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

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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 inter-actions in directing the phenotypes of distinct neuronal subtypes at different phases of their maturation.

Cerebellar granule neurons (CGNs) 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 granular 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_AR subunits 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_AR subunit 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 (Nfiec) (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.
NFI Controls GABRA6 Expression in Granule Neurons

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 PercollTM 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-lysine at a density of 2 × 10^5/ml in NeurobasalTM 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% CO2. Mouse cortical neurons were prepared from E18 CD1 pups. Dissected cortices were digested with trypsin at room temperature for 2 min. After mechanical trituration, the cells were plated on plastic dishes coated with 100 μg/ml poly-lysine at a density of 2 × 10^5/ml in the same NeurobasalTM/B27 medium used for CGNs.

Human embryonic kidney 293T cells and human chorionicarcoma 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). Human embryonic kidney 293T cells and human choriocarcinoma JEG3 cells were transduced with lentiviral expression vectors at multiple dilutions and assayed for luciferase activity. 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, GTTCCTTTGCTGAGCTGGTGA; antisense, TCCCGGTTGACTGATCATT) was used as an internal reference for normalization of GABRA6 (sense, TGGACGCCGAGATTGTTGTG; antisense, CAGGCTTGACTTTAAGATGTA) transcripts. The specificity of primers was confirmed by sequencing the RT-PCR products. Expression studies were repeated at least three times in different experiments and the presentation of results used the 2^-ΔΔCT method as previously described (24).

Immunohistochemistry and Western Blotting—Mouse brains were fixed by transcardial perfusion with 4% paraformaldehyde in phosphate-buffered saline at pH 7.4. Cerebella were dissected and kept in 30% sucrose at 4 °C for 10 h followed by overnight incubation in 30% sucrose at 4 °C. Frozen sections (16 μm) were examined for NFI proteins using a 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) (1:2000) that recognizes all NFI isoforms containing the NFI carboxy-terminal domain (specificity determined by western blotting of nuclear proteins. DNA competitors were used at a 100-fold excess relative to the DNA probe concentration. Antisera that recognize native NFI proteins (16) or pre-immune rabbit serum were used as supershift specific controls. All experiments were repeated at least three times in different experiments and average densitometry values were determined by analyzing genomic DNA from viral-infected JEG3 cells using competitive PCR for luciferase activity. The Student’s t-test was used for statistical analysis.

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 pCMVΔ1.91 and the pMD.G vesicular stomatitis virus G protein viral envelope expression vector into 293T cells. High titer viral stocks were prepared by ultracentrifugation as previously described (22). The titers of CMV-b-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 promoters 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 virus-infected JEG3 cells using competitive PCR for luciferase activity. The Student’s 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-dT12–18 primers or random hexamers using M-MLV reverse transcriptase (RT-PCR) (Invitrogen). Semi-quantitative RT-PCR assays are listed in the Supplemental Table. Quantitative RT-PCR products were analyzed by real-time RT-PCR. The expression of mGABRA6 was determined by real-time RT-PCR (Applied Biosystems). The expression of mGABRA6 was determined by real-time RT-PCR (Applied Biosystems). The expression of mGABRA6 was determined by real-time RT-PCR (Applied Biosystems). The expression of mGABRA6 was determined by real-time RT-PCR (Applied Biosystems). The expression of mGABRA6 was determined by real-time RT-PCR (Applied Biosystems).

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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 (Ctx). As shown in Fig. 1, the A3 sequence strongly competed 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). Nuclear extracts from 6 DIV CGNs were incubated without (∗) or with a 100-fold molar excess of unlabeled A region DNA (AN) or an unrelated competitor DNA (NS). Specific complexes are shown by an asterisk.

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, A1–A6, only one of which, A3, competed well for binding of 6 DIV CGN extracts to this region (Fig. 3A)). Further, specific binding to this region was confirmed in EMSAs using an A3 DNA probe and CGN extracts (Fig. 3B). As shown in Fig. 1, the A3 sequence contained 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). EMSAs using the proximal A region probe, including the majority of the transcription initiation sites (Fig. 2A), were competed with nuclear extracts from 6 DIV CGNs using seven different overlapping competitor sequences (A1–A6; see Fig. 1) or with a 100-fold molar excess of unlabeled A region DNA (AN) or an unrelated competitor DNA (NS). Specific complexes are shown by an asterisk.

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. A1–A6 boxes indicate the location of predicted transcription initiation sites (Fig. 2A). 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.

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 (AN) 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 NFi mut sequences were used as competitors. 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 (Ctx). As shown in Fig. 1, the A3 sequence strongly competed 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). Nuclear extracts from 6 DIV CGNs were incubated without (∗) or with a 100-fold molar excess of unlabeled A region DNA (AN) or an unrelated competitor DNA (NS). Specific complexes are shown by an asterisk.

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NFI Controls GABRA6 Expression in Granule Neurons

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 polyuridine 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 18S 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.

NFI proteins specifically bind to an NFI consensus site within the mouse GABRA6 proximal promoter and are enriched in CGN cultures that express a dominant repressor form of NFI comprising the engrailed repressor domain alone (EnR) or β-galactosidase (β-Gal). The asterisk indicates significantly different from β-galactosidase or EnR control (p < 0.01). The presence of NFI proteins in differentiated CGN cultures was further demonstrated by Western analysis (Fig. 3D). A shift in the GABRA6 band is observed upon NFI expression, indicating that NFI binds to the GABRA6 promoter.

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. 4B). Dominant repressor NFI/EnR strongly suppressed GABRA6 promoter activity when compared with control LacZ and EnR-alone expressed proteins (Fig. 4B). This directly implicated endogenous NFI as a critical regulator of GABRA6 expression in granule neurons.

1. W. Wang and D. L. Kilpatrick, unpublished observations.
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 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 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. Asterisk, significantly different from wild-type (p < 0.05).
were concentrated in mature CGNs within the internal granule cell layer (Fig. 5A), 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. 6A). Furthermore, transcript levels for all NFI isoforms were substantially reduced in cortical neurons as compared with CGNs (Fig. 6B). 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. 6C). 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. 7A). 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. 7B). 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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**REFERENCES**

1. Bertrand, N., Castro, D. S., and Guillemot, F. (2002) *Nat. Rev. Neurosci.* **3**, 517–530.
2. Goldowitz, D., and Hamre, K. (1998) Trends Neurosci. 21, 375-382
3. Wisden, W., Korpi, E. R., and Bahn, S. (1996) Neuropharmacology 35, 1139–1140
4. Brickley, S. G., Revilla, V., Cull-Candy, S. G., Wisden, W., and Farrant, M. (2001) Nature 409, 88–92
5. Kato, K. (1990) J. Mol. Biol. 214, 619–624
6. Gao, B., and Fritschy, J. M. (1995) Brain Res. 702, 1–16
7. Bahn, S., Wisden, W., Dunnett, S. B., and Svendsen, C. (1999) Eur. J. Neurosci. 11, 2194–2198
8. Gronostajski, R. M. (2000) Gene (Amst.) 249, 31–45
9. Grunder, A., Ebel, T. T., Mallo, M., Schwarzkopf, G., Shimizu, T., Sippel, A. E., and Schrewe, H. (2002) Mech. Dev. 112, 69–77
10. Steele-Perkins, G., Butz, K. G., Lyons, G. E., Zeichner-David, M., Kim, H. J., Cho, M. I., and Gronostajski, R. M. (2003) Mol. Cell. Biol. 23, 1075–1084
11. Bahn, S., Wisden, W., Dunnett, S. B., and Svendsen, C. (1999) Eur. J. Neurosci. 11, 2194–2198
12. Shu, T., Butz, K. G., Plachez, C., Gronostajski, R. M., and Richards, L. J. (2003) J. Neurosci. 23, 203–212
13. Hatten, M. E. (1985) J. Cell Biol. 100, 384–396
14. Liu, F., Tokesh, J., Persengiev, S. P., Ebert, K., and Kilpatrick, D. L. (1997) J. Biol. Chem. 272, 5056–5062
15. Beijersbergen, R. L., Carlee, L., Kerkhoven, R. M., and Bernards, R. (1995) Genes Dev. 9, 1340–1353
16. Puzianowska-Kuznicka, M., and Shi, Y. B. (1996) J. Biol. Chem. 271, 6273–6282
17. Zafferey, R., Dull, T., Mandel, R. J., Bukovsky, A., Quiroz, D., Naldini, L., and Trejo, D. (1998) J. Virol. 72, 9873–9880
18. Bahn, S., Jones, A., and Wisden, W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9417–9421
19. Barry, S. C., Harder, B., Brzezinski, M., Flint, L. Y., Seppen, J., and Osborne, W. K. (2001) Hum. Gene Ther. 12, 1103–1108
20. Chaudhry, A. Z., Lyons, G. E., and Gronostajski, R. M. (1997) Dev. Dyn. 208, 313–325
21. Kessler, D. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13017–13022
22. Reiser, J. (2000) Gene. Ther. 7, 910–913
23. Condit, R. C. (2001) in Principles of Virus Structure, 4th Ed., Fields Virology (Knipe, D. M., and P. M. H., ed) pp. 31–32, Lippincott Williams & Wilkins, Philadelphia, PA
24. Livak, K. J., and Schmittgen, T. D. (2001) Methods 25, 402–408
25. Luo, R. X., Postigo, A. A., and Dean, D. C. (1998) Cell 92, 463–473
26. McLean, P. J., Shepekt, D., Randzypadhyay, S., Russe, S. J., and Farh, D. H. (2000) J. Neurochem. 74, 1558–1569
27. Jones, A., Bahn, S., Grant, A. L., Kohler, M., and Wisden, W. (1996) J. Neurochem. 67, 907–916
28. Zheng, T., Santi, M. R., Bovolin, P., Marlier, L. N., and Grayson, D. R. (1993) Brain Res. Dev. Brain Res. 75, 91–103
29. Lin, X., and Bulleit, R. F. (1996) Neurosci. Lett. 220, 81–84
30. Inoue, T., Tamura, T., Furushi, T., and Mikoshiba, K. (1990) J. Biol. Chem. 265, 19065–19070
31. Adams, A. D., Choate, D. M., and Thompson, M. A. (1995) J. Biol. Chem. 270, 6975–6983
32. Behrens, M., Venkatraman, G., Gronostajski, R. M., Reed, R. R., and Margolis, F. L. (2000) Eur. J. Neurosci. 12, 1372–1384
33. Carletti, B., Grimaldi, P., Magrassi, L., and Rossi, F. (2002) J. Neurosci. 22, 7132–7146
34. Yacubova, E., and Komuro, H. (2002) J. Neurosci. 22, 5966–5981
35. Yamashita, T., Kawaji, K., Ono, K., Bito, H., Hirano, T., Osumi, N., and Komuro, H. (2002) J. Neurosci. 22, 2308–2319
36. Zhu, H., Hughes, D. E., Major, M. L., Yoo, K., Pesold, C., and Costa, R. H. (2001) Gene Expr. 9, 217–236
37. Hatten, M. E., Alder, J., Zimmerman, K., and Heintz, N. (1997) Curr. Opin. Neurobiol. 7, 40–47
38. Zhou, H., Hughes, D. E., Major, M. L., Yoo, K., Pesold, C., and Costa, R. H. (2001) Gene Expr. 9, 217–236
39. Hattie, M. E., Alder, J., Zimmerman, K., and Heintz, N. (1997) Curr. Opin. Neurobiol. 7, 40–47
40. Mill, S., Hartig, E., Jenneswein, P., Doppler, W., and Cato, A. C. (1992) Mol. Cell. Biol. 12, 4906–4918
41. Oriz, L., Azan-Blanc, P., Tannini, M., Cato, A. C., and Santisteban, P. (1999) J. Biol. Chem. 274, 15213–15221
42. Spitz, F., Salminen, M., Demignon, J., Kahn, A., Daegelen, D., and Maire, P. (1997) Mol. Cell. Biol. 17, 656–666