairs. Oligonucleotides for NFI wild-type (TTTTGGATTGAAGCCAATATGATAA) and NFI mutant (TTTTGGATTGAATAAAAATATGATAA; mutated bases are underlined) competitors were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The binding reactions were performed as previously described (14) using 0.5–1.25 μg of nuclear proteins. DNA competitors were used at a 100-fold molar excess relative to the DNA probe concentration. Antisera that recognize native NFI proteins (16) or pre-immune rabbit serum were used for supershift assays. Specific mammalian isoforms recognized by the NFI antiserum have not been determined.

Construction of Plasmids—The plasmids used for lentiviral vector production (pHR′-CMV-LUC-W-Sin18 and pHR′-CMV-LacZ-Sin18) have been previously described (17). Plasmid pmα 6IRES-LacZ6 containing the mouse GABRA6 (mGABRA6) promoter with additional upstream and downstream sequences (18) was used for generating mGABRA6 promoter viruses. An mGABRA6 promoter-IRES fragment was isolated from pmα 6IRES-LacZ6 by partial digestion with NcoI and SphI (blunted) and inserted into pGL3-Basic (Promega, Madison, WI) plasmid to yield pGL3-GABRA6-IRES. An NFI site mutant promoter fragment was generated by PCR using relevant primers (sense, CCATTCGAAGTCCGAACTAGCCGTG; antisense, CCAAATCCTTCATATTCCTCATCCCACTC) and cloned into pGL3-GABRA6-IRES via BstBI and PstI sites. The mGABRA6 promoter was removed from pGL3-GABRA6-IRES using SmaI and EcoRI to generate the promoter-less vector pGL3-IRES by re-ligation. Lentiviral plasmids containing wild-type and mutant mGABRA6 promoter-IRES-luciferase sequences as well as an IRES-luciferase reporter were prepared by digesting the relevant pGL3-IRES plasmids with MluI (blunted) and SalI and insertion of isolated fragments into pHR′-CMV-LacZ-Sin18.

For protein expression, the lentiviral vector pHR′-cPPT-CMV-W-Sin18 was generated by replacing the CMV promoter and luciferase reporter gene in pHR′-CMV-LUC-W-Sin18 with the cPPT-CMV fragment from pRRL-cPPT-CMV-X-PRE-SIN (19) using NotI and XhoI. Hemagglutinin-tagged mouse NFI-A, -B, and -X fragments were released from their expression vectors (20) by digestion with EcoRI (blunted)/SalI and cloned into pHR′-cPPT-CMV-W-Sin18. The transcription regulatory domain of NFI-X was removed using AflII (blunted) and XhoI and replaced by the *Drosophila engrailed* transcription repressor domain (an EcoRI (blunted)/XhoI fragment derived from pENG-N (21)) to generate dominant repressor NFI (NFI/EnR).

Transduction of CGNs and Analysis of Luciferase Activity—Vesicular stomatitis virus G protein-pseudotyped lentiviruses were generated by transient co-transfection of the vector construct, the packaging construct pCMVΔ8.91 and the pMD.G vesicular stomatitis virus G protein viral envelop expression vector into 293T cells. High titer viral stocks were prepared by ultracentrifugation as previously described (22). The titers of CMV-β-Gal and NFI expressing viruses were determined by infecting JEG3 cells with a viral serial dilution (23) and staining 48 h later using either 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Fisher Biotech, Pittsburgh, PA) or hemagglutinin antibodies (Cell Signaling, Beverly, MA). The viral stocks for promoter constructs were titered using an human immunodeficiency virus, type 1, p24 enzyme-linked immunosorbent assay kit (ZeptoMetrix Corp., Buffalo, NY) as well as by analyzing genomic DNA from viral-infected JEG3 cells using competitive PCR for luciferase sequences (sense, ATACGCCCTGGTTCCTG; antisense, AATGCCCATACTGTTGAGC).

For analysis of endogenous gene expression, CGNs were transduced with lentiviral expression vectors at a multiplicity of infection of 2. For promoter studies, CGNs were infected using a multiplicity of infection of 0.2–0.5. In co-infection studies, expression viruses were added at 1/10th the concentration of the promoter virus. Luciferase activity was determined in triplicate samples, and the results of at least three independent experiments were averaged to determine the mean ± S.D. for luciferase activity. The Students *t* test was used for statistical analysis.

Semi-quantitative and Real-time RT-PCR—Total RNA was extracted from tissues and cells using Tri reagent (Sigma). First strand cDNAs were synthesized with oligo-dT_{12-18} primers or random hexamers using the SuperScript™ RT-PCR system (Invitrogen). Primers for semi-quantitative RT-PCR assays are listed in the Supplemental Table. 18 S rRNA was assayed for normalization purposes, and serial dilutions were used to confirm linearity of the reactions.

For real-time PCR, whole brain cDNA samples from three individual wild-type and *Nfia* knockout mice (P16) (11) were analyzed in duplicate with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Green I (Applied Biosystems). Hypoxanthine guanine phosphoribosyl transferase mRNA (sense, GTTCTTTGCTGACCTGCTGGA; antisense, TCCCCCGTTGACTGATCATT) was used as an internal reference for normalization of GABRA6 (sense, TGGAGCGGAGATTGTTGTG; antisense, CAGGCGTCGATTTTAAGATGG) transcripts. The specificity of primers was confirmed by sequencing the RT-PCR products. Experiments were repeated twice. The ratio of transcripts for GABRA6 relative to hypoxanthine guanine phosphoribosyl transferase was determined using the $2^{-\Delta ct}$ method as previously described (24). The Student *t* test was used for statistical analysis.

Immunohistochemistry and Western Blotting—Mouse brains were fixed by transcardial perfusion with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4. Cerebella were dissected and kept in fixative for 10 h followed by overnight incubation in 30% sucrose at 4 °C. Frozen sections (16 μm) were examined for NFI proteins using anti-NFI antibody (sc-5567, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:400) that recognizes all NFI isoforms containing the NFI DNA binding domain. Antibodies to GABRA6 (Chemicon, Temecula, CA) were used at a 1:400 dilution. Bound antibodies were detected using Cy3-conjugated goat anti-rabbit IgG secondary antibody (for fluorescence) or horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Chemicon) and 3,3′-diaminobenzidine peroxidase substrate kit (Vector, Burlingame, CA), according to the manufacturer's instructions.

Protein extracts were separated on 8% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride transfer membranes (Millipore, Bedford, MA). The buffer used for dilution of antibodies and the blocking step was 1× phosphate-buffered saline buffer with 0.1% Tween 20 and 5% nonfat dry milk. Primary antibody for NFI (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added at 1:1000 dilution, and the blot was incubated at 4 °C for overnight. Bound antibodies were detected with a Western Lightning Chemiluminescence kit (PerkinElmer Life Sciences) according to the manufacturer's protocol.

Chromatin Immunoprecipitation Assay—ChIP assays were performed as previously described (25). Briefly, DNA-binding proteins were cross-linked to DNA by adding formaldehyde (1% final concentration) directly to the culture medium and incubating for 10 min at 37 °C. Cross-linking was stopped by adding glycine (125 mM), and the cells were collected and lysed in SDS buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture mix). Chromatin was sonicated to an average length of ~600 bp, and the NFI antibody used in supershift assays was added to the lysate. After overnight incubation at 4 °C, immune complexes were collected with protein A-Sepharose (Amersham Biosciences). The precipitated chromatin was treated with proteinase K and RNase A followed by overnight incubation to reverse the cross-linking. DNA was extracted and used as template for PCR assay of the

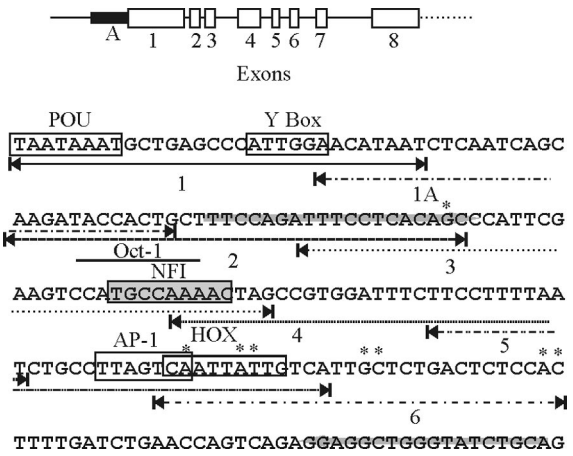


FIG. 1. **Characterization of mouse GABRA6 promoter sequences.** *Upper panel*, organization of the mouse gene and location of region A. *Lower panel*, DNA sequence of the proximal region A of the mouse promoter. *Boxes* indicate the location of predicted transcription factor binding sites (identified using the TRANSFAC 6.0 data base), with the *box* for the NFI site *shaded*. *Underlined sequences* (*solid*, *dotted*, or *dashed*) indicate the DNAs used in competitive EMSA experiments in Fig. 3*A*. The transcription initiation sites within this region previously identified by Jones *et al.* (27) are indicated by an *asterisk*. Primer sequences used for ChIP assays are shown by *shaded arrows*.

mouse GABRA6 promoter (see Fig. 1 for primer regions) or mouse brain factor-2 genomic sequences (sense, GGGGTTGGTTTCGTTC; antisense, AAGTCAGGGTTGCAGCATAG) as a negative control.

RESULTS

Identification of CGN-enriched Nuclear Proteins That Bind to the GABRA6 Promoter—Developmental and neuronal subtype-specific regulation of the mouse GABRA6 gene is largely determined at the level of gene transcription (18). The mouse GABRA6 promoter lacks a consensus TATA box and initiates transcription from multiple sites spanning 50–90 bp (26, 27) (Fig. 1). To screen for nuclear factors that might control GABRA6 expression in mouse CGNs, EMSAs were performed using multiple probes spanning different segments (150–200 bp in length) of the proximal promoter and 5′-flanking regions of the mouse gene. Nuclear extracts from adult and postnatal day-15 (P15) mouse cerebella, both of which express the GABRA6 gene (28), were compared with those from adult and P15 mouse cortex, which do not. This permitted an assessment of DNA binding factors potentially mediating expression of GABRA6 selectively in CGNs. One of these genomic segments (Fig. 1, *region A*) was bound by a prominent complex that was greatly enriched in cerebellar relative to cortical extracts (Fig. 2*A*). This 160-bp segment spans the proximal promoter region, including the majority of the transcription initiation sites (Fig. 1). The complex was specifically competed by homologous unlabeled competitor DNA but not unrelated sequences (not shown). We next examined whether these complexes also were present in nuclear extracts from cultured CGNs. GABRA6 gene expression is readily detectable in differentiated CGNs cultured for 6 days *in vitro* (6 DIV) (29), which was confirmed in the cultures used here (data not shown). EMSAs confirmed that nuclear extracts from 6 DIV CGNs also contained abundant amounts of the specific cerebellar-enriched A probe complex (Fig. 2*B*).

Next, competitive EMSAs were used to localize the binding site(s) for the CGN complexes within the A region. Seven overlapping competitors were tested (Fig. 1, *A1A–A6*), only one of which, A3, competed well for binding of 6 DIV CGN extracts to this region (Fig. 3*A*). Further, specific binding to this region was confirmed in EMSAs using an A3 DNA probe and CGN extracts (Fig. 3*B*). As shown in Fig. 1, the A3 sequence con-

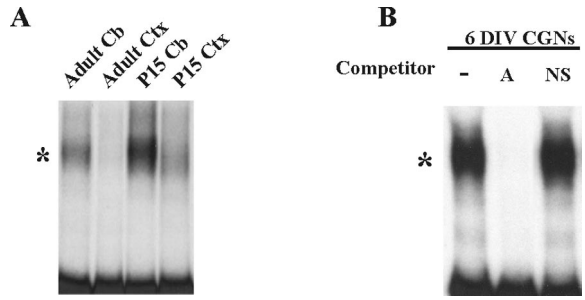


FIG. 2. **Detection of CGN-enriched nuclear proteins that bind the GABRA6 promoter.** *A*, EMSAs using the proximal A region probe, with the set of specific complexes indicated (*asterisk*). Nuclear extracts from adult and P15 mouse cerebellum (*Cb*) were compared with those from adult and P15 mouse cortex (*Ctx*). *B*, nuclear extracts from 6 DIV CGNs were incubated without (−) or with a 100-fold molar excess of unlabeled A region DNA (*A*) or an unrelated competitor DNA (*NS*). Specific complexes are shown by an *asterisk*.

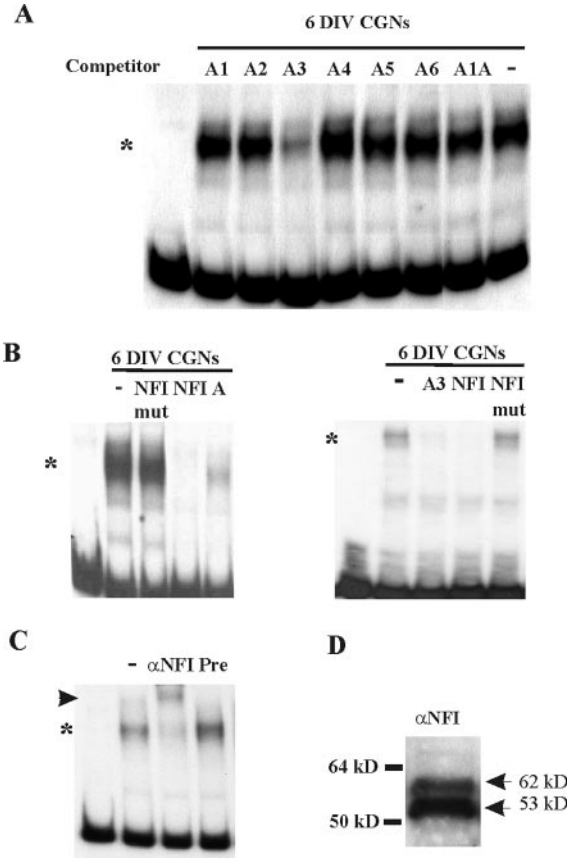


FIG. 3. **NFI is the major DNA-binding protein interacting with the proximal region of the mouse GABRA6 promoter *in vitro*.** *A*, to localize the A region binding site, competitive EMSAs were performed with nuclear extracts from 6 DIV CGNs using seven different overlapping competitor sequences (*A1–A6*; see Fig. 1). *B*, *left panel*, competitive EMSAs were performed using the A probe and 6 DIV CGN extracts without (−) or with a 100-fold molar excess of unlabeled A region, NFI consensus or mutant NFI site competitors. In other experiments, higher amounts of the A region sequence fully and specifically competed for binding to DNA-protein complexes (data not shown). *Right panel*, binding of CGN extracts to the A3 DNA probe. A 100-fold excess of unlabeled A3, NFI, or NFImut sequences were used as competitors. −, no competitor. Variable intensities between NFI complexes detected in the *left* and *right panels* likely reflect differences in the specific activities of the probe preparations used in these experiments. *C*, supershift experiments were performed using the A probe and nuclear extracts from 6 DIV CGNs in the presence of α NFI-specific antiserum or pre-immune serum (*Pre*). The *arrowhead* indicates the supershifted complex. The *asterisks* indicate the NFI complexes. *D*, Western analysis of NFI proteins in 6 DIV CGNs.

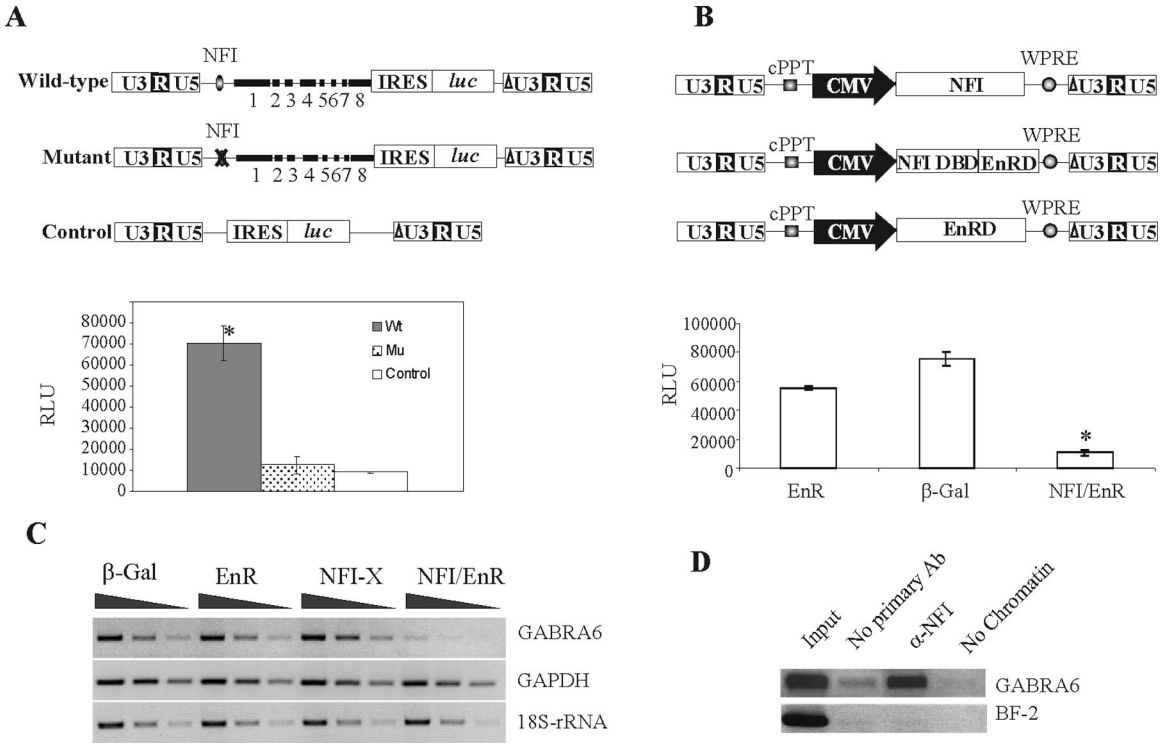


FIG. 4. **NFI proteins regulate GABRA6 gene transcription in CGNs and bind to its promoter *in vivo*.** *A*, *upper panel*, lentiviral vectors expressing wild-type or NFI site mutant mGABRA6 promoter constructs, as well as a promoterless control virus. *Black boxes* indicate exons 1–8. *Lower panel*, CGN cultures were infected with virus on 1 DIV and cells were extracted and assayed for luciferase activity on 6 DIV. The *asterisk* indicates significantly different from promoterless control ($p < 0.01$). *B*, *upper panel*, the structures of lentiviral vectors expressing NFI-A, -B, NFI-X, dominant repressor NFI (*NFI/EnR*) and *Drosophila engrailed* repressor domain alone (*EnR*). *cPPT*, central polypurine tract; *WPRE*, woodchuck hepatitis regulatory element. *Lower panel*, primary CGN cultures were co-infected on 1 DIV with viruses expressing the mGABRA6 promoter and either the dominant repressor NFI, *engrailed* repressor domain alone (*EnR*) or β-galactosidase (*β-Gal*). The *asterisk* indicates significantly different from β-galactosidase or EnR control ($p < 0.01$). *C*, semi-quantitative RT-PCR assays were performed with total RNAs extracted from CGNs infected on 1 DIV with viral vectors for EnR, NFI-X, or NFI/EnR dominant repressor. RNAs was extracted on 4 DIV, and 18 S rRNA and glyceraldehyde-3-phosphate dehydrogenase transcripts were used as internal controls. *D*, ChIP analysis of 6 DIV CGNs. Cross-linked chromatin was precipitated with α NFI antibody and then assayed by PCR for mGABRA6 genomic sequences using primers spanning the NFI site (see Fig. 1). Assays also were performed without added chromatin or primary antibody as negative controls. Amplification of mouse brain factor-2 genomic sequences lacking an NFI site demonstrated specific association of NFI with the mGABRA6 promoter.

tained consensus elements for two factors, NFI and Oct-1. In competition EMSAs, NFI consensus sequences fully competed for binding to the CGN complexes observed with either the A or A3 probes (Fig. 3*B*), whereas Oct-1 sequences did not (data not shown). Furthermore, mutation of the NFI binding site within either the NFI consensus competitor or the A3 sequence (not shown) resulted in loss of competition (Fig. 3*B*).

Supershift assays were performed to determine whether the CGN-enriched A probe complexes contained NFI proteins. A shifted complex was formed by the addition of antiserum that recognizes native mammalian NFI DNA-protein complexes (16), whereas control serum did not (Fig. 3*C*). Thus, NFI proteins specifically bind to an NFI consensus site within the mouse GABRA6 proximal promoter and are enriched in CGN cultures that express the GABRA6 gene. Competitive EMSAs and supershift assays also confirmed that the A probe complexes that were highly enriched in the P15 cerebellum (Fig. 2*A*) recognized NFI consensus sequences and contained NFI proteins (data not shown). The presence of NFI proteins in differentiated CGN cultures was further demonstrated by Western analysis (Fig. 3*D*). Two predominant NFI proteins were detected (~62 and 53 kDa), which may represent different NFI family members. This question was not further investigated here.

NFI Directly Regulates a Stably Integrated GABRA6 Promoter in CGNs—Promoter studies were initiated to examine the interaction between the mouse GABRA6 promoter and NFI proteins in cultured CGNs. Lentiviruses were employed because these vectors transduce postmitotic mouse CGNs with high efficiency and without significantly altering their differentiation, including the expression of GABRA6.[2] They also provide a means for expressing promoters as integrated transgenes within a more physiological, chromatin environment. Lentiviruses were generated that express a luciferase reporter under the control of either wild-type mouse GABRA6 genomic sequences or a mutant version harboring an inactive NFI site (Fig. 4*A*). We used a genomic fragment containing ~1 kb of 5′-flanking sequence, exons 1–8, and intervening introns of the mouse GABRA6 gene that are required for faithful and robust CGN-specific promoter activity *in vivo* (18). Infection of mouse CGN cultures confirmed that the wild-type promoter was expressed in a dose-dependent manner (data not shown). Mutation of the NFI binding site resulted in essentially complete loss of promoter activity in CGNs (Fig. 4*A*), indicating that this site and its cognate NFI proteins are critical determinants of GABRA6 transcription.

To further confirm this, lentiviral vectors were generated that express a dominant repressor form of NFI comprising the NFI-X DNA binding domain fused to the repressor domain for *Drosophila engrailed* (NFI/EnR) (Fig. 4*B*). Dominant repressor NFI/EnR strongly suppressed GABRA6 promoter activity when compared with control LacZ and EnR-alone expressed proteins (Fig. 4*B*). This directly implicated endogenous NFI as a critical

[2] W. Wang and D. L. Kilpatrick, unpublished observations.

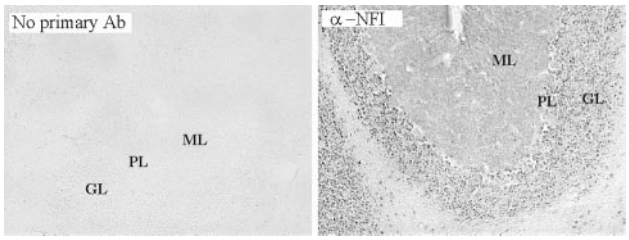
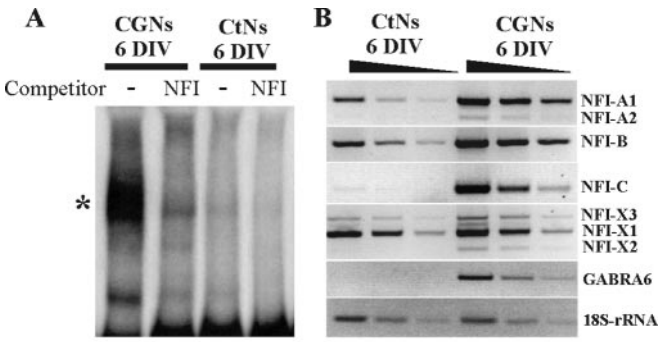


FIG. 5. **Immunohistochemical staining for NFI proteins in adult mouse cerebellum.** *Right panel*: elevated NFI expression is evident specifically in the granule cell layer (*GL*). *ML*, molecular layer; *PL*, Purkinje cell layer. *Left panel*, staining in the absence of primary antibody.



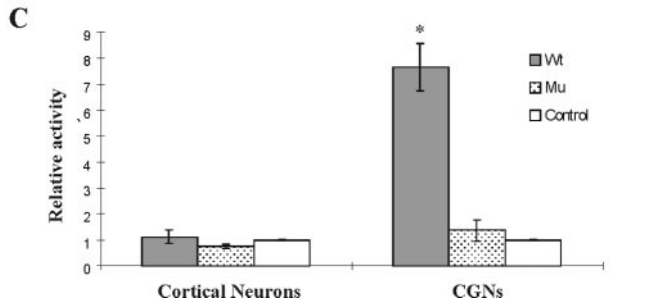
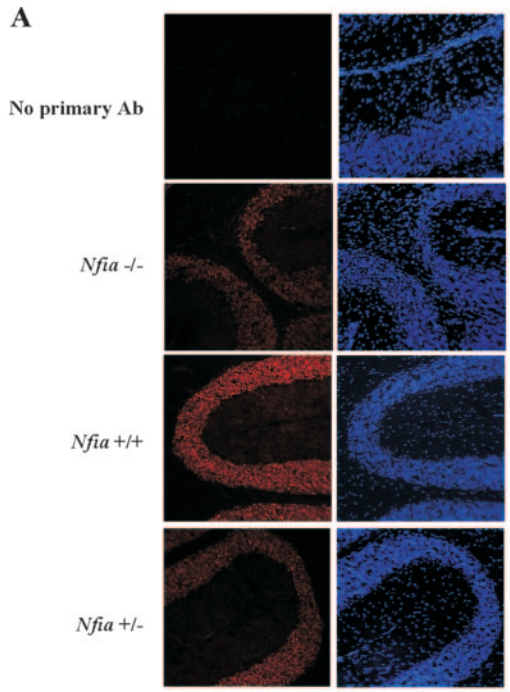


FIG. 6. **Comparison of NFI expression and activity in CGNs and cortical neurons.** *A*, DNA binding complexes in 6 DIV cultures of mouse CGNs and cortical neurons (*CtNs*). Nuclear extracts were incubated with A probe with or without a 100-fold molar excess of unlabeled NFI consensus site DNA. *Asterisk*, specific DNA-protein complexes. *B*, semi-quantitative RT-PCR was performed for mouse NFI isoforms and GABRA6 using total RNAs from 6 DIV CGNs or cortical neurons. *C*, primary cultures of CGNs or cortical neurons were infected with lentiviral vectors for wild-type (*Wt*) or NFI-site mutant (*Mu*) mGABRA6 promoters or promoterless control virus on 1 DIV. The infected cells were cultured to 6 DIV prior to luciferase assays. *Asterisk*, significantly different from promoterless control ($p < 0.01$).



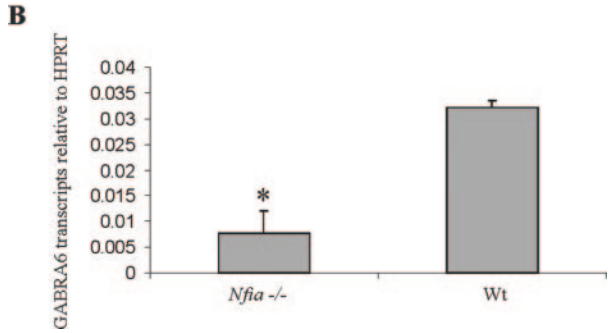


FIG. 7. **GABRA6 expression is greatly reduced in *Nfia*-deficient mice.** *A*, immunohistochemical staining of GABRA6 expression in P17 mouse cerebellum of *Nfia*(−/−), *Nfia*(+/−), and wild-type control mice. *Left panels*, sections were stained with anti-GABRA6 antibody. *Right panels*, nuclear staining of the same sections with bisbenzimide to identify the granule cell layer. *B*, real-time PCR analysis of GABRA6 mRNA abundance in brains from wild-type (*WT*) and *Nfia*(−/−) mice. *Asterisk*, significantly different from wild-type ($p < 0.05$).

regulator of the GABRA6 promoter in CGNs. Lentiviral vectors expressing wild-type NFI-B, NFI-A, and NFI-X proteins produced only a weak, insignificant stimulation of GABRA6 promoter activity (Fig. 4*B* and data not shown). Possible explanations for this are that NFI proteins are not greatly limiting for GABRA6 promoter activation in CGNs, or that one or more important co-regulators of NFI are limiting.

NFI Factors Regulate Endogenous GABRA6 Gene Expression in Mouse CGN Cultures—To establish the role of NFI in regulating endogenous GABRA6 transcription, CGN cultures were infected with lentiviruses expressing dominant repressor NFI/EnR or NFI-X at multiplicity of infection 2, which results in >80% transduction of CGN cultures (data not shown). NFI/EnR strongly inhibited the expression of the endogenous GABRA6 gene, whereas glyceraldehyde-3-phosphate dehydrogenase expression was unaffected (Fig. 4*C*). In contrast, NFI-X induced a slight increase in GABRA6 transcript abundance, similar to the effects of NFI proteins on transgene promoter activity. Together these findings indicated that NFI is an important regulator of GABRA6 gene transcription in CGNs.

Chromatin immunoprecipitation assays were next used to determine whether NFI proteins interact with the mouse GABRA6 promoter in differentiated CGNs *in vivo*. Primers were chosen that specifically amplified the proximal GABRA6 promoter region containing the NFI response element (see Fig. 1). Analysis of 6 DIV cultures using NFI antibodies demonstrated strong and specific NFI binding to this promoter region (Fig. 4*D*). Thus, NFI proteins directly interact with the endogenous GABRA6 promoter in CGNs, further supporting their importance in the physiological regulation of the GABRA6 gene.

NFI Gene Expression and DNA Binding Activity Are Enriched in CGNs—EMSAs showed that NFI DNA-binding complexes were elevated in P15 mouse cerebellum relative to cortex (see Fig. 2*A*), suggesting that this factor is involved in region-specific gene expression within the brain. Furthermore, the NFI binding site is located within a region of the GABRA6 gene previously implicated in its CGN-specific expression (26). We therefore examined the potential role of NFI in neuronal subtype-specific expression of the GABRA6 gene in CGNs. First, immunohistochemistry demonstrated that NFI proteins

were concentrated in mature CGNs within the internal granule cell layer (Fig. 5*A*), whereas staining in Purkinje and other cell types was much lower. Thus, CGNs are the major site of NFI expression within the adult mouse cerebellum.

Next, we compared the expression of NFI in cortical neurons, which do not express the GABRA6 gene, relative to CGNs. EMSAs revealed that NFI complexes were very low in nuclear extracts from 6 DIV cortical neurons (Fig. 6*A*). Furthermore, transcript levels for all NFI isoforms were substantially reduced in cortical neurons as compared with CGNs (Fig. 6*B*). These findings suggested that differential expression of NFI proteins was an important determinant of the selective expression of the GABRA6 gene in CGNs. Lentiviral promoter studies confirmed that the GABRA6 promoter was essentially inactive in infected cortical neurons (Fig. 6*C*). Co-expressed wild-type versions of different NFI family members did not increase promoter activity in cortical cultures (data not shown), indicating that additional cell-specific factors or mechanisms also are required for CGN-specific activation of the GABRA6 promoter by NFI proteins.

GABRA6 Expression in CGNs Requires NFI-A—NFI-A was originally identified as a cerebellar-enriched NFI protein (30), and mutant mice lacking this gene exhibit neurological defects (11). However, a direct role for NFI-A in cerebellar development has not been previously reported. We therefore examined the expression of GABRA6 in the cerebellum of *Nfia*-null mice at P16–P17, when GABRA6 expression becomes predominant in mouse CGNs. Immunohistochemistry revealed a marked decline in GABRA6 staining within the internal granule cell layer of *Nfia*-null mice relative to wild-type (Fig. 7*A*). This effect was not limited to certain neuronal sub-populations but occurred generally among CGNs. Furthermore, a partial decline in GABRA6 expression also was detectable in the cerebellum of *Nfia* (±) mice, indicating a gene dosage effect on receptor expression. Quantitation of these effects using real-time PCR of total RNA from P16 mouse brain showed that the *Nfia*-null mutation resulted in a 4-fold decline in GABRA6 transcripts relative to wild-type controls (Fig. 7*B*). Thus, NFI-A is a critical regulator of the *in vivo* expression of the GABRA6 gene in the mouse cerebellum.

DISCUSSION

NFI interacts with the promoters for numerous cell-specific and developmentally expressed genes in a diverse range of cell types, including neurons (31–33). Furthermore, gene knockout studies have demonstrated the necessity for *Nfia* in CNS midline glia formation (12). However, clearly defined roles and *in vivo* target genes for NFI proteins during neuronal differentiation remain elusive. To our knowledge, GABRA6 is the first neuronal gene shown to be a direct target of NFI *in vivo*. The present studies directly implicate NFI proteins as neuronal subtype-enriched transcriptional regulators of the GABRA6 gene in CGNs. Using a combination of functional approaches, we found that an NFI site within the GABRA6 proximal promoter is essential for its transcriptional activation in differentiated CGNs, and endogenous NFI proteins associate with this promoter *in vivo* and are important for GABRA6 gene expression in these cells. Furthermore, analyses of knockout mice showed that NFI-A is specifically required for GABRA6 gene activation. Residual expression of GABRA6 mRNA and protein in *Nfia*-knockout mice may reflect contributory roles for other NFI family members in regulating this gene. Expression of NFI-C, -B, and -X genes also is elevated in CGNs along with NFI-A, and thus these proteins may participate in GABRA6 gene regulation. Preliminary analyses of *Nfic*-null mice have found no major alterations in CGN expression of GABRA6 (data not shown), suggesting that this family member does not play a critical role. The involvement of NFIB and NFIX in GABRA6 expression cannot be assessed in knockout mice at this time, because *Nfib*-null animals die perinatally and *Nfix*-deficient mice are currently not available.

CGNs provide an excellent model for examining cell-intrinsic mechanisms responsible for neuronal subtype specification and the regulation of neuronal maturation. Based on transplantation studies, CGN progenitors in the postnatal cerebellum are already committed (34, 35), and thus much of their differentiation program is intrinsically driven. This includes initial cell polarization and cell migration events (36, 37) and the expression of differentiation-related genes (7, 29), including GABRA6. For example, GABRA6 expression persists in transplanted CGNs developing within ectopic CNS locations (7). Similarly, granule neurons of HNF-3β transgenic mice migrate inappropriately and do not form a normal internal granule cell layer, yet continue to express GABRA6 (38). Numerous studies also have shown that the expression of GABRA6 is retained in isolated CGN cultures (6, 29). The present studies demonstrate that NFI is an integral part of the intrinsic program directing CGN differentiation through its regulation of the GABRA6 gene.

Several transcription factors are involved in CGN lineage specification (reviewed in Ref. 39), including RU49/Zipro1, Engrailed-2, and Math1. NFI-A was originally identified as a cerebellar-enriched form of NFI (30); however, the present studies show that *all* NFI family members are expressed at elevated levels in CGNs. These results along with our functional studies implicate NFI in the neuronal subtype-specific expression of the GABRA6. The inability of exogenous NFI proteins to stimulate GABRA6 promoter activity in cortical neurons raises the possibility that additional, possibly CGN-enriched cofactors also are required for subtype-specific expression. This may involve transcription factors acting through other, as yet undefined regulatory regions of the GABRA6 gene. Interactions between NFI proteins and transcriptional co-regulators have been directly implicated in cell-specific gene regulation in several tissues and cell types (40–42).

NFI family members have been implicated in gene regulation within several neuronal types, including olfactory (33) and cortical and midbrain neurons (20). However, the enriched expression of NFI genes in CGNs suggests a specialized role in the differentiation and function of these cells. As discussed above, NFI sites are frequently found in gene promoters. These factors therefore may have a broader role in CGN differentiation, regulating multiple genes. Different NFI family members also may possess distinct, complementary functions in CGNs. Future analyses of NFI-null mice as well as dominant-negative studies will provide deeper insight into the roles and target genes for NFI proteins during the maturation of CGNs. The present findings also demonstrate that lentiviruses can serve as effective vectors for these studies. In particular, they provide highly efficient expression of both transcriptional regulators and transgene promoters in primary neurons. These approaches should provide further insight into the transcriptional mechanisms controlling neuronal specification and maturation.

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