GABAergic Differentiation Induced by Mash1 Is Compromised by the bHLH Proteins Neurogenin2, NeuroD1, and NeuroD2

During forebrain development, Mash1 directs γ-aminobutyric acid (GABA)ergic neuron differentiation ventrally in the ganglionic eminences. Repression of Mash1 in the cortex is necessary to prevent the formation of GABAergic interneurons. Negative regulation of Mash1 has been attributed to members of the Neurogenin family; the genetic ablation of Neurogenin2 (Ngn2) leads to the derepression of Mash1 and the formation of ectopic GABAergic neurons in the cortex. We have developed an in vitro system to clarify the importance of NeuroD proteins in the Mash1 regulatory pathway. Using a neurosphere culture system, we show that the downstream effectors of the Ngn2 pathway NeuroD1 and NeuroD2 can abrogate GABAergic differentiation directed by Mash1. The ectopic expression of either of these genes in Mash1-expressing cells derived from the lateral ganglionic eminence, independently downregulate Mash1 expression without affecting expression of distal less homeodomain genes. This results in a complete loss of the GABAergic phenotype. Moreover, we demonstrate that ectopic expression of Mash1 in cortical progenitors is sufficient to phenocopy the loss of Ngn2 and strongly enhances ectopic GABAergic differentiation. Collectively, our results define the compensatory and cross-regulatory mechanisms that exist among basic helix-loop-helix transcription factors during neuronal fate specification.

Keywords: fate, GABAergic, identity, LGE, Mash1, neural progenitor, NeuroD1, NeuroD2, Neurogenin2

Introduction

Fate specification and regional identity are the major components of neuronal subtype diversity. In the developing mammalian brain, directed neuronal differentiation is spatially and temporally governed by cascades of transcription factors, often involving the basic helix-loop-helix (bHLH) transcription factors (Falk et al. 2002; Lo et al. 2002; Hjalt 2004; Kanda et al. 2004; Helms et al. 2005; Anderson et al. 2006).

The proneural bHLH genes, Neurogenin2 (Ngn2)/MATH4a and the rodent member of the achaete/scute family, Ascl1 (also known as Mash1), are involved in neuronal differentiation, cell cycle withdrawal, production, and migration of neuroblasts (Farah et al. 2000; Ge et al. 2006; Guillemot et al. 2006; Nguyen et al. 2006). They have different regional expression patterns. During forebrain development, Ngn2 is expressed dorsally and elicits the generation of glutamatergic cortical neurons (Schuurmans et al. 2004). The expression of Mash1 is restricted to the ventral regions, specifically the lateral ganglionic eminences (LGEs) and medial ganglionic eminences, which originate γ-aminobutyric acid (GABA)ergic neuronal differentiation (Parras et al. 2004). The transcription cascade elicited by Ngn2 leads to the glutamatergic phenotype and involves the T-brain transcription factor-1 (Tbr1), as well as other members of the bHLH transcription factor family, including NeuroD1 and NeuroD2 (Englund et al. 2005; Hevner et al. 2006). By contrast, GABAergic differentiation is directed by Mash1 and distal less homeodomain genes 1 and 2 (Dlx1 and Dlx2; Petryniak et al. 2007; Long et al. 2009).

Previous work suggests that neuronal fate specification in the developing mouse neocortex takes place in 2 phases involving the Ngn2 or the paired box 6 (Pax6) genes (Schuurmans et al. 2004; Kroll and O'Leary 2005). Whether or not Ngn2 directly prevents Mash1 expression in the cortex is still not clear. It is possible that additional factors function downstream of Ngn2 to either downregulate Mash1 or to maintain its downregulation during glutamatergic differentiation and, as a result, prevent ectopic subcortical GABAergic differentiation. Recent work has shown that NeuroD1 may also regulate Mash1 expression, in both “Xenopus” explants and P19 mammalian carcinoma cell line (Seo et al. 2007). In addition, in the Ngn2/Ngn1 double knockout mice, Mash1-induced cortical GABAergic differentiation is greater than in Ngn2 knockout mice (Fode et al. 2000), which may be due to the almost complete absence of NeuroD proteins in the double knockout. Thus, we hypothesize that NeuroD proteins may participate in the molecular mechanism repressing GABAergic differentiation during cortical development.

To determine the relative roles of the NeuroD family and Ngn2 in the regulation of GABAergic differentiation in the dorsal forebrain, we developed an in vitro neurosphere-based assay. We examined the effects of NeuroD proteins in Mash1-induced GABAergic neuronal specification of LGE-derived progenitors. As shown previously, ectopic expression of Ngn2 in LGE-derived neural progenitors in vitro prevents GABAergic neuron formation. Similarly, we show that ectopic expression of either NeuroD1 or NeuroD2 also prevents GABAergic differentiation. Finally, we demonstrate that repression of GABAergic differentiation is associated with Mash1 down-regulation in LGE cells, at least in part, and does not affect other GABAergic neuronal determinants such as the Dlx genes. These studies provide evidence that NeuroD proteins participate in regulating Mash1-induced GABAergic differentiation.

Material and Methods

Animals
We used E14.5 rat and E14 mouse embryos isolated from Sprague-Dawley pregnant rat (B&K Stockholm, Sweden) and NeuroD1-LacZ pregnant mice (Miyata et al. 1999), respectively. We housed animals in groups with ad libitum access to food and water during a 12 h light-dark
cycle. The Ethical Committees at Lund University, Sweden and at Columbia University, New York, USA approved all experimental procedures in this study. NeuroD1 mice were genotyped as previously described (Chao et al. 2007).

**Subcloning, Virus Production, and Titer Measurement**

We used the Moloney leukemia-derived retroviral vectors pCMMP-IR-E2S2GP-WPRE, pCMMP-Ngn2-IRES2GP-WPRE, pCMMP-Mash1-IRES2-GFP-WPRE, pCMMP-NeuroD1-IRES2GP-WPRE, and pCMMP-NeuroD2-IRES2GP-WPRE (Hofstetter et al. 2005; Roybon et al. 2008; Roybon, Deierborg, et al. 2009). To generate the construct pCMMP-Mash1-IRES2GP-WPRE and pCMMP-NeuroD2-IRES2GP-WPRE, we amplified the mouse Mash1 and NeuroD2 cDNAs from a pcDNA1-Mash1 plasmid (kindly provided by Professor François Guillemot, London, UK) and pYX-AscNNeuroD2 plasmid (Open Biosystems, Huntsville, AL) by polymerase chain reaction to introduce the restriction sites PmeI in 5′ and Xhol in 3′. Amplification of cDNAs was performed as previously described (Roybon et al. 2008). We verified the constructs by enzymatic restriction and by DNA sequencing using BigDye 3.1 (Applied Biosystems, Foster City, CA). We produced all infectious particles using the producer cell line 293SV-G as we previously described (Ory et al. 1996; Roybon et al. 2008). We measured the titer of each retrovirus by flow cytometry based on green fluorescent protein (GFP) expression 3 days postinfection of HT1080 cells (ranging from 0.5 x 10^5 to 2.1 x 10^5 transducing units/ml).

**Neurospheres Formation, Transduction, and Differentiation**

Pregnant Sprague-Dawley rats were anesthetized by an overdose of sodium pentobarbital (intraperitoneal, 60 ng/ml). Embryos at stage embryonic day E14.5 (plug day as day 0) were collected in Hank’s balanced salt solution (Invitrogen-Gibco, Stockholm, Sweden) on ice. Neurospheres were generated and cultured as previously described (Roybon et al. 2005, 2008).

NeuroD1 mutant animals were previously described (Miyata et al. 1999). To generate neurospheres from NeuroD1 mutant animals, the LGE regions were dissected from each individual (homozygous wild type, heterozygous, and homozygous mutants) and processed separately. The LGE were mechanically dissociated into single-cell suspensions and seeded separately in T25 flasks, into 5 ml of proliferation medium, composed of DMEM:HAMS-F12 (at 3:1; Invitrogen-Gibco), B27 (2%; Invitrogen-Gibco), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (both 20 ng/ml each; both from R & D Systems, Minneapolis, MN), Heparin (5 μg/ml; Sigma, Stockholm, Sweden), and penicillin/streptomycin (50 μg/ml/50 μg/ml; Sigma). In parallel, tails from the embryos were processed and genotyped (Fig. 5B; Chao et al. 2007). Thereafter, cells that belong to NeuroD1 wild type or null mutant animals were pooled into T75 flasks and grown as described above (Fig. 5B). Cells from heterozygote animals were discarded. Mitogens were added (EGF and bFGF; Sigma) and grown as described (Ory et al. 1996; Roybon et al. 2008). We measured the titer of each retrovirus by flow cytometry based on green fluorescent protein (GFP) expression 3 days postinfection of HT1080 cells (ranging from 0.5 x 10^5 to 2.1 x 10^5 transducing units/ml).

**Immunocytochemistry and Immunohistochemistry**

For immunocytochemistry, cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS) for 20 min and washed with PBS 3 times. Immunocytochemistry was carried out using standard protocol, with primary antibody incubation for an overnight, at room temperature, followed by incubation with secondary antibody for an hour. Primary antibodies and dilutions were as follows: mouse anti-β-tubulin III (1:500, monoclonal, Sigma), mouse anti-microtubule-associated protein-2 (MAP2; 1:500, monoclonal, Sigma), rabbit anti-gliarial fibrillary acidic protein (GFAP; 1:1000, polyclonal, Dako, Glostrup, Denmark), mouse anti-2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPase; 1:1000, monoclonal, Millipore/Chemicon, Solna, Sweden), mouse anti-Mash1 (1:100, monoclonal, BD PharMingen, Stockholm, Sweden), rabbit anti-Ngn2/MATH4 (1:200, polyclonal, Millipore/Chemicon), rabbit anti-GAD65 (1:200, polyclonal, Millipore/Chemicon), mouse anti-GAD67 (1:200, monoclonal, Millipore/Chemicon), rabbit anti-DARP2-32 (1:500, polyclonal, Millipore/Chemicon), rabbit anti-glutamate (1:200, polyclonal, Sigma), rabbit anti-VGlut1 (1:1000, polyclonal, Synaptic System, Germany), goat anti-NeuroD1 (1:200, polyclonal, Santa Cruz, Heidelberg, Germany), rabbit anti-Tbr1 (1:1000, polyclonal, Millipore/Chemicon), rabbit anti-ND2 (1:200, polyclonal, ABCAM, Cambridge, UK), rabbit anti-Dlx1 and anti-Dlx2 (1:100; polyclonal, ABCAM), and chicken anti-GFP (1:200, polyclonal, Millipore/Chemicon). The secondary antibodies Cy3, Cy2, FITC, Cy5, and Cy5 (1:200) were purchased from Jackson IR laboratories (Suffolk, UK); Alexa-fluor-488, -568, and -647 from Invitrogen/Molecular Probes (Paisley, UK); all used at 1:200. Cultures were counterstained with 4′,6-Diamidino-2-phenylindole (DAPI; 5 μl/ml, Molecular Probes, Paisley, UK) for 30 min and washed before mounting in polyvinyl alcohol-DABCO. All antibodies for GABAergic and glutamatergic markers have been tested using immunohistochemistry on adult Sprague-Dawley 8-12 weeks old rats, as positive controls (data not shown).

**Microscopy and Quantification**

Specimen analyses were performed using either a fluorescence microscope (Leica DMIRE) with a 40× magnification objective or a Leica confocal microscope (Leica software), equipped with a GreNe and a HeNe laser, using the following lines of excitation: 488, 594, and 647 nm (laser at 20% power; 200-μm pinhole aperture), using 20× and 63× objectives. For high-power images (using 63× objective), we performed sequential scans for each specimen, for each line of excitation, through the Zaxis (8-12 scans for 6-10-μm thickness). Images were composed in CANVAS-X software.

For each condition, at least 3 independent experiments were performed. For each experiment, immunocytochemistry was performed separately. For each experiment, 6 to 7 randomly chosen different fields of view representative of the whole cultures were counted. Approximately 3000-5000 cells were counted for each independent experiment. When we observed that the marker of interest was completely absent following transgene expression (e.g., at day 4: a complete absence of Mash1 expression) the number of cells counted was decreed to 2000, per experiment. The differences we observed in those fields, for each of the conditions, are showed as percentage of transduced cells coexpressing the transgene of interest and the reporter eGFP. We performed statistical analysis using 1- or 2-factor analysis of variance (ANOVA) with transgene and time variables, followed by Scheffe’s post hoc test when we observed significant differences, using Statview 5.0.
software (SAS institute Inc.). *P* value was considered significantly different when $P < 0.05$. For each diagram, the level of significance ($P$ value) is represented as follows: $P < 0.05 = \ast$, $P < 0.01 = \ast\ast$, and $P < 0.001 = \ast\ast\ast$. All data are expressed as ± standard error of the mean (SEM).

**Results**

**Ngn2 Directs Differentiation of LGE Neural Progenitors into Neurons and Induces Cell Cycle Exit**

To gain insight into how Ngn2 prevents GABAergic differentiation in the cortex (Schuurmans et al. 2004), we developed an in vitro neurosphere-based system using LGE progenitors, which naturally express Mash1 and are competent to form GABAergic neurons. To validate our approach, we first tested whether the ectopic expression of Ngn2 would direct neuronal differentiation of expanded neural precursors isolated from rat LGE at embryonic day 14.5 postcoitum (E14.5). We grew the LGE neural precursors as neurospheres and transduced them with retrovirus that expressed either Ngn2 and GFP or GFP only (Fig. 1A,B). We allowed the cultured cells to attach and differentiate for 2–12 days (Fig. 1C–D'). Cells that overexpressed Ngn2 adopted a bipolar neuron-like morphology as early as 2 days after transduction (Fig. 1C). Four days after transduction, the majority of cells (>95%) transduced with Ngn2 expressed the neuronal marker β-III-tubulin, whereas only 6% (±0.6%, SEM) became neurons upon transduction with the control vector (Fig. 1D,D'). We obtained similar results using immunocytochemistry for the mature neuronal marker MAP2 (Supplementary Fig. 1B). Ngn2-expressing cells were not immunopositive for the astrocytic marker GFAP or the oligodendrocyte marker CNPase (Supplementary Fig. 1C). In contrast, cells from control cultures differentiated into a mixture of β-III-tubulin-, GFAP-, and CNPase-immunopositive cells (Supplementary Fig. 1D–F).

We next tested whether Ngn2-transduced cells become postmitotic. We differentiated cells for 7 days and then pulse labeled them with the 5-bromo-2-deoxyuridine analog CldU for 72 h, prior to fixation. We used an extra long pulse in order to make sure all cells even those dividing slowly would be quantified. Only a few of the transduced LGE neural progenitors (<3%) incorporated CldU (Fig. 1E and Supplementary Fig. 2A,A') indicating that they were postmitotic before we added CldU. Approximately 30% of cell transduced with the
GFP-control vector incorporated CldU (Fig. 1E and Supplementary Fig. 2B–D’). Taken together, these experiments demonstrate that Ngn2 is sufficient to initiate neuronal differentiation of E14.5 LGE neural precursors.

**Ngn2 Prevents GABAergic Differentiation of LGE Neural Progenitors**

We then characterized the neuronal phenotype of Ngn2-transduced LGE cells after differentiation. First, we assessed the endogenous GABAergic differentiation in LGE cultures not subjected to any viral vectors. We found that the number of GAD65-expressing neurons increased (from 0.5% to 13.0% between days 4 and 12) over time (ANOVA, *F* value = 8.23; *P* value < 0.01, Supplementary Fig. 3A). When we transduced LGE cultures with Ngn2, we found that the transduced cells did not express GAD65 (Fig. 2B), GAD67 (Fig. 2D), or the dopamine-receptor related phosphoprotein with a molecular weight of 32 kDa (DARPP-32; Fig. 2F). By contrast, the cells that were not transduced by the Ngn2 vector (and cells in control cultures) displayed a GABAergic phenotype and were immunopositive for GAD65 (Fig. 2A, B, and E), GAD67 (Fig. 2C, D), or DARPP-32 (Fig. 2F). DARPP-32 positive cells were only found at very low numbers starting at day 12 (Fig. 2D). These results show that although Ngn2 stimulates differentiation of LGE precursors into neurons (Fig. 1), it does not induce a GABAergic phenotype. Therefore, our in vitro system mimics the Ngn2-induced repression of GABAergic differentiation observed during cortical development.

**NeuroD1 Prevents GABAergic Differentiation of LGE Neural Progenitors**

We next asked whether NeuroD1, a downstream substrate of Ngn2 (Mattar et al. 2004), plays a role in GABAergic differentiation. We tested whether NeuroD1 would have the capability of repressing GABAergic differentiation of LGE progenitors. As expected, NeuroD1 induced LGE progenitors to differentiate into β-III-tubulin neurons (>95% of GFP* cells 4 days after differentiation; Fig. 3B), and these differentiated neurons became postmitotic by culture day 7 (Fig. 3C). Moreover, NeuroD1 completely abolished the expression of the GABAergic markers GAD65 and GAD67, throughout the time course of the study (Fig. 3D–F). Cumulatively, our data show that both Ngn2 and NeuroD1 can direct the neuronal differentiation of LGE progenitors and prevent GABAergic differentiation (Fig. 3F).

**GABAergic Differentiation Mediated by Mash1 Is Compromised by Ngn2 and NeuroD1**

To gain insights into the underlying mechanisms by which Ngn2 and NeuroD1 prevent LGE neural progenitors from differentiating into GABAergic interneurons, we performed immunocytochemistry for Mash1, assuming that Ngn2 and NeuroD1 may regulate Mash1 in a direct or indirect manner during the process of GABAergic differentiation (Seo et al. 2007). We detected Mash1 in more than 70% of cells transduced with the control virus after 4 days of differentiation in vitro (Fig. 4A, D, and Supplementary Fig. 4). In contrast, endogenous Mash1 expression was not detected in LGE neural precursors transduced with either Ngn2 or NeuroD1 (Fig. 4B, C, and D; 1-factor ANOVA, effect of gene; *F* value = 2492.93; *P* value < 0.0001), indicating that Ngn2 and NeuroD1 repress Mash1 expression.

Dlx genes play a role in GABAergic neuronal differentiation (Petryniak et al. 2007). Interestingly, overexpression of either Ngn2 or NeuroD1 did not affect Dlx1 (Fig. 4E, F) and Dlx2 expression (data not shown). Virtually all cells transduced with Ngn2 or NeuroD1 vector continued to express Dlx proteins whereas losing Mash1 expression. This suggests that Mash1 plays an essential role during GABAergic fate specification, independent of Dlx regulation. This finding is consistent with a recent study demonstrating that Dlx genes and Mash1 can function in parallel pathways to promote the formation of GABAergic neurons (Long et al. 2009). We also demonstrate that Ngn2 and NeuroD1 appear to have an instructive repressing role when expressed in E14.5 LGE neural precursors. Ngn2 and NeuroD1 induce Mash1 downregulation, which consequently blocks GABAergic differentiation.

![Figure 2](image-url)
Figure 3. NeuroD1 directs neuronal differentiation and prevents GABAergic differentiation of LGE neural progenitors. (A) E14.5 rat LGE neural progenitors transduced with NeuroD1 retrovirus express both transgene and GFP reporter gene, after 2 days differentiation in vitro. (B) Efficient generation of βIII-tubulin-expressing neurons in LGE cultures infected with NeuroD1 retrovirus, after 4 days differentiation in vitro. (C) LGE progenitors infected with NeuroD1 retrovirus become postmitotic neurons after 7 days cultures, as mark by the lack of CldU incorporation following 72 additional hours of CldU chase. White arrowhead points at a double-labeled CldU/GFP cell. (D and E) The ectopic expression of NeuroD1 in LGE progenitors alters their GABAergic differentiation into GAD65- or GAD67-positive neurons. (F) Summary. Scale bars in: 50 μm (B-E) and 25 μm (A).

Figure 4. Ngn2 and NeuroD1 repress endogenous expression of MASH1 in LGE-transduced cells. (A–F) Four days differentiated E14.5 rat LGE neurospheres upon infection with control, Ngn2, and NeuroD1 retroviruses. (A) Cells transduced with control retrovirus retain Mash1 endogenous expression. (C and D) LGE cells transduced with Ngn2 or NeuroD1 retrovirus display abolished Mash1 expression. (D) Bar diagram showing the proportion of LGE-transduced (GFP+) cells expressing Mash1 at 4 days differentiation in vitro (error bars represent the SEM; ***correspond to P value < 0.0001). (E and F) Ngn2- and NeuroD1-transduced LGE neural progenitors still express Dlx1 gene, at 4 days differentiation in vitro. Bottom and right panels of A–C, E, and F pictures represent a Z-projection of 12–20 stack pictures at the level of intersection of the white crosshair. Scale bars: 50 μm (A–C, E, and F).
Mash1 Is Sufficient to Direct GABAergic Differentiation of both LGE Progenitors and Cortical Progenitors

To confirm that GABAergic differentiation is Mash1 dependent and that Mash1 is sufficient to promote GABAergic neuron differentiation, we first analyzed whether Mash1 expression would increase the production of GABAergic neurons from LGE progenitors. This experiment was based on the assumption that overexpression of Mash1 in cortical progenitors is sufficient to phenocopy the Ngn2-lacking animals, which display an increase in GABAergic neuron differentiation, presumably due to the derepression of Mash1. We generated a retroviral vector driving the expression of Mash1 (Fig. 5A). Mash1 expression was observed in more than 97% of both LGE- and cortical-transduced progenitors 4 days after infection (Fig. 5 and data not shown). Neuronal differentiation directed by the ectopic expression of Mash1 in LGE progenitors increased overtime (Fig. 5B,C; 1-factor ANOVA, effect of time; F-value: 120.48; P value < 0.0001). In agreement with previous reports (Miyoshi et al. 2004), we observed that Mash1 was sufficient to improve GABAergic neuron differentiation of LGE progenitors (Fig. 1E and Fig. 5D–F). The proportion of GAD65-expressing cells in Mash1-transduced LGE cultures increased dramatically over time (Fig. 5F; 1-factor ANOVA, effect of time; F-value: 95.71; P value < 0.0001), and resulted in a 10-fold increase in the proportion of GFP+/GAD65+ cells compared with GFP-transduced cultures, for each of the time points.

Figure 5. Mash1 is sufficient to direct GABAergic differentiation of both LGE and cortical progenitors. (A) E14.5 rat LGE neural progenitors transduced with Mash1 retrovirus express both transgene and GFP reporter gene, after 2 days differentiation in vitro (>97%). (B and C) Neuronal differentiation directed by Mash1 in LGE progenitors increases overtime. Bar diagram shows the proportion of LGE-transduced (GFP+) cells expressing β-III-tubulin, 4, 8, and 12 days upon retroviral infection (day 4: 15%, day 8: 80%, and day 12: 93%). (D–F) Mash1 is sufficient to improved GABAergic neuron differentiation of LGE progenitors. Bar diagram shows the proportion of LGE-transduced (GFP+) cells expressing GAD65, 4, 8, and 12 days upon retroviral infection (day 4: 1%, day 8: 24%, and day 12: 67%). (G–I) Mash1 expression in cortical progenitors results in the generation of GAD65-expressing GABAergic neurons. Bar diagram shows the proportion of cortical-transduced (GFP+) progenitors expressing GAD65, 4, 8, and 12 days upon retroviral infection (day 4: 3%, day 8: 15%, and day 12: 31%). Error bars represent the SEM. ***, **, and * correspond to P < 0.0001, P < 0.01, and P < 0.05, respectively. Bottom and right panels in A represent a Z projection of 12 stack pictures at the level of intersection of the white crosshair. Scale bars: 50 μm (A, B, D, E, G, and H).
studied. Moreover, the single overexpression of Mash1 in cortical progenitors resulted in the generation of GAD65-expressing GABAergic neurons (Fig. 5F; 1-factor ANOVA, effect of gene; F value: 20.99; P value < 0.001). The proportion of GFP+/GAD65+ cells present in Mash1-transduced cortical cultures increased significantly overtime (Fig. 5F; 1-factor ANOVA, effect of time; F value: 11.19; P value = 0.001; 2-factor ANOVA, effect of time × gene; F value: 6.06; P value = 0.01). These data further confirm the importance of Mash1 during GABAergic neuron subtype specification and suggest that the derepression of Mash1 leads to ectopic GABAergic differentiation.

Ngn2 Represses Mash1 in a NeuroD1-Independent Manner

NeuroD1 is a direct downstream target gene in the Ngn2 pathway and can be induced in response to Ngn2 overexpression in various progenitors isolated from different parts of the developing brain (Roybon, Hjalt, et al. 2009). We therefore analyzed NeuroD1 expression in Ngn2-transduced LGE cultures. We observed that all cells transduced with Ngn2-activated NeuroD1 whereas losing Mash1 (Fig. 6A), suggesting that downregulation of Mash1 triggered by Ngn2 may be mediated by NeuroD1. Indeed, the overexpression of NeuroD1 was sufficient to initiate the downregulation of Mash1 (Fig. 4C).

We further examined whether the repression of Mash1 by Ngn2 was NeuroD1 dependent. We transduced Ngn2 into LGE progenitors that were isolated from NeuroD1 knockout E14.5 mouse embryos and subsequently grown as neurospheres (Fig. 6B). Interestingly, Ngn2 could still prevent Mash1 expression (Fig. 6D,F).

NeuroD2 Expression Directed by Ngn2 and NeuroD1 Efficiently Prevents Mash1 Expression

NeuroD2 is another member of the NeuroD family that, together with NeuroD1, induces ectopic neuronal differentiation (Farah et al. 2000). Because NeuroD2 is also a downstream effector of the Ngn2 pathway (Mattar et al. 2004), it was possible that NeuroD2 could substitute NeuroD1 function in the differentiation pathway. Therefore, we analyzed whether Ngn2 would also induce NeuroD2 expression in wild type and NeuroD1 null LGE progenitors. We detected NeuroD2 in almost all cells transduced with Ngn2 and NeuroD1 vectors (>95%), as early as 4 days after differentiation in both wild type and NeuroD1 null LGE progenitors (Fig. 7A-D), suggesting functional redundancy between the NeuroD family members in this regulatory pathway.

We then tested whether expression of NeuroD2 alone would downregulate Mash1 expression in LGE-derived progenitors. Transduction of NeuroD2 did not induce the expression of NeuroD1 (Fig. 7E) but was sufficient to prevent Mash1 expression (Fig. 7F). Furthermore, we found that NeuroD2, similar to Ngn2 and NeuroD1, had no effect on repressing Dlx1 and Dlx2 expression (Fig. 7G and data not shown). Additionally, we found that NeuroD2 also prevents GABAergic neuronal fate (Fig. 7H and Supplementary Fig. 5A,B), which we hypothesize is due to downregulation or repression of Mash1.
Ngn2 and NeuroD1 Can Partially Re-Specify LGE-Derived Progenitors Toward a Glutamatergic Fate

In order to examine the phenotype of the neurons that have lost the GABAergic fate, we assessed whether the signals mediated by Ngn2 and NeuroD1 would be sufficient to redirect the fate of LGE progenitors toward a glutamatergic phenotype. Thus, we performed immunocytochemistry for glutamate and vesicular glutamate transporter-1. None of the cells were immunopositive for these markers, regardless of whether they had been transduced with Ngn2 or NeuroD1 vectors, even following 16 days of differentiation (data not shown). The addition of Wnt-3A, a growth factor required for glutamatergic neuronal specification of neocortical progenitors (Braun et al. 2003), hippocampal progenitors (Lie et al. 2005), and mouse embryonic stem cells (Watanabe et al. 2005), had no additional effect in LGE progenitors on directing glutamatergic differentiation (data not shown). However, we observed the presence of cells expressing the Tbr1 in Ngn2 (29.6 ± 3.3%) and NeuroD1-transduced cultures (32.9 ± 3.4%) 8 days after differentiation, but barely in control and NeuroD2-transduced cultures (Fig. 8A–C and data not shown; 1-factor ANOVA, effect of gene, F value: 38.84, P value < 0.0001). Furthermore, we observed abolishment of Tbr1 expression in cortical cells transduced with Mash1 (Fig. 8D).

Taken together, our data show that Ngn2 and NeuroD proteins prevent Mash1-directed GABAergic differentiation and that the expression of either Ngn2 or NeuroD1 alone can partially respecify LGE-derived progenitors toward a glutamatergic fate (Fig. 8E). Our data further demonstrate that the misexpression of Mash1 in cortical progenitors prevents glutamatergic differentiation while leading to respecification toward a GABAergic fate (Fig. 5G–I), as observed when Mash1 is derepressed in Ngn2 null mutants.

Discussion

Efficient Neuronal Differentiation Induced by Ngn2 and NeuroD1 of LGE Progenitors

In the developing nervous system, Ngn2 is a proneural gene that is expressed in the ventral mesencephalon, dorsal telencephalon, thalamus, and dorsal spinal cord (Gradwohl et al. 1996; Sommer et al. 1996). In juvenile and adult animals, Ngn2 expression is restricted to the 2 regions that retain a neurogenic potential under normal conditions, the subgranular zone of the hippocampal dentate gyrus and the subventricular zone lining the lateral ventricle (Ozen et al. 2007; Roybon, Deierborg, et al. 2009; Roybon, Hjalt, et al. 2009). Previous work has shown that Ngn2 coordinates neuronal differentiation and inhibits glial differentiation (Nieto et al. 2001). Moreover, overexpression of Ngn2 can induce retinal pigment epithelial cells to become retinal neural cells (Yan et al. 2001) and enhance survival and differentiation of neural precursor cells after transplantation, via neurotrophin3/TrkC-mediated signaling (Yi et al. 2008). In the present study, we demonstrate that retroviral-mediated expression of Ngn2 in cells derived from E14.5 rat LGE significantly increased neuronal differentiation. Nearly all transduced cells became...
β-III-tubulin- and MAP2-immunopositive and postmitotic neurons, following transgene expression. We obtained similar results following overexpression of NeuroD1. As Ngn2 induces NeuroD1 expression, we hypothesized that NeuroD1 directs the neuron-inducing effect mediated by Ngn2. A plethora of studies support this hypothesis. Ngn2 null mutants display a marked reduction of neurons in the developing spinal cord, ventral mesencephalon, hippocampus, and dorsal forebrain. In these tissues, the production of neurons is reduced, but not abolished, because of the absence of Ngn2 and instructive signals of NeuroD1 (Mattar et al. 2004; Helms et al. 2005; Andersson et al. 2006; Hevner et al. 2006; Seo et al. 2007; Galichet et al. 2008; Roybon, Hjalt, et al. 2009).

Loss of Mash1 in LGE Progenitors following Ectopic Expression of Ngn2 and NeuroD1/2 Proteins Results in Loss of GABAergic Phenotype

In Ngn2 null mutants, the expression profile of Mash1 extends to the dorsal forebrain (Fode et al. 2000). Mash1 expression is also increased in the cortical tissue in Ngn2/Ngn1 double-mutant mice (Fode et al. 2000). The Ngn2KIMash1 mutant animals, which express Mash1 under the Ngn2 promoter, display GABAergic interneurons located ectopically in the dorsal telencephalon. However, there are no ectopic glutamatergic neurons in the LGE in the Mash1KINgn2 mutant (Ngn2 expression under the control of the Mash1 promoter), which was in contrast to the data reported by Mattar et al. 2008 showing an upregulation of Nsc12, BlhblB5, NeuroD, NeuroD2, Math2, Tbr1, FezL1, VGlut2, transcripts in the LGE following Ngn2 ectopic expression at E12.5, in utero (Mattar et al. 2008). Previous work proposed a dual-phase model of neuronal fate specification in the mouse neocortex, depending on Ngn1/2 in the early phase of embryonic neocortiogenesis (before E14.5, in mice; Schuurmans et al. 2004). Our results demonstrate that Ngn2, NeuroD1, and NeuroD2 prevent GABAergic subtype specification. When we overexpressed these transcription factors in LGE neural cells, none of the transduced cells become GABAergic interneurons. Thus, unlike the permissive effect ascribed to Ngn2 in vivo in the Mash1KINgn2 mutants, its overexpression in LGE progenitors in vitro leads to an instructive signal resulting in downregulation of Mash1, as observed following ectopic expression of NeuroD proteins. This phenomenon is Dlx-1 and -2 independent. As a result, the LGE neurons lose their GABAergic phenotype. It is possible, however, that Ngn2 and NeuroD1 ectopic expression altered an unknown factor that blocks GABA differentiation by LGE progenitors despite the presence of Dlx 1 and 2 or simply induced pan-neuronal maturation. Parras and coworkers hypothesized that the permissive effect of Ngn2 when expressed under the control of the Mash1 promoter reflects the weakness of the Mash1 promoter, as opposed to that of Ngn2 (Parras CM et al. 2002). The difference between this study showing that Ngn2 had a noninstructive role compared with Mash1 in the LGE, and our results could be due to the higher expression level of Ngn2 by our retroviral vector. It is also possible that the virally mediated expression of Ngn2 in differentiating LGE neurons persists, whereas Ngn2 expression is downregulated in Mash1KINgn2 knock-in mice. Taken together, these data suggest that above a certain level of expression, Ngn2 represses GABAergic differentiation and leads to glutamatergic differentiation, at least in the dorsal forebrain. We have shown that overexpression of Ngn2, NeuroD1, or NeuroD2 alone leads to Mash1 downregulation and loss of GABAergic phenotype. Interestingly, a lack of functional Wnt or Pax6 proteins, the upstream soluble and transcriptional regulators of the Ngn2 pathway in the dorsal telencephalon, also leads to an upregulation of Mash1 and Dlx1 in the cortical ventricular zone (Braun et al. 2003; Kroll and O’Leary 2005). Moreover, the ectopic expression of Mash1 is associated with...
that of Dlx1 and Dlx2, leading to the notion that their upregulation in the cortex of Ngn2 null mutant is Mash1 dependent. Our data are in-line with those from Parras and Schuurmans studies and show that GABAergic repression is Mash1 dependent. Furthermore in E14.5 Pax6<sup>Sey/Sey</sup> animals, which are mutant for the Pax6, the expression of Ngn2 in the ventrolateral dorsal telencephalon exhibits a modest dorsal shift; Ngn2 displays strong superficial expression compared with wild-type animals where the highest expression is in the ventricular zone. Thus, one could speculate that the delocalization of Ngn2 in Pax6<sup>Sey/Sey</sup> mutants leads to a failure to repress Mash1. Further investigation is required to determine whether or not the NeuroD proteins are upregulated in these mutants. Thus, it is possible that Pax6 initially delimits the appropriate proliferative zone for the genesis of GABAergic interneurons and regulates the number and distribution of these neurons by repressing the ventral fates of dorsal telencephalic progenitors via Ngn2 and the NeuroD proteins.

**Ectopic Expression of Mash1 in Cortical Progenitors Directs GABAergic Differentiation, In Vitro**

We showed that viral expression of Mash1 was sufficient to redirect the phenotype of cortical progenitors. GABAergic differentiation was increased by 5-fold, whereas Tbr1 expression completely abolished. In contrast, viral expression of Ngn2, NeuroD1, and NeuroD2 completely block GABAergic differentiation of LGE progenitors; however, only Ngn2 and NeuroD1 simultaneously cause partial induction of glutamatergic differentiation. We only detected the early marker of glutamatergic neurons, Tbr1, but not the mature markers, VGluT1 and glutamate, in those cells. The absence of VGluT1 and glutamate markers was surprising as Mattar and coworkers showed that 4 days after electroporation of Ngn2 in LGE tissue, VGluT2 and other cortical markers were upregulated, in utero (Mattar et al. 2008). It is possible that we did not wait long enough to see an upregulation of those markers in Ngn2- and NeuroD1-transduced cultures (Berninger et al. 2007). Alternatively, it is possible that environmental cues may be missing in our cultures to promote a complete maturation of Ngn2- and NeuroD1-expressing LGE cells.

We recently demonstrated that Ngn2 and NeuroD1 are expressed in a subpopulation of Mash1-expressing subventricular zone progenitors, during the initial phase of postnatal neuronal differentiation (Roybon, Dieierborg, et al. 2009). Ngn2 and NeuroD1 expression precede that of Tbr1 and NeuroD2. At this stage, the cells are newborn migrating neuroblasts. Interestingly, Ngn2 and NeuroD1 overexpression in adult subventricular zone progenitors grown as neurospheres leads to generation of calbindin- and calretinin-expressing neurons, 2 of the 3 periglomerular olfactory bulb neuronal subtypes generated postnatally (Batista-Brito et al. 2008; Chesler et al. 2008). In addition, when Ngn2 is overexpressed in neural progenitors isolated from other brain regions, for example, ventral mesencephalic neurospheres, it does not lead to dopaminergic differentiation, although it results in an upregulation of NeuroD1 and expression the vesicular monoamine transporter-2 (Roybon et al. 2008; Roybon, Hjalt, et al. 2009). Thus, it is likely that during neocorticosgenesis, Ngn2 and NeuroD1 need to be combined to intrinsic cofactors that are restricted to the neocortical tissue to form a transcriptional code, which will result in the activation of transcriptional cascades specifying glutamatergic neuronal subtype fate.

**Concluding Remark**

We have generated an in vitro system to analyze the effect of transcription factors involved in GABAergic differentiation. Our findings indicate that Ngn2 and NeuroD proteins play important roles during neuronal subtype specification. Our in vitro data reinforce previous in vivo observations (Mattar et al. 2008) as well as the idea that compensatory and cross-regulatory mechanisms exist among the bHLH factors during neuronal fate specification in vivo. Furthermore, our observations support the notion that neuronal subtype specification relies on specific combinations of transcription factors that define fate specification and regional identity.

**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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