

Generation of neurons by transient expression of neural bHLH proteins in mammalian cells

Mohamed H. Farah^{1,2}, James M. Olson^{3,4}, Holly B. Susic¹, Richard I. Hume^{2,5}, Stephen J. Tapscott⁶ and David L. Turner^{1,2,*}

¹Mental Health Research Institute, ²Neuroscience Program, and ⁵Department of Biology, University of Michigan, Ann Arbor, MI 48104-1687, USA

³Division of Pediatric Oncology, University of Washington, Seattle, WA 98105, USA

⁴Clinical Division and ⁶Division of Molecular Medicine, Fred Hutchinson Cancer Research Center, Seattle WA 98109, USA

*Author for correspondence (e-mail: dltturner@umich.edu)

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SUMMARY

Basic helix-loop-helix (bHLH) transcription factors are known to function during mammalian neurogenesis. Here we show that transient transfection of vectors expressing *neuroD2*, *MASH1*, *ngn1* or related neural bHLH proteins, with their putative dimerization partner *E12*, can convert mouse P19 embryonal carcinoma cells into differentiated neurons. Transfected cells express numerous neuron-specific proteins, adopt a neuronal morphology and are electrically excitable. Thus, the expression of neural bHLH proteins is sufficient to confer a neuronal fate on uncommitted mammalian cells. Neuronal differentiation of transfected cells is preceded

by elevated expression of the cyclin-dependent kinase inhibitor *p27^{Kip1}* and cell cycle withdrawal. This demonstrates that the bHLH proteins can link neuronal differentiation to withdrawal from the cell cycle, possibly by activating the expression of *p27^{Kip1}*. The ability to generate mammalian neurons by transient expression of neural bHLH proteins should create new opportunities for studying neurogenesis and devising neural repair strategies.

Key words: Basic-helix-loop-helix, Transcription, Neuron, *p27*, Mouse

INTRODUCTION

MASH1, *neuroD*, *neuroD2*, *MATH1-3* and *neurogenin1-3* (*ngn1-3*) are basic helix-loop-helix (bHLH) transcription factors expressed during mammalian neuronal determination and differentiation (Johnson et al., 1990; Bartholoma and Nave, 1994; Akazawa et al., 1995; Lee et al., 1995; Shimizu et al., 1995; Ma et al., 1996; McCormick et al., 1996; Sommer et al., 1996; Takebayashi et al., 1997). Targeted disruptions of *MASH1*, *MATH1*, *ngn1*, *ngn2* or *neuroD* in mice lead to the loss of specific subsets of neurons (Guillemot et al., 1993; Ben-Arie et al., 1997; Fode et al., 1998; Ma et al., 1998; Miyata et al., 1999). Although the neural bHLH proteins encoded by these genes are essential for neuron formation, their functions during neurogenesis remain unclear. Forced expression of either *neuroD* in *Xenopus* neural ectoderm (Lee et al., 1995) or *MASH1* in rat neural crest stem cells (Lo et al., 1998) leads to premature neuronal differentiation, suggesting that the neural bHLH proteins may regulate the formation of neurons from neural precursors. In *Xenopus*, zebrafish or chicken embryos, ectopic expression of *neuroD*, *ngn2* or other neural bHLH proteins leads to the formation of neurons from a subset of non-neural cells (Turner and Weintraub, 1994; Lee et al., 1995; Ma et al., 1996; Blader et al., 1997; Perez et al., 1999), showing that neural bHLH proteins can impose a neuronal fate

on some cells. In mammalian systems, however, expression of neural bHLH proteins has not led to the formation of neurons from non-neural cells (Johnson et al., 1990, 1992). Thus, it is not clear whether neural bHLH proteins are sufficient to direct mammalian cells to adopt a neural fate, or whether they can only regulate neuronal differentiation in mammals.

We have investigated the ability of neural bHLH proteins to direct neuron formation from pluripotent mouse P19 embryonal carcinoma cells. P19 cells were derived from a mouse embryo and have been used extensively as a model system for in vitro differentiation (McBurney, 1993; Bain et al., 1995). After treatment with retinoic acid (RA) and aggregation, P19 cells differentiate into neurons and glia while, under other conditions, these cells can form skeletal or cardiac muscle, endoderm or other cell types. Numerous genes are activated in P19 cells in response to RA treatment, including genes encoding several neural bHLH proteins (Johnson et al., 1992; McCormick et al., 1996; Boudjelal et al., 1997; Itoh et al., 1997). Here we show that transient expression of *neuroD2*, *MASH1*, *ngn1* or related bHLH proteins is sufficient to convert P19 cells into differentiated neurons in the absence of RA or aggregation. Moreover, we find that expression of neural bHLH proteins can initiate cell cycle withdrawal, accompanied by elevated expression of the cyclin-dependent kinase inhibitor (CDKi) *p27^{Kip1}* (Polyak et al., 1994). These observations

demonstrate that the neural bHLH proteins can impart a neuronal fate on mammalian cells and that these proteins can link neuronal differentiation to cell cycle regulation.

MATERIALS AND METHODS

Expression plasmids

pCS2+MTXneuroD (Lee et al., 1995), pCS2+MTXngn2 (pCS2+MTXNGNR1a) (Ma et al., 1996), pCS2+ngn1/neuroD3 (McCormick et al., 1996) and pCS2+ mouse MyoD (Rupp et al., 1994) have been described previously. cDNAs for other mouse neural bHLH proteins (see Introduction for references), rat MASH1 (Johnson et al., 1990), hamster BETA3 (Peyton et al., 1996), mouse E12 (Walker et al., 1990) and EGFP (Clontech) were cloned into pCS2+ or pCS2+MT (for myc epitope tag fusions; Rupp et al., 1994; Turner and Weintraub, 1994). Details are available upon request. pCS2+ Hand1/Thing1, pCS2+Hand2/Thing2, and pCS2+Epicardin/Halo1 were provided by Stan Hollenberg (Hollenberg et al., 1995). For the data in Table 1, pCS2+ constructs were used, except for neuroD and Xngn2, which were expressed in pCS2+MT. However, MATH1-3 and neuroD2 gave similar results in either pCS2+ or pCS2+MT (Fig. 1A-C and unpublished observations).

The neuroD2^{DBM} mutation changes E131R132 to A131Q132. These residues are conserved in almost all bHLH proteins and are required for DNA binding in MyoD (Davis et al., 1990). An identical mutation in the *Xenopus* XASH3 protein is non-functional in *Xenopus* embryos (Turner and Weintraub, 1994). pCS2+ neuroD2^{DBM} was generated by PCR of pCS2+neuroD2 with Pfu1 (Stratagene), using the oligos GCCAATGCGCGAGCTCAGAACC GCATGCACGAC and GCATGCGGTTCTGAGCTCGCGCATTGGCCTTCT.

Cell culture and transfections

P19 cells were cultured in MEM α with 7.5% calf serum and 2.5% fetal bovine serum (HyClone) and maintained subconfluent prior to transfection (McBurney et al., 1988). With cells maintained under these growth conditions, we observed less than one spontaneous TuJ1-labeled cell per million untransfected P19 cells (0-5 TuJ1-positive cells per confluent 60 mm dish, 4 days after plating). For transfections, cells were plated on murine laminin (Gibco) or poly-L-lysine (Sigma)-coated dishes (Corning). Transfections were performed by the BES-buffered saline (BBS) CaPO4 method (Chen and Okayama, 1987) with 5% CO₂, or with FuGene6 (Roche) as directed by the manufacturer. For a 60 mm dish, BBS transfections included 7.5 μ g neural bHLH vector, 2.5 μ g E12 vector and 5 μ g GFP vector. For FuGene transfections, one third as much DNA was used. When necessary, a vector that expresses a nuclear localized multimerized myc tag, pCS2+nlsMT (Rupp et al., 1994), was added to maintain a constant total amount of DNA. Cotransfection efficiency was at least 90%, based on a comparison of GFP with a cotransfected myc epitope-tagged protein (pCS2+nlsMT).

For longer term culture of transfected P19 cells, media and serum were replaced 2 days after transfection with Neurobasal media and B27 supplement (Gibco), without glutamine or glutamate, plus 15 μ g/ml cytosine arabinoside (araC)(Sigma). 2 days later, the media was changed to Neurobasal media and B27 supplement with 0.5 mM glutamine and 15 μ g/ml araC. Media was changed every 2 days thereafter. Most untransfected P19 cells die after 1-2 days in these conditions.

10T1/2 cells were grown in DMEM with 10% calf serum. Primary rat astrocytes were grown in DMEM with 10% fetal bovine serum and 33 mM glucose. Transfections and immunohistochemistry were performed as described for P19 cells.

Immunohistochemistry and antibodies

Cells were fixed for 8-20 minutes with either 3.7% formaldehyde or 4% paraformaldehyde in phosphate-buffered saline (PBS). For amino

acid neurotransmitter antibodies, 1% glutaraldehyde was included. Primary antibodies are listed followed by the antigen, with the dilution used in parentheses. TuJ1 neuron-specific tubulin (Babco, 1:1000). From Chemicon International, Inc.: AB1515 internexin (1:1000), MAB1621 neurofilament-M (1:1000), MAB 1615 neurofilament-L (1:250), MAB1623 neurofilament-H (1:300), AB1778 calbindin (1:1000), AB133 glutamate (1:200-500), AB141 GABA (1:200), AB1566 substance P (1:200-1000, preabsorbed on untransfected P19 cells), MAB363 NMDAR1 (1:200). From the Developmental Studies Hybridoma Bank (supernatants diluted 1:3; ascites 1:500): 5A5 NCAM, 40.2D6 islet1, 4F2 LIM1+2, RC2 and 3CB2 radial glia, 2H3 neurofilament-M, and MF20 myosin heavy chain. Other antibodies: rabbit anti-GFAP (Sigma, 1:500), rabbit anti-synapsin 1 (1:500, gift of T. Ueda), M6 (1:10, gift of C. Lagenaur), and 16A11 HuC/D (1:500, gift of M. Marusich). For p27^{Kip1}, similar results were obtained with three different antibodies (Santa Cruz sc397, 1:1000; Neomarkers DCS72F6, 1:600; Transduction Laboratories K25020, 1:250).

Secondary antibodies were Cy3- or rhodamine-conjugated anti-rabbit or anti-mouse (1:500-1000, Jackson or Chemicon). Because of glutaraldehyde-induced autofluorescence, GABA and glutamate expression was confirmed with alkaline phosphatase-conjugated goat anti-rabbit (Roche).

Cells were photographed with a 35 mm film camera or a color video camera and images were digitized. Double-label images were assembled in Adobe Photoshop. To determine the percentage of TuJ1-labeled cells (Table 1), digitized video images were superimposed in Photoshop and scored on a computer monitor.

Reporter assays

The reporter EB7Luc was cloned by inserting three copies of a double-stranded oligonucleotide AGAGAGTGACAGATGCGCGGGGT into the *Sma*I site of pT81-Luc. This oligo contains an E-box (underlined) from the neuroD2 promoter (J. M. O. and S. J. T., unpublished observations). Reporter activity was assayed 2 days after transfection. To control for transfection efficiency, luciferase activity was normalized to β -galactosidase activity from cotransfected pCS2+c β gal (Turner and Weintraub, 1994) using the Tropix Dual Light system. Data shown is an average from three experiments.

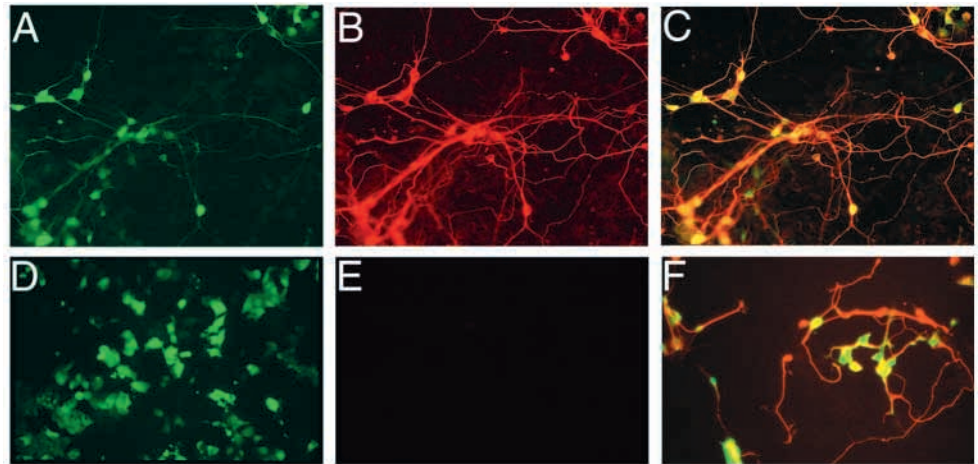
Electrophysiology

Tight-seal, whole-cell recordings from cells in culture 3-7 days after transfection were made using standard techniques. Transfected cells were identified by GFP fluorescence. The pipette solution contained (in mM) KCl 140, NaCl 5, MgCl₂ 1, Na₂EGTA 10, Hepes 10, pH 7.4. In voltage-clamp experiments in which the ionic dependence of the voltage-dependent inward current was tested, extracellular solutions were: normal sodium solution (in mM): NaCl 140, KCl 1, MgCl₂ 1, CaCl₂ 1, Hepes 10, pH 7.4. choline solution: cholineCl 139, NaCl 1, KCl 1, MgCl₂ 1, CaCl₂ 1, Hepes 10, pH 7.4. The choline solution was applied to the cell under study from a 0.9 mm diameter pipette placed close to the cell. The shift in reversal potential (see Results) indicated that the solution exchange was about 95% effective. In all other experiments, the external solution used contained (in mM) 132 NaCl, 5.3 KCl, 1.3 NaH₂PO₄, 1.7 MgSO₄, 5.4 CaCl₂, 12 Hepes, 6.3 glucose and 0.3 phenol red (pH, 7.4). To assess excitability, voltage responses obtained under current clamp were classified as non-regenerative or regenerative. Constant current was passed so that the resting potential was near -80 mV; 2.5 msec depolarizing current pulses of increasing amplitude were given, until a regenerative response was elicited or until the cell was depolarized to 0 mV. A response was considered regenerative if it deviated by +2 mV or more from that predicted by passive decay.

Proliferation studies

For BrdU experiments, transfected P19 cells were fixed as described under Immunohistochemistry and antibodies. BrdU (final 15 ng/ml,

Fig. 1. Generation of neurons by transfection of *neuroD2*. (A-C) P19 cells transiently cotransfected with vectors for myc epitope-tagged *neuroD2*, E12 and GFP were fixed 4 days after transfection. (A) GFP fluorescence revealed that transfected cells adopted a neuronal morphology. (B) Most transfected cells expressed a neuron-specific β -tubulin isoform, detected by indirect immunofluorescence with the antibody TuJ1. (C) Combination of GFP fluorescence (green) and indirect immunofluorescence for TuJ1 (red). Overlapping signals are yellow or orange. (D,E) P19 cells cotransfected with E12 and GFP maintained the normal morphology of P19 cells as revealed by GFP fluorescence (D), and were not labeled by TuJ1 (E). (F) P19 cells transfected with *neuroD2* and GFP without E12 adopted a neuronal morphology and were labeled by indirect immunofluorescence for TuJ1. Colors are the same as in C.



Sigma) was added from 24-36 or 48-60 hours after transfection. Rat anti-BrdU supernatant (Accurate Chemical & Scientific) was diluted 1:40 in PBS with 1 mM CaCl₂, 1 mM MgCl₂ and 100 units per ml DNase (Promega), and applied to the cells for 1 hour at 37°C (modification of Amersham BrdU-labeling kit protocol). Secondary antibody was Texas Red-conjugated donkey anti-rat (Jackson) at 1:200, with 2% calf serum.

RT-PCR analysis

Transfections included the GFP vector and transfection efficiency was monitored by observation of GFP in live cells prior to RNA harvest. RT-PCR was performed essentially as described (Rupp and Weintraub, 1991) with 0.5 μ g of total RNA for each RT-PCR. Specific PCR primer pairs were designed for mouse *neuroD*, *neuroD2*, *MATH2* and *MATH3*. Primer pairs generate products that span the first intron of each target gene (to facilitate the identification of mRNA-derived products). These primer pairs do not generate a PCR product from the corresponding neural bHLH expression vectors used for transfections (since bHLH coding regions in the vectors start in the second exon of each gene). The product of the control primer pair for the ubiquitous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA spans three introns. Primer sequences are available upon request. PCR was performed for 18 cycles for GAPDH and 28 cycles for neural bHLH genes.

RESULTS

Generation of neurons from P19 cells by transfection of *neuroD2*

P19 cells were transiently cotransfected with plasmid expression vectors for mouse *neuroD2*, a bHLH dimerization partner E12 (Murre et al., 1989), and green fluorescent protein (GFP). Cells were maintained in monolayer culture under growth conditions, without RA. Transfected cells could be identified by GFP fluorescence. By 4 days after transfection, most GFP-expressing cells adopted a neuronal morphology with round cell bodies and one or more long processes. These cells expressed a neuron-specific class III β -tubulin protein, detected by indirect immunofluorescence with the antibody TuJ1 (Easter et al., 1993; Fig. 1A-C; Table 1). In contrast, cells transfected with only the E12 and GFP expression vectors

Table 1. Neuron formation and reporter activation in P19 cells transfected with bHLH plasmids

bHLH class	Transfected bHLH cDNA(s)	TuJ1+/GFP+ (%)	E-box reporter (fold activation)
Neuronal differentiation	<i>neuroD2</i> +E12	64	89
	<i>neuroD2</i>	49	36
	<i>neuroD</i> +E12	6	71
	<i>MATH2</i> +E1	<1	57
	<i>MATH3</i> +E12	2	43
	<i>neuroD2</i> ^{DBM} +E12	0	3
Neural precursor	<i>MATH1</i> +E12	12	32
	<i>ngn1</i> +E12	8	309
	<i>Xngn2</i> +E12	<1	266
	<i>ngn3</i> +E12	<1	149
Neural precursor (achaete-scute family)	<i>MASH1</i> +E12	36	202
	<i>MASH1</i>	31	29
	<i>D. m. scute</i> +E12	<1	36
Neuronal differentiation (other bHLH families)	<i>NSCL1</i> +E12	<1	3
	<i>Hand1</i> +E12	0	1
Repressor	<i>BETA3</i> +E12	0	2
Non-neural	<i>MyoD</i> +E12	0	32
	<i>Hand2</i> +E12	0	1
	<i>Epicardin</i> +E12	0	3
Dimerization partner	<i>nlsMT</i> +E12	0	1
Vector Control	<i>nlsMT</i>	0	1

Neuron formation was assessed by indirect immunofluorescence with TuJ1 4 days after transfection. The percentages of TuJ1 labeled cells (>1%) are from a representative experiment, with TuJ1 expression scored for 300-1100 GFP-labeled cells per vector. The E-box reporter column shows relative activation of a luciferase reporter by each neural bHLH vector (see text for details).

neuroD2^{DBM} contains a mutation in the DNA-binding domain (see text).

maintained the morphology of undifferentiated P19 cells and were not labeled by TuJ1 (Fig. 1D,E). Cotransfection of the *neuroD2* and GFP vectors without E12 also generated neurons (Fig. 1F), although fewer cells were TuJ1 positive (Table 1), and most processes on these cells were shorter than those generated by *neuroD2* with E12. The correlation between GFP

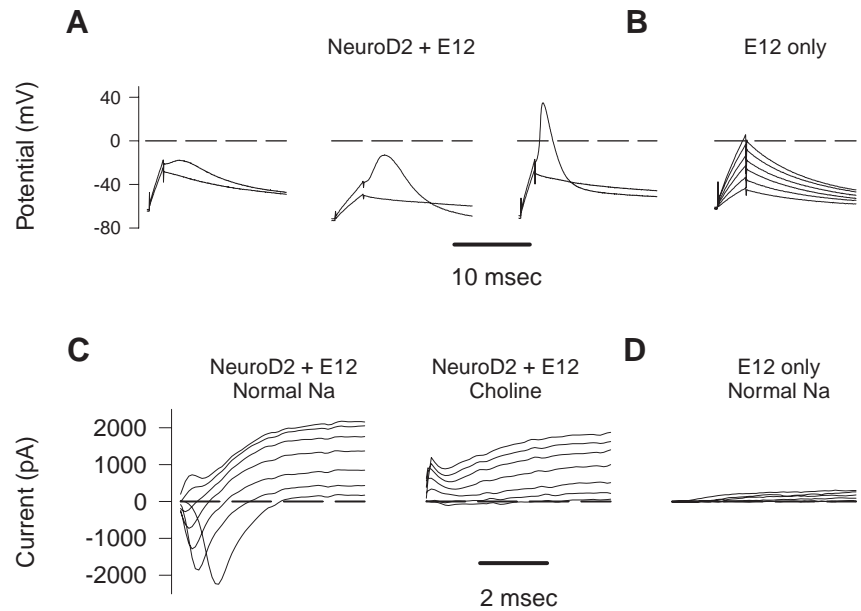


Fig. 2. Electrical properties of transfected P19 cells. (A) Range of current-clamp responses observed at 4–6 days after transfection with neuroD2, E12 and GFP. Each panel shows the response of a different cell to current pulses just below and just above threshold for eliciting a regenerative response. (B) No regenerative response was observed in a cell transfected with only E12 and GFP vectors. (C) Voltage-clamp responses from a neuroD2 and E12 transfected cell in normal sodium and choline external solutions. The cell was held at -100 mV, and then stepped over the range of -40 mV to $+80$ mV in 20 mV increments. (D) Voltage-clamp responses from an E12 transfected cell. The same pulse paradigm was used as in C.

expression and neuron formation (Fig. 1C) suggests that the generation of neurons after neuroD2 transfection is cell autonomous. We also tested a mutant of neuroD2 in which two essential residues in the DNA-binding basic region were altered (see Materials and Methods). Cotransfection of this mutant with the E12 plasmid into P19 cells did not generate neurons (Table 1), indicating that an intact DNA-binding domain is required for generation of neurons by the neuroD2 protein.

Since one hallmark of neurons is the ability to propagate electrical signals, we determined whether P19 cells transfected with neuroD2 and E12 vectors had the electrophysiological properties of neurons. Under current-clamp, transfected cells showed regenerative responses to depolarizing current steps in all cases ($n=14$; Fig. 2A). Cells transfected with only the E12 vector showed no regenerative responses ($n=10$; Fig. 2B). Under voltage-clamp with normal external sodium, all tested cells cotransfected with neuroD2 and E12 had rapidly activating, voltage-dependent, transient currents, which were inward at potentials negative to $+40$ mV ($n=25$; Fig. 2C), while none of ten E12 transfected control cells studied had fast activating inward currents (Fig. 2D). The rapidly activating currents were due to flow through voltage-dependent sodium channels, since the reversal potential decreased by 77 ± 5 mV when extracellular sodium was replaced with choline (Fig. 2C). Cells transfected with E12 alone or with neuroD2 and E12 showed slowly activating, voltage-dependent, sustained outward currents (Fig. 2C,D). However, the conductance increase associated with the outward current was about seven times larger in cells transfected with neuroD2 and E12 (peak conductance of 7.0 ± 1.1 nS, $n=25$, compared with 1.2 ± 0.3 nS, $n=10$, for cells transfected with only E12). Thus, expression of neuroD2 and E12 confers P19 cells with the electrical signaling properties of neurons.

Other neural bHLH proteins can generate neurons from P19 cells

To determine whether neural bHLH proteins other than neuroD2

could generate neurons from P19 cells, we transfected vectors expressing the MASH1, neuroD, ngn1–3, MATH1–3 or NSCL1 (Begley et al., 1992) bHLH proteins, in combination with the E12 plasmid. All of these transfections led to the generation of TuJ1-positive neurons from a subset of the transfected cells (Fig. 3A,D; Table 1), although the percentage of transfected cells that differentiated as neurons varied more than 100-fold (Table 1 and data not shown). Interestingly, MATH2 and most of the other neural bHLH plasmids that generated neurons infrequently were able to activate a cotransfected multimerized bHLH DNA-binding site (E-box) reporter (Table 1), indicating that these proteins were expressed and functional. The ability to generate neurons in P19 cells is an evolutionarily conserved characteristic of the neural bHLH proteins, since vectors expressing *Xenopus* ngn2 or *Drosophila melanogaster* (*D.m.*) scute also produced neurons when cotransfected with the E12 vector (Table 1). As observed for neuroD2, cotransfection of E12 was not essential for neuron formation by any of the neural bHLH proteins, but it did increase the number of TuJ1-positive cells and these cells often had more extensive processes (Table 1 and unpublished observations).

In contrast to the neural bHLH proteins, transfection of expression plasmids for the myogenic bHLH protein MyoD (Weintraub et al., 1991), the non-neural bHLH protein epicardin (Robb et al., 1998), or the predominantly non-neural bHLH proteins Hand1 and Hand2 (Cserjesi et al., 1995; Hollenberg et al., 1995), each with the E12 plasmid, failed to generate neurons (Table 1). BETA3, a protein structurally related to neuroD but reported to function as a repressor (Peyton et al., 1996), also did not produce neurons. Although MyoD activated the E-box reporter, other non-neural bHLH proteins activated the reporter weakly, if at all. These proteins may prefer a different E-box, they may not be transcriptional activators (e.g. BETA3 and epicardin), or they may be expressed at lower levels. Nonetheless, our results indicate that the ability to generate neurons from P19 cells is specific to the neural-specific bHLH proteins and is unlikely to result from promiscuous bHLH dimerization or other nonspecific interactions.

Characterization and persistence of neuronal differentiation after transient neural bHLH expression

To further characterize the extent of neuronal differentiation of P19 cells transfected with neural bHLH vectors, we assessed the expression of various neuron-specific proteins. Pan-neuronal markers such as neurofilament-M, the HuC/D RNA-binding proteins (Wakamatsu and Weston, 1997), M6 (Yan et al., 1996) and synapsin I were present in most or all cells with neuronal morphology (Table 2; Fig. 3B,C,G). Moreover, subsets of the transfected cells were immunoreactive for the neurotransmitters GABA and glutamate (Fig. 3E), the GABA synthetic enzyme glutamic acid decarboxylase (GAD), the neuropeptide substance P (Fig. 3F), NMDA receptor 1, and the transcription factors Islet-1 or LIM1/2 (Table 2). At present, we do not know if the subsets of cells that express these proteins overlap. We have observed the same constellation of neurotransmitters, receptors and other markers of mature neuronal subtypes regardless of which neural bHLH vector was transfected. We did not detect expression of glial fibrillary acidic protein (GFAP), or markers of radial glia, in any transfected cells (Table 2).

Neurons generated by transient transfection of P19 cells with plasmids for MASH1, neuroD2 or ngn1 each cotransfected with the E12 plasmid, survived for at least 13 days after transfection (Fig. 3G) when cultured under conditions that eliminate most untransfected cells (see Materials and Methods). Cells maintained neuronal morphologies and continued to express neuron-specific proteins. Epitope-tagged versions of neuroD2 or MATH1 could be detected in the nucleus of most transfected cells 1 or 2 days after transfection, but were detectable in only a few cells at 4 days, and were undetectable at 10 days (data not shown). The cotransfected GFP marker also was undetectable by 10 days after transfection (data not shown). This suggests that the neural bHLH proteins initiate a stable program of neuronal gene expression that is maintained after the introduced bHLH proteins are no longer present.

Cell cycle withdrawal and elevated p27^{Kip1} expression in P19 cells transfected with neural bHLH vectors

During neurogenesis, neuronal differentiation is preceded by withdrawal from the cell cycle. We asked whether neurons generated by expression of bHLH proteins withdrew from the cell cycle by using bromodeoxyuridine (BrdU) incorporation to label proliferating cells. 3 days after transfection, 84% of the GFP-expressing control transfected cells incorporated BrdU, while only 16% of the GFP-expressing cells cotransfected with neuroD2 and E12 incorporated BrdU (Fig. 4A-C). Other transfected neural bHLH cDNAs also reduced the fraction of cells that incorporated BrdU (Fig. 4C). The degree to which different neural bHLH proteins reduced BrdU incorporation paralleled the ability of each bHLH protein to generate neurons. Since transfected cells can survive for more than 10 days in the presence of cytosine arabinoside, a nucleotide analog that kills dividing cells (Fig. 3G), it is likely that cell cycle withdrawal triggered by the neural bHLH proteins is permanent.

The myogenic bHLH protein MyoD also can initiate cell cycle withdrawal, and this is likely to be mediated in part by a

Table 2. Neural antigens detected by indirect immunofluorescence after transfection of P19 cells with neural bHLH plasmids

Antigen type	Antigen	Expression	Notes
panneuronal	HuC/HuD (16A11)	+++	1,2
	internexin	+++	1,2
	neurofilament-M	+++	1,2
	neurofilament-L	+	1,2
	neurofilament-H	-	1,2
	synapsin1	+++	1,2
	M6	+++	1,2
	NCAM (siacylated form)	-	
Neuronal subpopulations	calbindin	++	1
	glutamate	+	1,2
	GABA	+	1,2
	GAD	+	1,2
	substance P	+	1,2
	NMDA receptor 1	+	1,2
	islet1	+	1,2
LIM1/2	+	1,2	
glial cells	GFAP	-	3
	radial glia (RC2, 3CB2)	-	1
muscle	myosin heavy chain	-	

P19 cells transfected with neuroD2, ngn1, or MASH1, each with E12 and GFP plasmids, were tested for all antigens. Cells were fixed at 4 to 7 days after transfection.

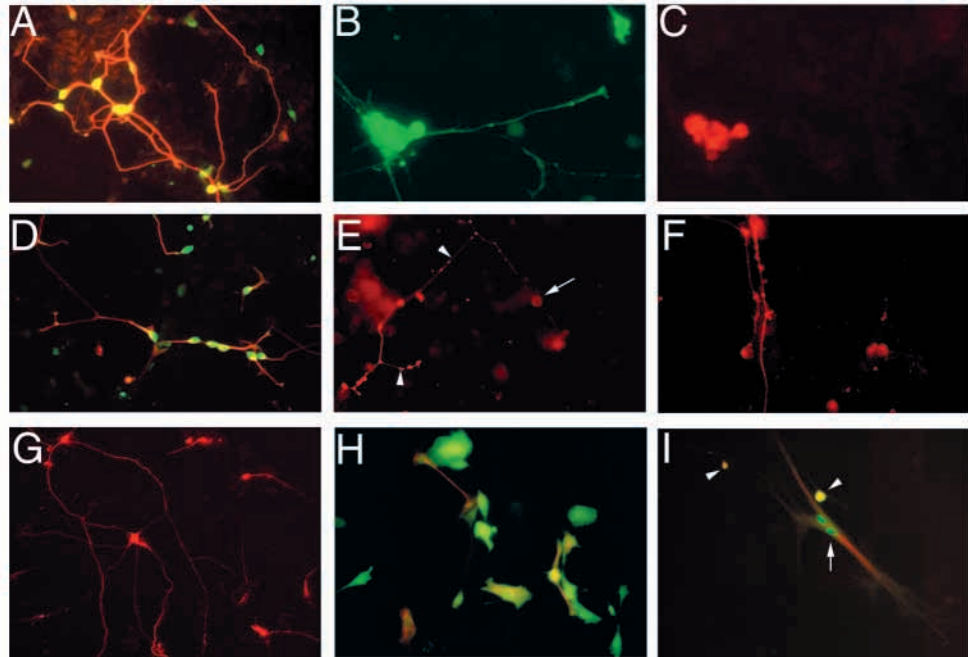
Expression: +++, most or all transfected cells with neuronal morphology (75-100%); ++, 10-25%; +, <10%; -, none.

Notes: similar results with (1) MATH1 and E12, (2) neuroD and E12, or (3) MATH1, MATH2, or MATH3 and E12.

direct activation of the CDKi p21 (Halevy et al., 1995; Otten et al., 1997). Recently, neuroD has been reported to inhibit cell cycle progression and activate p21 expression in a transfected non-neuronal cell line (Mutoh et al., 1998). We tested whether P19 cells transfected with the neural bHLH vectors and E12 expressed elevated levels of the CDKi proteins p21 or p27^{Kip1}. We could not detect p21 expression in P19 cells after transfection of either neuroD or other neural bHLH cDNAs (data not shown). However, P19 cells transfected with any of the neural bHLH vectors with E12 expressed the CDKi protein p27^{Kip1}, as assessed by indirect immunofluorescence with three different antibodies (Fig. 4D,E,H and data not shown; see Materials and Methods). In contrast, only occasional cells had detectable p27^{Kip1} expression in P19 cells transfected with a control vector (Fig. 4H).

To further characterize the relationship between p27^{Kip1} expression, cell cycle withdrawal and neuronal differentiation, we compared p27^{Kip1} expression with BrdU incorporation and TuJ1 expression after cotransfection of P19 cells with the neuroD2, E12 and GFP vectors. Most cells that expressed p27^{Kip1} did not incorporate BrdU (Fig. 4F,G). In addition, the percentage of transfected cells that expressed p27^{Kip1} was essentially the same as the percentage of cells that did not incorporate BrdU (Fig. 4H). These observations indicate that the transfected cells with elevated p27^{Kip1} did not enter the cell cycle. This is consistent with the possibility that the neural bHLH proteins drive cell cycle withdrawal by elevating the level of p27^{Kip1}. We also observed that almost all cells labeled by TuJ1 expressed p27^{Kip1} (Fig. 4I-K), and the percentage of TuJ1 and p27^{Kip1} double-labeled cells increased with time (Fig. 4K). This suggests that p27^{Kip1} expression (and cell cycle

Fig. 3. Generation of neurons from P19 cells by neural bHLH proteins and expression of neuronal proteins in transfected cells. (A) Transfection of *ngn1*, E12 and GFP into P19 cells generated TuJ1-labeled neurons: GFP (green), TuJ1 (red), overlaps are yellow. (B,C) The nuclei of cells transfected with MASH1, E12 and GFP labeled by indirect immunofluorescence with the antibody 16A11, which recognizes the neuron-specific HuC/D proteins: (B) GFP and (C) 16A11. (D) TuJ1-labeled neurons (red) generated from MASH1-, E12- and GFP-transfected P19 cells (green). (E) P19 cells transfected with *ngn1* and E12 labeled by indirect immunofluorescence with an antisera to the neurotransmitter glutamate (arrow denotes labeled cell body, arrowheads denote labeled neurites). (F) Neurons from *neuroD2* and E12 transfected P19 cells were labeled by indirect immunofluorescence with anti-substance P. (G) P19 cells cotransfected with MASH1, E12 and GFP and cultured for 13 days after transfection maintained a neuronal morphology and were labeled by indirect immunofluorescence with an antibody to neurofilament-M. (H) 10T1/2 fibroblasts transfected with MASH1, E12 and GFP (green) did not adopt a neuronal morphology, but were labeled by indirect immunofluorescence with TuJ1 (red; overlap is yellow or orange) 4 days after transfection. (I) Primary rat astrocytes transfected with *neuroD2*, E12 and GFP (green) labeled by indirect immunofluorescence with TuJ1 (red; overlap with GFP is yellow or orange). Most transfected astrocytes maintained a flat morphology (arrow), but in rare instances transfected cells with a neuronal morphology were observed (arrowheads).



withdrawal) preceded neuronal differentiation in the transfected cells.

Endogenous bHLH gene expression in P19 cells transfected with neural bHLH vectors

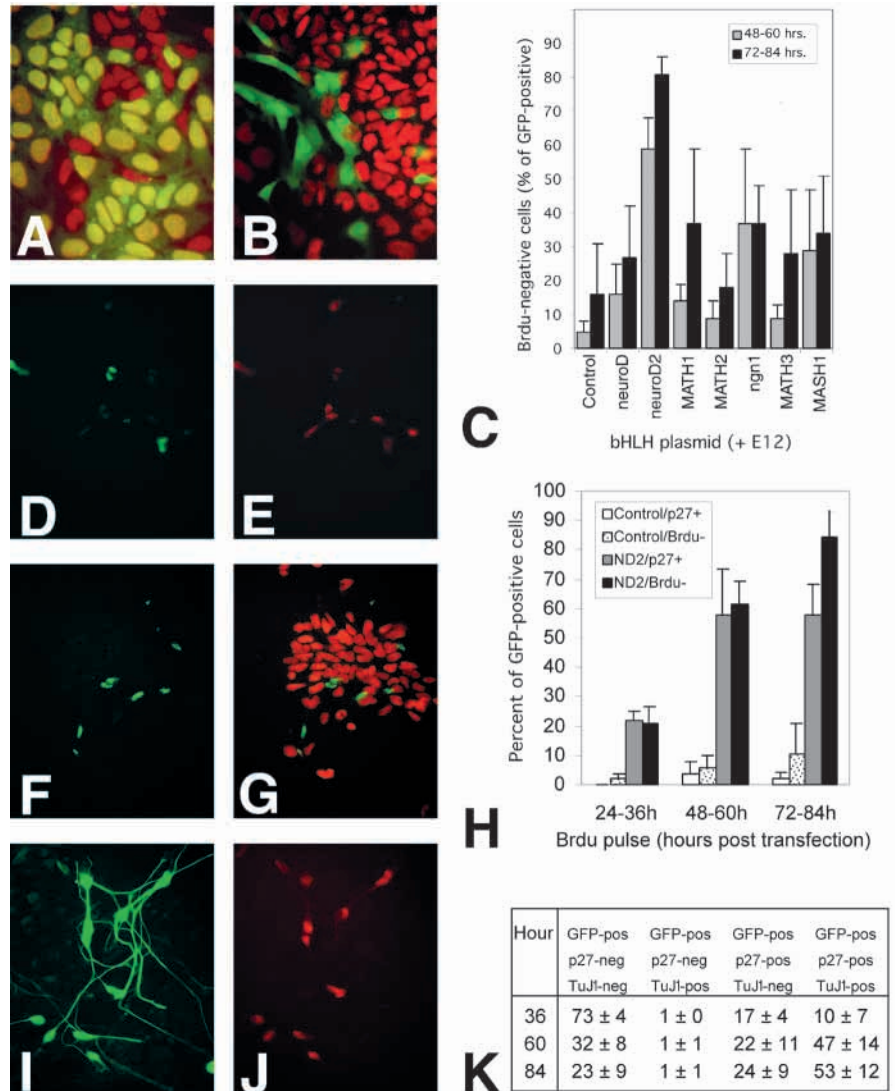
Sequential activation of neural bHLH gene expression has been demonstrated during vertebrate neurogenesis, suggesting that these genes function in a cascade (Lee et al., 1995; Ma et al., 1996; Cau et al., 1997). We used RT-PCR analysis (Rupp and Weintraub, 1991; Boudjelal et al., 1997) to examine whether transfection of P19 cells with vectors for the neural bHLH proteins activated transcription of the endogenous neural bHLH genes. We focused on *neuroD*, *neuroD2*, *MATH2* and *MATH3*, since these genes are expressed during neuronal differentiation. P19 cells did not express detectable *MATH3* after transfection of a control vector and E12. However, *MATH3* mRNA was present 2 or 5 days after the transfection of vectors expressing either precursor- or differentiation-specific neural bHLH proteins (Fig. 5 and data not shown). P19 cells transfected with a control vector and E12 expressed detectable *neuroD* mRNA, consistent with a previous report that undifferentiated P19 cells express a low level of *neuroD* mRNA (Boudjelal et al., 1997). However, P19 cells transfected with neural bHLH vectors and E12 had increased levels of *neuroD* mRNA relative to control transfected cells at 2 days after transfection (Fig. 5). Interestingly, introduced *MATH3* activated the endogenous *MATH3* gene and introduced *neuroD* increased the expression of the endogenous *neuroD* gene modestly,

suggesting that these proteins can autoactivate their own transcription. We did not detect expression of *MATH2* or *neuroD2* mRNA 2 days after transfection with any of the neural bHLH vectors, but *neuroD2* mRNA was detectable at 5 days after transfection of MASH1 or *ngn1* and E12 (data not shown). These results indicate that the introduced bHLH proteins activate the expression of one or more endogenous neural bHLH genes.

Expression of neuronal markers in other cell types after transfection of neural bHLH vectors

To determine whether mammalian cell types other than P19 could generate neurons in response to neural bHLH protein expression, we transfected the *neuroD2*, MASH1 or *ngn1* vectors, together with the E12 and GFP vectors, into several cell lines and primary rat astrocytes. Most cell lines, including NIH3T3, CHO, NT2, PC12 and HaCAT, were not labeled by TuJ1 after transfection (unpublished observations). However, 10T1/2 fibroblasts and rat astrocytes were immunoreactive for TuJ1 after cotransfection of any of the three neural bHLH vectors with E12 (Fig. 3H,I and data not shown). Although we occasionally observed one or two TuJ1-labeled 10T1/2 cells or astrocytes per dish with a neuronal morphology, most transfected cells did not adopt a neuronal morphology (Fig. 3H,I), nor did they express other neuronal markers or detectable p21 or p27^{Kip1} proteins (unpublished observations). Thus, efficient generation of neurons by transfection of neural bHLH vectors was limited to P19 cells.

Fig. 4. Cell cycle withdrawal and elevated p27^{Kip1} expression in P19 cells transfected with neural bHLH vectors. (A) BrdU (red) was incorporated by most P19 cells transfected with E12 and GFP (green; overlap with BrdU is yellow-green). (B) In contrast, most P19 cells transfected with neuroD2, E12 and GFP (green) did not incorporate BrdU (red) 2 days after transfection. (C) Quantitation of BrdU incorporation in GFP-expressing cells after transfection of P19 cells with bHLH expression vectors. All transfections included E12 and GFP plasmids. Control denotes empty pCS2+ expression vector used in place of the neural bHLH plasmid. Data is an average from four experiments. Standard deviations are indicated. (D,E) p27^{Kip1} expression in P19 cells transfected with neuroD2, E12 and GFP plasmids; (D) GFP fluorescence and (E) indirect immunofluorescence for p27^{Kip1}. (F-H) NeuroD2-induced p27^{Kip1}-positive cells withdraw from cell cycle. (F) indirect immunofluorescence for p27^{Kip1} in neuroD2/E12-transfected cells (green). (G) BrdU (red) was incorporated by most cells, but neuroD2-transfected cells expressing p27^{Kip1} (green, same field as F) did not incorporate BrdU. (H) Quantitation of p27^{Kip1} and BrdU co-labeling in control- or neuroD2-transfected cells demonstrated concordance between the fraction of neuroD2-transfected cells that were p27^{Kip1}-positive and those that no longer incorporated BrdU. To determine whether p27^{Kip1} induction was linked to neuronal differentiation, the same plates scored for p27^{Kip1} in H were labeled with TuJ1 and a fluorescein-conjugated secondary antibody (I-K). (I) GFP-positive (green nuclei) and TuJ1-positive cells (green processes) are also positive for p27^{Kip1} (J; red), 60 hours after transfection with neuroD2/E12/GFP. (K) Quantitation of these plates revealed a shift from p27^{Kip1}-negative/TuJ1-negative cells at 36 hours (73% of GFP-positive cells) to p27^{Kip1}-positive/TuJ1-positive cells at 84 hours (53% of GFP-positive cells).



DISCUSSION

Our results demonstrate that transient expression of neural bHLH proteins is sufficient to initiate a stable program of neuronal differentiation in pluripotent mouse P19 cells. This program includes alterations in morphology, neurite extension, expression of neuron-specific proteins, cell cycle withdrawal and the capacity for electrical signaling. Thus, the neural bHLH proteins can function as master regulators for mammalian neurogenesis, in that they are sufficient to initiate a program of neuronal differentiation in non-neural cells. The only other mammalian proteins known to have similar abilities in cell culture are the MyoD family of bHLH proteins, which can direct myogenesis (Weintraub et al., 1991; Molkenin and Olson, 1996), and PPAR γ , which can direct adipogenesis (Tontonoz et al., 1994). Studies of MyoD and related bHLH proteins in cell culture have led to considerable progress in understanding the regulation of myogenesis. The ability of the neural bHLH proteins to generate neurons in culture from P19

cells should similarly facilitate studies on the molecular regulation of neurogenesis.

Neural bHLH proteins link neuronal differentiation, CDKi expression and cell cycle withdrawal

Neuronal differentiation of P19 cells transfected with neural bHLH vectors was preceded by cell cycle withdrawal, providing direct evidence that the neural bHLH proteins can link neuronal differentiation and cell cycle regulation. These findings are consistent with the observation that expression of neuroD from a retroviral vector introduced into rat retinal progenitors reduced clone sizes in vivo (Morrow et al., 1999). The expression of p27^{Kip1} CDKi protein in the transfected P19 cells suggests that the neural bHLH proteins may direct cell cycle withdrawal by elevating the level of p27^{Kip1} or other CDKi proteins. Such a mechanism would be analogous to that proposed for the myogenic bHLH proteins during muscle formation (Halevy et al., 1995). Although we did not observe increased expression of the CDKi p21 in the transfected cells,

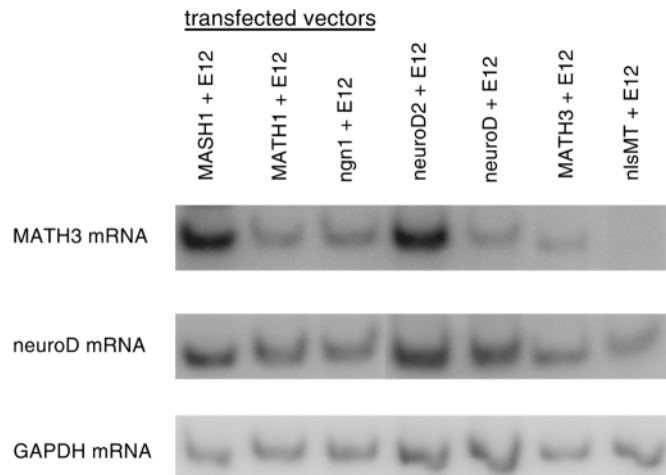


Fig. 5. RT-PCR analysis of endogenous *MATH3* and *neuroD* expression in P19 cells transfected with neural bHLH, E12 and GFP vectors. *MATH3* mRNA expression was detected in P19 cells 2 days after transfection of neural bHLH vectors, but not in P19 cells transfected with a control vector (pCS2+nlsMT) and E12. While *neuroD* mRNA was present in cells transfected with the control vector, its expression increased after transfection of neural bHLH vectors. The ubiquitous *GAPDH* mRNA was used as an internal control.

a transfected *neuroD* vector can drive cell cycle withdrawal accompanied by increased p21 expression in HeLa cells (Mutoh et al., 1998). This suggests that there may be cell-type-specific mechanisms of CDKi activation by the neural bHLH proteins.

Specificity and persistence of bHLH-mediated neuron formation

The ability to generate neurons from P19 cells was restricted to bHLH proteins expressed primarily in the nervous system. All of these proteins are known to function as transcriptional activators. For *neuroD2*, we demonstrated an intact DNA-binding domain was required for neuron formation. These observations strongly suggest that *neuroD2* and the other neural bHLH proteins initiate neuronal differentiation in P19 cells by activating the expression of specific target genes, rather than by titrating a dimerization partner or another protein. A potential application of the P19 transfection assay is the identification of target genes for the neural bHLH proteins.

While expression of any of the neural bHLH proteins led to the formation of neurons from the transfected P19 cells, the percentage of transfected cells that differentiated as neurons varied, depending on which bHLH protein was introduced. For example, *neuroD2* or *MASH1*, in combination with E12, produced at least 100-fold more neurons than *MATH2* and E12. This difference is intriguing since the *MATH2* and *neuroD2* proteins have similar amino acid sequences and both are expressed during RA-induced P19 differentiation (our unpublished observations; McCormick et al., 1996). *MATH2* and most of the neural bHLH proteins that produced few neurons were expressed and functional, since they activated a cotransfected reporter efficiently. Possibly, cofactors present in the P19 cells cooperate more efficiently with *neuroD2* and *MASH1* to activate neuron formation. Although cotransfection

of E12 with the neural bHLH vectors was not required for neuron formation, it increased the frequency of neurons among the transfected cells, and it increased the elaboration of processes by the neurons. We speculate that cotransfected E12 may enhance neuronal differentiation by augmenting limited amounts of endogenous E12 or related proteins.

Neurons generated from P19 cells by the neural bHLH proteins maintained neuronal characteristics for at least 13 days after transfection. Since cells did not express detectable levels of protein from the transfected vectors by 10 days after transfection, it seems likely that the introduced neural bHLH proteins activated endogenous regulatory factors, which maintained the program of neuronal gene expression. Such factors could include neural bHLH proteins encoded by endogenous genes. Consistent with this, we observed increased levels of mRNA from the endogenous *MATH3*, *neuroD* and *neuroD2* genes after the transfection of various neural bHLH vectors with E12. Expression of the *MATH3* and *neuroD* genes may be maintained in the transfected cells by autoactivation, since the *MATH3* and *neuroD* proteins each activated the expression of the corresponding endogenous gene. It has been proposed that the neural bHLH proteins participate in a cascade of gene expression to regulate sequential determination and differentiation steps during neuron formation (Lee et al., 1995; Ma et al., 1996; Cau et al., 1997). A similar stepwise process may occur in the transfected P19 cells, since introduction of precursor-specific bHLH proteins such as *MASH1*, *MATH1* or *ngn1* activated the expression of endogenous neural bHLH genes that are expressed during differentiation.

Restrictions on neural bHLH protein function

Transfection of cell types other than P19 with neural bHLH vectors did not lead to the formation of neurons at a significant frequency. The activation of a neuron-specific tubulin in 10T1/2 cells and astrocytes indicates that the neural bHLH proteins are functional in these cell types, despite their inability to activate other aspects of the neuronal differentiation program. Similar restrictions on neural bHLH function may exist in some cells in *Xenopus* embryos, since expression of neural bHLH proteins in these embryos only converts a subset of ectodermal cells to a neuronal fate (Turner and Weintraub, 1994; Lee et al., 1995). The observations presented here suggest that either the bHLH-activated neuronal differentiation program is dependent on cofactors preferentially expressed in P19 cells, or it is repressed by factors present in other cells. Comparison of P19 cells with cell types that fail to support bHLH-driven neurogenesis should provide a basis for identifying cofactors or regulators of the neural bHLH proteins.

Neuronal identity and bHLH proteins

The role of neural bHLH proteins in determining the identities of specific neuronal subtypes remains unclear. *MASH1* is required for noradrenergic differentiation of many CNS and PNS neurons. However, expression of *MASH1* from a retroviral vector did not confer noradrenergic properties on neurons, although it did activate expression of the *Phox2a* homeobox protein, which is associated with noradrenergic differentiation (Hirsch et al., 1998; Lo et al., 1998). In *Xenopus* and mouse retinas, as well as chick neural tube, expression of neural bHLH proteins can bias cells toward specific neuronal fates (Kanekar et al., 1997; Morrow et al., 1999; Perez et al.,

1999). However, the widespread expression of the *neuroD* and *neurogenin* gene families suggests that these genes are unlikely to specify neuronal identities by themselves. We observed that neurons generated from P19 cells transfected with different neural bHLH vectors expressed similar or identical patterns of neurotransmitters and other neuronal proteins. This suggests that the various bHLH family members have the capacity to generate neurons with similar characteristics, under the conditions tested here. Interestingly, while aggregated P19 cells treated with RA give rise to both neurons and glial cells, we did not observe expression of astrocyte or radial glial markers after transfection of P19 cells with neural bHLH vectors. It has also been reported that neuroD expression inhibited Müller glia formation in the retina (Morrow et al., 1999). Taken together, these observations suggest that formation of glia may be controlled by regulatory factors distinct from the neural bHLH proteins. Other bHLH proteins may be involved in this process, since we have identified a novel subfamily of bHLH proteins expressed in glial cells (H. B. S. and D. L. T., unpublished observations).

One long-term goal of this research is to develop strategies for replacing neurons lost from disease or injury. P19 cells have properties similar to embryonic stem cells isolated from mice and humans (Thomson et al., 1998). Thus, it may be possible to use transient expression of neural bHLH proteins to direct human embryonic stem cells to adopt a neuronal fate. Since neurodegenerative diseases such as Amyotrophic Lateral Sclerosis and Parkinson's disease lead to the loss of specific populations of neurons, an essential aspect of any neural replacement strategy will be the ability to generate specific types of neurons. Recent work suggests that the identities of photoreceptors, motor neurons and, possibly, other neuronal subtypes may be regulated by individual homeobox transcription factors (Furukawa et al., 1997; Tanabe et al., 1998). By expressing proteins that regulate the formation of specific types of neurons in combination with neural bHLH proteins, it may be possible to generate neurons with defined identities.

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