Differential actions of the proneural genes encoding Mash1 and neurogenins in Nurr1-induced dopamine neuron differentiation

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Accepted 23 February 2006
Journal of Cell Science 119, 2310-2320 Published by The Company of Biologists 2006
doi:10.1242/jcs.02955

Summary
The steroid receptor-type transcription factor Nurr1 has a crucial role in the development of the mesencephalic dopamine (DA) neurons. Although ectopic expression of Nurr1 in cultured neural precursor cells is sufficient in establishing the DA phenotype, Nurr1-induced DA cells are morphologically and functionally immature, suggesting the necessity of additional factor(s) for full neuronal differentiation. In this study, we demonstrate that neurogenic basic helix-loop-helix (bHLH) factors Mash1, neurogenins (Ngn) and NeuroD play contrasting roles in Nurr1-induced DA neuronal differentiation. Mash1, but not Ngn2, spatially and temporally colocalized with aldehyde dehydrogenase 2 (AHD2), a specific midbrain DA neuronal progenitor marker, in the early embryonic ventral mesencephalon. Forced expression of Mash1 caused immature Nurr1-induced DA cells to differentiate into mature and functional DA neurons as judged by electrophysiological characteristics, release of DA, and expression of presynaptic DA neuronal markers. By contrast, atonal-related bHLHs, represented by Ngn1, Ngn2 and NeuroD, repressed Nurr1-induced expression of DA neuronal markers. Domain-swapping experiments with Mash1 and NeuroD indicated that the helix-loop-helix domain, responsible for mediating dimerization of bHLH transcription factors, imparts the distinct effect. Finally, transient co-transfection of the atonal-related bHLHs with Nurr1 resulted in an E-box-independent repression of Nurr1-induced transcriptional activation of a reporter containing Nurr1-binding element (NL3) as well as a reporter driven by the native tyrosine hydroxylase gene promoter. Taken together, these findings suggest that Mash1 contributes to the generation of DA neurons in cooperation with Nurr1 in the developing midbrain whereas atonal-related bHLH genes inhibit the process.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/11/2310/DC1

Key words: Nurr1, bHLH, Mash1, Neurogenin, Tyrosine hydroxylase (TH), Midbrain dopamine neuron differentiation

Introduction
During brain development, neural precursor cells differentiate into various neuronal subtypes with distinct cellular and physiological properties. Dopamine-secreting (DA) neurons represent one of the major neuronal subtypes in the CNS. A major population of DA neurons resides in the midbrain, and these cells are known to play crucial roles in the control of motor and reward behaviors as well as other cognitive functions (Graybiel et al., 1994; Berke and Hyman, 2000; Nieoullon, 2002). Consistently, dysfunction of midbrain DA neurons is strongly correlated with the development of Parkinson’s disease, schizophrenia and drug addiction. Despite the clinical significance of the midbrain DA system, our knowledge of DA neuronal development in the midbrain is still limited.

Nurr1 is an orphan nuclear receptor-type transcription factor whose onset of expression in the developing rodent midbrain precedes that of the DA neuronal phenotype, as examined by tyrosine hydroxylase (TH) induction, the rate-limiting enzyme for DA biosynthesis (Zetterstrom et al., 1996). Gene-targeting experiments have clearly demonstrated the specific and crucial requirement of Nurr1 in DA neuronal development (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998; Castillo et al., 1998). Gain-of-function studies in mouse embryonic stem cells (Chung et al., 2002; Kim et al., 2002; Sonntag et al., 2004) and rat nestin-positive neural precursor cells (Kim, J. Y., et al., 2003; Sakurada et al., 1999) have demonstrated that Nurr1 could prominently induce the DA phenotype. However, it is probable that additional factors are
required for differentiation of neural precursor cells into mature DA neurons. For example, forced expression of Nurr1 in some cells resulted in either undifferentiated non-neuronal cells (Sonntag et al., 2004; Sakurada et al., 1999) or morphologically and functionally immature DA neurons (Kim, J. Y. et al., 2003). Therefore, identification of such additional factors is a prerequisite to further our understanding of the regulatory cascade underlying the differentiation of midbrain DA neurons.

The transcription factors belonging to the basic Helix-Loop-Helix (bHLH) gene family are known to regulate crucial developmental processes in various mammalian tissues. Neural bHLH transcription factors such as Neurogenin 1 (Ngn1), Ngn2, NeuroD, and Mash1 are expressed in both the central and the peripheral nervous systems during development and promote early neuronal differentiation (for reviews, see Kageyama and Nakanishi, 1997; Lee, 1997; Bertrand et al., 2002; Ross et al., 2003). In addition to the generic neurogenic action, several lines of evidence have shown that they are also involved in specifying neuronal subtype identities. In fact, it has been reported that the achaete-scute homolog Mash1 and the atonal-related genes Ngn1 and Ngn2 are expressed in a complementary pattern in the developing telencephalon, spinal cord and neural crest, and specify distinct neuronal subtype identities (Fode et al., 2000; Parras et al., 2002; Hirsch, 1998; Perez, 1999). It has also been recently reported that a cooperative or synergistic action of proneural bHLHs with homeodomain factors specifies the subtype identity of particular neurons. For example, Mash1 with Phox2b (Dubreuil et al., 2002; Pattyn et al., 2004), and Ngn2 with LIM-homeodomain protein (Lee and Pfaff, 2003) specify the ventral hindbrain and spinal cord motor neurons, respectively. These findings prompted us to ask if such combined action between bHLH and Nurr1 is necessary for a more complete midbrain DA neuronal differentiation.

In this study, we report that Mash1 and atonal-related bHLH factors exert contrasting effects on Nurr1-induced DA neuronal differentiation. Forced expression of the Mash1 protein, whose expression is seen in early DA neuronal progenitor cells in the developing mesencephalon, promoted differentiation of immature Nurr1-induced DA cells toward mature and functional DA neurons with pre-synaptic DA neuronal markers. By contrast, atonal-related Ngn1, Ngn2 and NeuroD markedly repressed Nurr1-induced acquisition of DA phenotypes. These findings provide the first evidence for the specific and discrete roles of bHLH transcription factors in the development of midbrain DA neurons.

Results
Expression patterns of Mash1 and Ngn2 in the developing ventral midbrain
We first examined the expression patterns of Mash1 and Ngn2 proteins in the ventral domain of rat embryonic midbrain where DA neurons develop between embryonic days 11-13 (E11-E13). Mash1 expression was already detectable throughout the ventral mesencephalon by E11 and E12 (Fig. 1B-D). Aldehyde dehydrogenase 2 (AHD2), a specific marker for committed midbrain DA neuronal progenitor cells colocalizing with Nurr1 mRNA in the early ventral mesencephalon (Wallen et al., 1999), showed an overlap with Mash1. Specifically, a part of the Mash1-expressing domain in the more ventral part of the neural tube containing the floor plate showed AHD2 expression (Fig. 1B). At the cellular level, a major subpopulation of AHD2-positive DA neuronal progenitor cells also expressed Mash1 at this stage (Fig. 1B-D). Cells immunoreactive to TH, one of the terminal DA neuronal markers, were not detected in the E11-E12 mesencephalons. TH expression begins to appear at E13 and is restricted to the cells with differentiated neuronal morphology in the mantle area of the ventral mesencephalic neural tube (Fig. 1E,F). Mash1 expression was restricted to the ventricular zone of the TH-expressing domain in the E13 ventral mesencephalon. This result is consistent with the previous finding that TH expression is always external to the proliferative zone marked by Mash1.
expression in the basal ganglia (Marin et al., 2005). By contrast, Ngn2 expression was barely detected in the mesencephalon of embryos at E11-12 (Fig. 1G), but observed in the ventral domain of E13 midbrain (Fig. 1H,I). In the ventricular zone of E13 ventral midbrain, Ngn2-positive cells were intermingled with Mash1-positive cells (Fig. 1H), and subpopulations of AHD2-positive cells expressed Mash1 or Ngn2 (Fig. 1I). The similar overlapping pattern of the Mash1 and Ngn2 localizations has been demonstrated in dorsal spinal cord interneurons (Helms et al., 2005).

Mash1 induces neuronal differentiation of Nurr1-induced TH cells

We have previously demonstrated that Nurr1 sufficiently induces the acquisition of DA neuron phenotype in the cultured neural precursor cells isolated from rat embryonic brains (Kim, J. Y. et al., 2003). This result suggests that experimental systems using the neural precursor cell cultures could be used to investigate the midbrain DA neuron development. The above regional and chronological patterns of Mash1 protein expression suggest a specific role of Mash1 in the development of the midbrain DA neurons. Thus, we examined the effect of co-expressing Mash1 with Nurr1 in embryonic neural precursor cells compared with cells expressing Nurr1 only. The retroviral vector system showed a consistent transduction efficiency of >90% for all the constructs as examined by immunocytochemical analysis of transgene expression (supplementary material Fig. S1). Basal expression of Mash1 was marginal in the naive precursors when analyzed by both immunocytochemistry and RT-PCR, and was greatly upregulated by retroviral transduction (supplementary material Fig. S1). Forced expression of Mash1 resulted in extensive neurite outgrowth from Nurr1-induced TH-positive cells (17.9±11.1 µM versus 294.8±101.7 µM in the cultures for cortical precursor cells, Fig. 2A-C) without significant alterations in the number of cells positive for TH (44.5±14.4% versus 40.1±14.8%; n=20, P=0.13) and TH protein expression (Fig. 2D). MAP2, a late neuronal marker, was expressed in 50.3±8.6% of TH-positive cells in the cultures transduced with Nurr1 and Mash1 whereas only 11.0±11.7% of TH cells were positive to MAP2 in the control cultures transduced with Nurr1 and LacZ (Fig. 2E-G). Furthermore, expression of mRNAs encoding presynaptic marker proteins (i.e. synaptophysin and synapsin) and related to growth cone formation (GAP43) was significantly upregulated by Mash1 (Fig. 2H). The effects of Mash1 in the maturation of Nurr1-DA cells were similarly observed in the precursor cells isolated from other brain regions tested, such as the lateral ganglionic eminences (LGEs) and ventral midbrain at E13 and E14 (supplementary material Fig. S2 and data not shown). The following data were obtained in the cultures for E14 cortical precursor cells unless noted.

We next evaluated mature DA neuronal functions of the cells transduced with Nurr1 along with Mash1 or LacZ by estimating DA release, specific DA uptake, and electrophysiological properties assessed by single-cell recordings. HPLC analysis demonstrated that DA levels in the medium conditioned for 24 hours was more than ten times greater in the Nurr1 and Mash1-transduced cultures (359.4±32.4 pg/ml of medium), compared with those transduced with Nurr1 and LacZ (34.7±8.5 pg/ml). An important functional aspect of mature presynaptic DA neurons

Fig. 2. Mash1-induced neuronal differentiation of Nurr1-TH cells. Mash1 or LacZ (control) was co-expressed with Nurr1 in the precursor cells isolated from rat embryonic cortices using a retroviral vector system. Differentiation of the precursors was induced and immunocytochemical, immunoblot and RT-PCR analyses were performed at day 3 (A-D,H) and at day 15 (E-G) of differentiation. (A,B) Representative images of TH-immunoreactive cells in the cultures transduced with Nurr1 and LacZ (A) and Nurr1 and Mash1 (B) at day 3 of in vitro differentiation. Insets, DAPI nuclear staining of the same field. (C) Morphometric analyses to compare the dendritic lengths of Nurr1-TH cells in the control and Mash1-transduced cultures. Box and bar in the graph represent mean and s.e.m. of total dendritic lengths, respectively. (D) Western blot analyses combined with the findings in Fig. 5R,S demonstrate that Mash1 increased expression of generic neuronal marker TuJ1, without any effect on the Nurr1-induced TH expression. (E-G) Localization of the mature neuronal marker MAP2 in Nurr1-induced TH’ cells. Representative images of MAP2 and TH immunocytochemistry of control (E) and Mash1-transduced (F) cultures at in vitro differentiation day 15. Insets, DAPI nuclear staining of the same field. (G) Percentages of double-immunoreactive cells for TH and MAP2 (TH+/MAP2+) out of total TH’ cells. (H) Semi-quantitative RT-PCR analyses for the mRNAs specific to synaptic formation (synaptophysin and synapsin) and growth cone development (GAP43). *Significantly different from the controls with P<0.001. Bars, 10 µm.
is the ability to release DA in response to membrane depolarization. Conditioning cells in the medium only for 15 minutes did not yield detectable levels of DA by HPLC in both cultures. However, treatment with high potassium (56 mM) induced DA release from Nurr1-transduced cells in both groups of cultures. The DA levels evoked by KCl-depolarization stimuli for 15 minutes were tenfold greater in the cultures transduced with Nurr1 and Mash1 (1101.7±40.7 pg/ml versus 106.4±15.3 pg/ml).

DAT-mediated high-affinity reuptake of DA transmitter is another crucial function of mature presynaptic DA neurons. We could scarcely observe DA uptake in cells transduced with Nurr1 and LacZ (control, 0.23±0.01 fmol/minute/well). By contrast, cells transduced with Nurr1 and Mash1 displayed an avid DA uptake (2.90±0.61 fmol/minute/well, Fig. 3B).

Electrophysiological properties of Nurr1-expressing cells were examined in the differentiated cultures transduced with Nurr1 and Mash1. Individual Nurr1-expressing cells could be identified based on the expression of GFP from the Nurr1-IRES-GFP bicistronic expression construct. The Nurr1-GFP-expressing cells with a differentiated neuronal shape were chosen for electrophysiological analysis using the conventional whole-cell recording technique. Recordings in the current-clamp configuration allowed us to determine the active membrane characteristics of these cells. Prolonged depolarizing current injections (Fig. 3C, left panel) demonstrated the capability to fire fast action potentials. The cells also exhibited voltage-dependent membrane currents (Fig. 3C, right panel). Depolarizing voltage steps elicited both large outward potassium currents and fast inward sodium currents. These action potential and inward sodium currents were completely blocked by tetrodotoxin (TTX) treatment (3 μM) (Fig. 3D). These results indicate the presence of well-developed sodium and potassium channels, characteristic of differentiated neurons. In addition, membrane potential changes were investigated in cells subjected to hyperpolarizing current injections. As the intensity of the hyperpolarizing current was increased, there was a time-dependent reduction in

**Fig. 3.** Acquisition of presynaptic neuronal function by Nurr1-induced DA cells with Mash1. Precursor cells from E14 cortices were transduced with Nurr1 and Mash1 (N+M) or Nurr1 and LacZ (N, control), and the functional analyses were performed after 10 days of in vitro differentiation. (A) HPLC quantification of DA release. The graph shows DA levels in the medium conditioned for 24 hours (24 hr medium) and released by KCl-evoked depolarization in N2 with 56 mM KCl for 15 minutes (KCl 15min), n=3. (B) DA uptake. The graph shows the specific DA uptake of the cells transduced with Nurr1 and Mash1 or Nurr1 and LacZ (n=5 for each value). Specific DA uptake was calculated by subtracting non-specific uptake (with nomifensine) from value without nomifensine. *Significantly different from the controls at \( P<0.001 \). (C-E) Electrophysiological properties of cells differentiated from the precursor cells transduced with Nurr1 and Mash1. (C) Typical recordings of electrophysiological properties of a differentiated neuron. Left panel, Current-clamp recordings during prolonged depolarizing current injections. Top traces represent current injections, whereas bottom traces indicate voltage recordings. Depolarizing current injections elicited fast action potentials. Right panel, voltage-dependent membrane currents. Depolarizing voltage steps (top traces) elicited outward K⁺ currents and fast inward Na⁺ currents (bottom traces). (D) Effect of tetrodotoxin (TTX) on action potential and voltage-dependent Na⁺ currents. TTX completely inhibited the action potential evoked by depolarizing current injections. Inset, Na⁺ current by depolarizing voltage steps. (E) Current-clamp recordings during prolonged hyperpolarizing current injections. The cells display the time-dependent anomalous rectification that is a characteristic of midbrain DA neurons after a hyperpolarizing pulse.
the membrane deflection (Fig. 3E), indicating an anomalous rectification characteristic of mesolimbic (Rayport et al., 1992) and nigrostriatal (Rohrbacher et al., 2000) DA neurons. Such rectification, however, could hardly be observed in the cells transduced with Nurrl alone (or Nurrl and LacZ), although the other electrophysiological properties described above could be detected in the Nurrl-transduced cells with well-differentiated neuronal shape.

Inhibitory role of atonal related bHLHs in Nurrl-induced expression of DA phenotype

We carried out similar experiments with atonal-related bHLH genes, Ngn1, Ngn2 and neuroD. To our surprise, co-expression of each of these genes with Nurrl dramatically repressed Nurrl-induced TH expression by 79-98%, as estimated by the number of cells positive for TH (Fig. 4A-E). Consistent with this finding, our immunoblotting analysis showed that TH protein levels were also significantly downregulated (Fig. 4F). This is in clear contrast to cells infected with Mash1-expressing virus (Fig. 2D), which showed no changes in TH expression. Importantly, overexpression of Ngn1, Ngn2 and NeuroD markedly diminished Nurrl-induced expression of mRNAs specific to DA phenotype such as DAT, AADC, and VMAT2 as well as TH (Fig. 4G), suggesting an inhibitory action of the atonal bHLH proteins in DA phenotype expression. The inhibitory roles of neurogenins and NeuroD were also observed in the cultures of different neural precursors tested, such as those isolated from the cortices, LGEs and ventral midbrains at either E13 or E14 (supplementary material Fig. S2 and data not shown).

Helix-Loop-Helix domains are responsible for the specificity of the bHLH effects in Nurrl-induced DA differentiation

Basic helix-loop-helix (bHLH) transcriptional factors bind to DNA via the basic regions of bHLH. The HLH portion is responsible for the protein-protein interaction between bHLH proteins or with other proteins (Ma et al., 1994; Ellenberger et al., 1994). Mutant derivatives of atonal-related bHLH genes, Ngn1/AQ, NeuroD/AQ, and NeuroD/G whose DNA binding capacity is abolished, did not display the inhibitory effect on the TH expression seen with wild-type genes (Fig. 5A-H), suggesting that DNA binding is required for the inhibitory activity of the atonal-related bHLH proteins. These mutations also resulted in the loss of general neuronal differentiation activity as estimated by TuJ1 expression (Fig. 5H), consistent with the notion that bHLH-mediated neurogenesis occurs via direct binding to target promoters and promoting transcriptional initiation.

To identify the important domain of the bHLH factors that impart distinct activities in Nurrl-induced DA differentiation, we generated a series of chimeric proteins by interchanging HLH domains between NeuroD and Mash1 and between NeuroD and muscle-specific bHLH factor MyoD. Forced expression of MyoD, a skeletal-muscle-specific myogenic bHLH gene, in the Nurrl-infected cultures did not alter the proportion of TH+ cells, compared with the control cultures transduced with Nurrl and LacZ retroviral vectors, despite the fact that total cell number was reduced in the MyoD-infected culture, presumably by decreased cell proliferation and/or viability. A reduction in the number of TH+ cells and in the level of TH protein, comparable to levels seen with the wild-type NeuroD, was observed by introducing chimeric protein MD, which is composed of Mash1 basic region and NeuroD HLH domain (Fig. 5J-S). Such data indicate that it is the HLH domain of NeuroD which is responsible for the inhibitory activity of NeuroD. Consistently, the substitution of the basic region in NeuroD with that of MyoD (in the chimera YD) did not affect NeuroD-induced inhibition of TH expression. We also examined the effect of DM which contains the basic region of NeuroD and the HLH domain of Mash1. In the cultures transduced with DM-bHLH, no significant alterations in TH+ cell number or in TH protein level was observed. Furthermore, as seen with the wild-type Mash1, enhanced levels of neurite

![Image](https://example.com/image.png)
outgrowths from Nurr1-induced TH cells were observed by forced expression of the chimera DM (data not shown). The chimera DY showed an indistinguishable effect from that of wild-type MyoD. Taken together, these results indicate that although DNA binding through the basic region is a prerequisite for their respective activities, HLH domains, but not the basic domains, of Mash1 and NeuroD contain the information that specifies distinct roles of each bHLH gene in Nurr1-induced DA differentiation.

Inhibitory activity of Ngn1 in TH expression is not attributable to either cell cycle arrest or inhibition of JAK/STAT signal

The inhibitory effects of Ngn1, Ngn2 and NeuroD were qualitatively and quantitatively similar although NeuroD was slightly less effective than neurogenins. The subsequent experiments were thus mostly carried out with Ngn1. We confirmed that the effect of Ngn1 expression was intracellular autonomous in nature. The degree of reduction of TH+ cells was the same whether the cells were transduced with bicistronic Nurr1-IRES-Ngn1 construct or with separate Ngn1 and Nurr1 constructs. Neither co-culture with Ngn1-infected cells nor treatment with conditioned medium prepared from Ngn1-infected cultures elicited an inhibitory effect on the Nurr1-induced TH expression (data not shown). These findings confirm the intracellular cell-autonomous mechanism of Ngn1 activity.

TH+ cell number in Nurr1 and Ngn1-transduced cultures was significantly less than the cultures transduced with Nurr1 alone from 1 day after infection (data not shown), suggesting that the inhibitory action of Ngn1 is not due to a delayed response of persistent Ngn1 overexpression. Ngn1 infection 1 day before Nurr1 infection was also effective in inhibiting Nurr1-induced TH expression. By contrast, when cultures were infected with Ngn1 1 day after Nurr1 infection, there was no clear decrease in TH+ cell numbers (data not shown). It has been reported that Nurr1-induced TH expression in the C17.2 neural stem cell line was observed only in cells with a high proliferation capacity (Wagner et al., 1999). Since proneural bHLH-induced neuronal differentiation is concomitant with the cell-cycle withdrawal, the inhibitory effect of Ngn1 in DA differentiation might be a consequence of neurogenin-induced cell-cycle arrest. To test this possibility, we used the FGF receptor blocker SU5402 (Mohammadi et al., 1997) and the MAPK inhibitor PD98059 (Alessi et al., 1995), which are inhibitors of bFGF signaling

Fig. 5. Essential structural components of Ngn1 and NeuroD required for the inhibitory actions on Nurr1-induced TH expression.

(A-H) Effect of the abolishment of DNA-binding capacity of Ngn1 and NeuroD. Each of the wild types and the DNA-binding mutants of Ngn1 and NeuroD was introduced into Nurr1-transduced precursors by co-infection as described in the Materials and Methods, and TH+ cell numbers and TH protein levels were determined at day 3 of differentiation using immunocytochemical (A-G) and western blot analyses (H), respectively. Note that the abolishment of the DNA-binding capacity in Ngn1 and NeuroD abrogates not only their generic neurogenic activities (TuJ1 protein level in H), but also the inhibitory activity on Nurr1 and TH expression. (I) Schematic diagrams of the chimeric proteins of NeuroD(D) and Mash1(M) or NeuroD and MyoD (Y) used to identify the domains for the differential roles of Mash1 (or MyoD) and NeuroD. Co-transduction of each of the chimeric proteins with Nurr1 was carried out as described above, and the effects of the chimeric bHLHs on Nurr1-TH expression were determined at day 3 of differentiation by immunocytochemical (J-R) and immunoblot (S) analyses.

(J-S) Analyses of the chimeric proteins reveal that the HLH domains are responsible for the differentiation actions of Mash1 and NeuroD. Note that the effects of the chimera YD and MD, which hold NeuroD HLH domain, on Nurr1-induced TH+ cell numbers and TH protein levels are not distinguishable from those of wild-type NeuroD, whereas the inhibitory activity of NeuroD is abolished by substituting the HLH domain with that of Mash1 or MyoD (in chimera DM or DY). Graphs G and R represent the mean percentage (± s.e.m.) of TH+ cells out of total DAPI+ cells. *Significantly different from the controls at P<0.001. Bars, 10 μm.
that is required for neural precursor cell proliferation. The treatment of these bFGF signal blockers indeed resulted in a decrease of cell proliferation, as estimated by the BrdU+ cells (supplementary material Fig. S3B). Importantly, although the inhibitory effect of SU5402 on the cell proliferation was much greater than that of Ngn1 (%BrdU+ cells of total cells; 0.6±0.2% in Ngn1-infected versus 10.0±0.8% in SU5402-treated cultures; \( n=20, P<0.01 \), TH+ cell number, compared with untreated control cultures, only slightly decreased in the cultures treated with SU5402. The effect of Ngn1 on the decrease in the number of TH+ cells was much greater than that of SU5402 (TH+/Nurr1+ cells; 3.9±0.6% in Ngn1-infected versus 0.9±0.3% in SU5402-treated cultures; \( n=20, P<0.01 \) ) (supplementary material Fig. S3C). This result indicates that Ngn1-mediated repression of TH expression is not attributable to the reduced cell proliferation.

Ngn1 inhibits astrocytic differentiation by blocking the activation of STAT transcription factor that is necessary for gliogenesis and by sequestering CBP-p300, which forms an active complex with Smad1, away from astrocyte differentiation genes (Sun et al., 2001). Thus, we tested whether Ngn1-mediated inhibitory activities on gliogenesis and DA differentiation share common mechanisms. The JAK-STAT pathway can be activated and inhibited by treatment with LIF (Fukada et al., 1996) and AG490 (Gazit et al., 1991) respectively. Neither the activation of the STAT signaling by LIF nor the inhibition by AG490 had any influence on Nurr1-induced TH+ cell yields (data not shown). We also asked if Nurr1 requires CBP-p300 for the transactivation of TH gene and the reduced availability of CBP-p300 by Ngn1-mediated sequestration is the underlying mechanism for Ngn1-induced inhibition of TH expression. If this were the case, the inhibitory activity would be restored by introducing extra CBP-p300. However, co-expression of CBP-p300 did not restore the inhibitory activity of Ngn1 (data not shown). Taken together, these data indicate that Ngn1-mediated regulation of STAT and CBP-p300 is not likely to be responsible for the repression of TH expression in the Ngn1-transduced cultures.

Atonal-related bHLH factors repress Nurr1-induced transactivation of TH promoter activity

We noted that both protein and mRNA levels of TH were markedly suppressed by Ngn1 (Fig. 4G), suggesting that Ngn1 may act at the transcriptional level in TH gene regulation by Nurr1. Although the TH promoter contains multiple sequence motifs homologous to the Nurr1-binding motif, only NL3, positioned from –872 to –865 of the transcription initiation site, showed a high affinity to Nurr1 (Kim, K. S. et al., 2003). Forced expression of Nurr1 in the cortical precursor cells markedly increased reporter gene expression driven by the native TH promoter (>1000-fold increase compared with the control cultures transfected with empty pEF1α plasmid). Interestingly, co-expression of Ngn1, 2 or NeuroD with Nurr1 resulted in a potent repression of Nurr1-induced activation of the promoter (% activities relative to Nurr1 and LacZ control: 5.1±0.8, 5.7±0.4 and 10.7±3.8 in the cultures co-tranduced with Ngn1, Ngn2, and NeuroD, respectively; Fig. 6A). By contrast, no significant change in TH promoter activity was observed by Mash1 co-expression. The existence of a consensus binding sequence (E-box; CANNTG) for bHLH proteins within the tested TH promoter (from –186 to –181) suggested that the inhibitory role of the atonal-related bHLHs might be mediated by binding to this E-box. However, Nurr1-induced increase of NL3-driven reporter activity, without the E-box, was similarly blocked by all of the atonal-related bHLHs tested (Fig. 6B), suggesting that the binding of the bHLHs to the E-box is not necessary for the inhibitory effect.

Similar to the findings of the effect on TH+ cell yield (Fig. 5), DNA-binding mutants Ngn1/AQ, NeuroD/AQ and NeuroD/G did not elicit inhibitory activities on the Nurr1-induced transcription activation in the TH reporter assay (Fig. 6A). Substituting the HLH region of NeuroD with that of Mash1 (chimera DM) also abolished the repressive activity of NeuroD on the Nurr1-induced transactivation, whereas Nurr1-induced activation of TH promoter and NL3 reporter activities were significantly repressed by co-expressing the chimera MD which contains the NeuroD HLH region. Taken together, these results indicate that atonal related bHLH genes repress the TH gene by binding to DNA as well as to their specific transcriptional co-regulator. Furthermore, the fact that the E-box within TH is not required for their inhibitory effect raises the possibility that inhibition may be indirect (see Discussion).
Discussion
An emerging theme in developmental neurobiology is that combinatorial actions of different transcription factors are crucial for the development and specification of subtypes of neuronal lineages (Goridis and Rohrer, 2002; Shirasaki and Pfaff, 2002). We have previously demonstrated that Nurr1 overexpression in cultured embryonic cortical precursor cells induced the expression of the markers specific to DA neurons including the genes involved in biosynthesis of DA (TH and AADC) as well as its storage (VMAT2) and uptake (DAT) (Kim, J. Y. et al., 2003). However, we noted that the resulting cells could not be qualified as mature DA neurons by morphological, immunocytochemical or presynaptic-functional criteria, strongly suggesting the requirement of additional transcription factor(s). In this report, using the same embryonic cortical precursor cultures as the model system, we investigated whether bHLH transcription factors are involved in the potential combined actions with Nurr1 for differentiation of precursor cells to mature and functional DA neurons.

Although mammalian atonal-related andachaete-scute homolog bHLHs share a general neurogenic activity, they play discrete and non-overlapping roles in the neuronal subtype specification in certain brain regions and in peripheral ganglia. Consistent with this established paradigm, we found that there is a striking difference in the effect of these bHLHs in the differentiation of midbrain DA neurons induced by Nurr1: whereas Mash1 facilitates neuronal differentiation of cortical precursor cells into functional and mature DA neurons in the presence of Nurr1, the atonal-related bHLHs inhibits the Nurr1-mediated induction of DA phenotypes.

Mash1 is expressed in the ventral domains of the embryonic brain and specifies ventral neuronal subtypes such as GABAergic neurons in the forebrain (Fode et al., 2000; Parras et al., 2002), brachiofacial- and visceromotor neurons (Dubreuil et al., 2002; Pattyn et al., 2004) and serotoninergic neurons (Pattyn et al., 2004) in the hindbrain. Mash1 is also a determinant for noradrenergic neurons in the locus coeruleus (Goridis and Brunet, 1999; Pattyn et al., 2000) and sympathetic neurons in the peripheral ganglia (Hirsh et al., 1998; Lo et al., 1998; Pattyn et al., 1999; Goridis and Rohrer, 2002). Our new findings furthermore suggest that Mash1 may also play a crucial role for midbrain DA neuron development.

In the generation of specific subtypes of neurons, proneural bHLHs not only play the role of a lineage determinant but also assume the responsibility of activating subtype-specific genes. However, because the subtype determination by the bHLHs occurs in a context-dependent manner in the specific brain regions, the bHLH proteins usually cooperate with other region-specific factors such as homeodomain genes for the neuronal subtype specification. For instance, it has been demonstrated that Mash1 acts in the sympathetic ganglia in a combinatorial manner with a determinant of the noradrenergic phenotype, the homeodomain protein Phox2b, to induce the expression of genes specific for noradrenergic phenotype (Hirsh et al., 1998; Lo et al., 1998; Pattyn et al., 1999; Goridis and Rohrer, 2002). Another example of cooperation between bHLH and homeodomain factors has been shown in the specification of motor neuronal fate in the embryonic spinal cord: Ngn2-NeuroM interacts with LIM-homeodomain factors to form an active and efficient transcription complex which then activates transcription of motor-neuron-specific genes (Lee and Pfaff, 2003). We have shown here that Mash1, but not neurogenin, is expressed in the ventral domain of early embryonic midbrain before the appearance of TH+ DA cells and that the expression of Mash1 is seen in AHD2+ DA neuronal progenitor cells (Fig. 1). In addition, Mash1 in vitro leads to neuronal maturation and acquisition of pre-synaptic functionality in the cells that acquired DA neuronal phenotypes through Nurr1 activity. These findings suggest an involvement of Mash1 in the development of midbrain DA neurons. However, unlike other cases of cooperation involving bHLH genes, Mash1 is not likely to have a cooperative or synergistic role with Nurr1 in the DA phenotype specification. This is because Mash1 does not have an additive or cooperative effect on the Nurr1-induced expression of DA phenotype genes such as TH, AADC, VMAT2 and DAT (Fig. 2A,B and data not shown) or in TH promoter assay (Fig. 6). Mash1-mediated improvement in DA release and uptake, without induction of the related gene expression, could be mediated by the role of Mash1 in the functional maturation of VMAT2 and DAT proteins, such as in the processes of protein modification and proper subcellular localization. The mechanisms need to be clarified in further studies. Furthermore, Mash1 did not activate the expression of the genes specific to midbrain DA neuronal markers (Nurr1, Ptx3) or to midbrain development markers (engrailed-1, Pax-2) (data not shown). Thus, although Mash1 and Nurr1 apparently work together in the midbrain DA development, they probably play independent roles. Specifically, Mash1 induces neuronal differentiation and maturation whereas Nurr1 mediates DA-specific phenotype expression. A similar mode of non-interactive involvement in a common developmental pathway has been previously observed in the generation of neuronal subtypes in the retina. Mash1 apparently determines only the generic neuronal fate in the retina without activating subtype-specific genes in a layer identified and specified by Chx10, a homeodomain gene (Hatakeyama et al., 2001).

Interestingly, atonal-related bHLH genes display an inhibitory effect in the Nurr1-induced expression of the genes specific to DA phenotype. We have ruled out several mechanistic possibilities of this inhibition, such as Ngn-mediated inhibition of the JAK/STAT signal, and sequestering p300-CBP away from participating in transcriptional activation of the TH gene. Importantly, atonal-related bHLH genes dramatically repressed the Nurr1-induced transactivation of TH promoter activity (Fig. 6). The findings obtained from the TH promoter assays using the DNA-binding mutants and chimeric proteins of the bHLHs indicated that DNA binding is absolutely required for the inhibitory roles of the atonal-related bHLHs and that interaction with specific co-regulator is likewise necessary. Furthermore, the unique E-box within the TH promoter does not appear to be functional in the inhibition by the atonal-related bHLH genes. Thus, the detailed mechanism still needs to be elucidated. It is possible that a new cis-element within the TH promoter is specified by a transcription complex that atonal-related bHLH genes form. It is also possible that the effect is indirect. In other words, the bHLH gene may be inducing the expression of another gene product whose presence leads to the inhibition of TH promoter.

The inhibitory role of neurogenins demonstrated using in vitro culture systems has certain elements of agreement with DA neuronal development in vivo. The complementary pattern
of Mash1 and Ng2 expression seen in the telencephalon and spinal cord is not observed in the embryonic midbrain (Fig. 1H). Rather, Ng2 expression shows a lag and could not be detected in the ventral midbrain during the early embryonic ages (E11 and E12). By E13, when Ng2 is seen in the ventricular zone of AHD2-positive domain in the ventral midbrain (Fig. 1I), a population of cells in the mantle zone are already TH positive (Fig. 1E,F). The similar expression pattern and opposite roles of Mash1 and Ng2 have recently been demonstrated in the generation of dorsal spinal cord interneurons: Mash1 is required for the formation of dorsal interneuron 3 (d13) and d15 neurons, whereas Ng2 acts to limit the number of these neuronal populations (Helms et al., 2005). Similarly, the late appearance and inhibitory action of Ng2 in the ventral midbrain may imply that the role of Ng2 in this region is to confine the number of midbrain DA neurons, which is consistent with the effect seen in vitro. Further studies could clarify the significance of the inhibitory actions of the bHLH proteins in midbrain DA neuronal development in vivo.

In summary, using embryonic neural precursor culture, we found that neurogenic BHLH factors Mash1, neurogenins and NeuroD have contrasting roles in Nurri-induced DA neuronal differentiation. Mash1 was expressed in midbrain DA neuronal progenitors and its forced expression caused immature Nurri-induced DA cells to differentiate into mature and functional DA neurons with presynaptic DA neuronal markers. By contrast, atonal-related bHLHs (Ng1, Ng2 and NeuroD) repressed Nurri-induced expression of DA neuronal markers. This new information will be useful not only for our understanding of transcriptional regulatory cascade underlying the midbrain DA neuron development, but also for future in vitro generation of functional DA neurons from stem and/or progenitor cells for cell therapy of Parkinson’s disease, which is caused by the loss of midbrain DA neurons.

Materials and Methods

Primary CNS neural precursor cultures

Time‐pregnant Sprague‐Dawley rats were purchased from KOATECH (Seoul, Korea). Embryonic CNS tissues were dissected from the cortex, lateral ganglionic eminence (LGE, anlage of striatum), and ventral midbrain of rat embryos at embryonic day 13 (E13) or E14 and mechanically dissociated in Ca2+/Mg2+‐free Hank’s balanced salt solution (CMF‐HBSS; Invitrogen, Carlsbad, CA). Cells were plated at 8000 cells/cm2 on 10 cm tissue culture dishes (Corning, Corning, NY) pre‐coated with poly‐L‐ornithine (15 μg/ml, Sigma, St Louis, MO) at 37°C overnight followed by fibronectin (1 μg/ml, Sigma) for at least 2 hours. Neural precursor cells were allowed to proliferate in the presence of 20 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN) in serum‐free medium (N2) (John et al., 1996) for 4-6 days. The bFGF‐expanded precursor cells were dissociated by incubation for 1 hour with CMF‐HBSS followed by mechanical pipetting, re‐plated onto freshly coated coverslips (12 mm diameter; Carolina Biological Supply Company, Burlington, NC) or culture plates, and grown in N2 medium with bFGF. When cultures were 50-60% confluent, cells were subjected to retroviral infection as described below. Cell differentiation was induced by withdrawal of bFGF. Cultures were maintained at 37°C in 5% CO2, media changes were carried out every other day, and bFGF was supplemented daily. In certain experiments, leukemia inhibitory factor (20 ng/ml, ESGRO®, Chemicon, Temecula, CA) and AG490 (15 μM, Calbiochem, San Diego, CA) were added to cultures.

Retroviral vector construction and virus production

The retroviral vectors expressing Nurri1 were constructed by inserting Nurri1 cDNA into the monocistronic retroviral vector pCL or into the bicistronic vector pIRES-NeuroD. The retroviral vectors expressing Nurr1 were constructed by inserting Nurr1 cDNA into the monocistronic retroviral vector pCL or into the bicistronic vector pIRES-NeuroD (Ng et al., 2001) and NeuroD/AQ (modeled after NgN1AQ), were generated by changing two amino acids NR, located in the basic region of the bHLH proteins and crucial for DNA binding, into AQ using the QuickChange® II XL site‐directed mutagenesis kit (Stratagene, La Jolla, CA) system. For the generation of another NeuroD DNA binding mutant NeuroD/G, amino R and S of the basic region was replaced by G (in a similar fashion to the previously described mutant for Mash1/G (Ellienerger et al., 1994; Ma et al., 1994; Nakada et al., 2004). The basic region of NeuroD was fused to the HH domains of Mash1 (M) and MyoD (Y), to generate the chimeric proteins DM and DY, respectively. Similarly, the chimeras M and YD were generated by fusing the basic regions of Mash1 or MyoD (by respectively fusing the basic regions of Mash1 and MyoD) to the NeuroD HH domain. Conventional methods for PCR amplification, digestion with restriction enzymes and ligation reactions were used for the production of the chimeras. All the constructs generated were sequence verified before use. The precise sequences for the bHLH regions used for the chimeric proteins and mutants are shown in Fig. 5. The retroviral vectors were introduced into the retrovirus packaging cell line 293gg (Ory et al., 1996) by transient transfection with Lipofectamine® (Invitrogen). After 72 hours, the supernatants were harvested and used for infecting cultured precursor cells as described above. Cells were incubated with virus in the presence of polybrene (1 μg/ml) for 2 hours. For co‐expression of the bHLHs or the bHLH mutants with Nurri1, each of the viruses of the monocistronic pCL constructs (pCL‐Mash1, pCL‐Ng1, pCL‐Ng2, pCL‐NeuroD, bHLH mutant constructs, and pCL‐LuAZ (control)) was mixed with that of pCL‐Nurri1 (1:1) (tv) and applied to cells for infection. In certain experiments, viruses of the bicistronic vectors pNurri1‐IRES‐Mash1, pNurri1‐IRES‐Ng1, pNurri1‐IRES‐NeuroD and pNurri1‐IRES‐LuAZ (control) were used for co‐expression. In most cases, differentiation of the infected precursors was induced in N2 medium 1 day after infection.

Immunostaining of cultured cells and brain slices

Rat embryos at E11‐E13 were cut at the level of the isthmus (midbrain‐hindbrain junction). The brain rostral parts were soaked in 4% paraformaldehyde at 4°C overnight and cryopreserved in 20% (w/v) sucrose solution. The brain tissues were subsequently frozen in Tissue‐Tek® (Sakura Finetek, Torrance, CA) solution and sectioned with cryostat at 15 μm as shown in Fig. 1A. Cultured cells or cryosectioned brain slices were fixed in 4% paraformaldehyde/0.15% picric acid in phosphate‐buffered saline (PBS) and were incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: anti‐rabbit‐aldehyde dehydrogenase 2 (AHD2, a kind gift from G. Duster, Burnham Institute for Medical Research, La Jolla, CA, 1:300), TH (Pel‐Freeze, Rogers, AR, 1:2500), neuro‐specific class III β‐tubulin (TuJ1, Covance, Richardson, CA, 1:2000), Nurri1 (Chemicon, 1:100), anti‐mouse‐Mash1 and Ng2 (a kind gift from D. J. Anderson, Howard Hughes Medical Institute, Pasadena, CA; 1:1000 and 1:5, respectively), TH (Sigma, 1:10000), Bromodeoxyuridine (BrdU, Accurate Chemical, Westbury, NY, 1:400), mouse antibodies associated with TH (Sigma, 1:200), and β‐galactosidase (Promega, Madison, WI, 1:400). Appropriate fluorescein‐tagged (Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibodies were used for visualization. Cells and tissue sections were mounted in Vectashield® with DAPI (Vector Laboratories, Burlingame, CA) mounting medium for fluorescence and photographed using an epifluorescence microscope (Nikon, Melville, NY).

RNA extraction and RT‐PCR analysis

Total cellular RNA was prepared using TRI REAGENT® (Molecular Research Center, Cincinnati, OH) following the manufacturer’s recommendations. For the synthesis of cDNA, a Superscript kit (Invitrogen) was used. The PCR reactions were carried out following standard protocols. Optimal MgCl2 concentrations and cycle numbers for linear amplification range were determined. Primer sequences (forward and reverse), number of cycles, annealing temperature and product sizes (base pairs) were as follows: GAPDH (5′‐GCGATGTGCTTGCTAATGACAA‐3′ and 5′‐AGGGCTCTCTTCCTTGTGTCTC‐3′, 25 cycles, 66°C, 165 bp); synapsin I (5′‐CCACCCC‐ CAAAGGCGACGACA‐3′ and 5′‐GTTCCGCGCCGACGACGATG‐3′, 29 cycles, 58°C, 512 bp); synaptophysin (5′‐TGGTATCTCACTCCGGCTT‐3′ and 5′‐ACTCACTCTCATAGGCT‐3′, 26 cycles, 58°C, 379 bp, GAPD (5′‐AGGAA‐ GCAGCCAAGCTGAGAGG‐3′ and 5′‐CAGGAGGACGAGGTTCAGTGG‐3′, 35 cycles, 58°C, 167 bp), VMAT2 (5′‐TGCTCAGGAGGCCGCCGACG‐3′ and 5′‐CACAGAGGCCGCAATCAGAA‐3′, 35 cycles, 60°C, 412 bp), DAT (5′‐GGAACATGTTCCTCGTGAGGCC‐3′ and 5′‐GAATTTGAGTTGGTTTGC‐3′, 33 cycles, 55°C, 208 bp), VMAT2 (5′‐GTTTTGAGTTGTTTGGAC‐3′ and 5′‐GCTTCTGTGGGCTCATGATG‐3′, 35 cycles, 58°C, 167 bp), VP2 (5′‐CACAGGGGAGTGTGTCC‐3′ and 5′‐GGATTGTTGGGAGATG‐3′, 28 cycles, 55°C, 291 bp), GAPD (5′‐CAGGACGA‐ GAAGACGAGCAA‐3′ and 5′‐GGTTGAACTTCTGGTACGCAAG‐3′, 35 cycles, 58°C, 273 bp), GABRB3 (5′‐GGCAGAGGGCGAGGACGCAAG‐3′ and 5′‐GGGACGAGGAGGAGGAGGAGG‐3′, 35 cycles, 58°C, 273 bp), NeuroD (5′‐CTCAGACGAGGAGGAGGAGGAGG‐3′ and 5′‐GGGACGAGGAGGAGGAGGAGG‐3′, 35 cycles, 58°C, 273 bp).
Western blot analysis
Cultured cells were lysed in 20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.5% Triton X-100, 1 mM PMSE, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. Protein concentration was determined using Bio-Rad Protein Assay® (Bio-Rad, Hercules, CA). A total of 20 µg protein for each sample was run on an 8% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Whatman S&S, Portland, OR). The blot was probed with an anti-mouse-TuJ1 (1:1000), TH (1:5000), anti-β-actin (Abcam, Cambridge, UK) 1:5000 or anti-rabbit Nurr1 (1:100) antibodies, followed by anti-mouse or anti-rabbit IgG conjugated with peroxidase (Cell Signaling Technology, Beverly, MA) 1:2000. Bands were visualized with ECL detection kit (Amer sham Pharmacia, Buckinghamshire, UK).

Morphometric analysis for neurite outgrowth of Nurr1-induced TH cells
The length of neurites from TH-immunoreactive cells was measured as previously described (Kim, J. Y. et al., 2003). Briefly, TH+ cells from randomly selected areas of at least five cultures from three independent experiments were photographed, and morphometric analysis was carried out using an Axiosvert phase-contrast microscope equipped with an Axioimager digital camera system and Axiovision image analyzer (Carl Zeiss, Zena, Germany). The length of a neurite was defined as the distance from the soma to the tip of the branch. The total neurite outgrowth was defined as the combined length of all neurites per cell.

Quantification of DA release
DA release in the medium conditioned by the differentiated precursor cells was detected using reverse-phase HPLC. Differentiated precursor cells in 24-well plates were incubated with 50 nM [3H]DA in PBS (5 µCi/ml) for 20 minutes (20°C) in the same medium without 56 mM KCl (evoked) for 15 minutes. The media were then collected and stabilized with 0.1 N perchloric acid containing 0.1 mM EDTA and analyzed for DA. The method used for the alumina extraction for dopamine analysis is generally described (Kim, J. Y. et al., 2003). Briefly, TH+ cells from randomly selected areas of at least five cultures from three independent experiments were photographed, and morphometric analysis was carried out using an Axiosvert phase-contrast microscope equipped with an Axioimager digital camera system and Axiovision image analyzer (Carl Zeiss, Zena, Germany). The length of a neurite was defined as the distance from the soma to the tip of the branch. The total neurite outgrowth was defined as the combined length of all neurites per cell.

DA uptake assay
DA uptake assays were conducted according to the methods previously described with minor modifications (Lee et al., 2000). Cells were washed with PBS and incubated with 50 nM [3H]DA in PBS (5 µCi/ml) for 20 minutes (20°C) in the same medium without 56 mM KCl (evoked) for 15 minutes. The media were then collected and stabilized with 0.1 N perchloric acid containing 0.1 mM EDTA and analyzed for DA. The method used for the alumina extraction for dopamine analysis is generally described (Kim, J. Y. et al., 2003). Briefly, TH+ cells from randomly selected areas of at least five cultures from three independent experiments were photographed, and morphometric analysis was carried out using an Axiosvert phase-contrast microscope equipped with an Axioimager digital camera system and Axiovision image analyzer (Carl Zeiss, Zena, Germany). The length of a neurite was defined as the distance from the soma to the tip of the branch. The total neurite outgrowth was defined as the combined length of all neurites per cell.

Cell counting and statistical analysis
Cell counting was performed in microscopic fields, using an eye-piece grid at a final magnification of 200× or 400×. Five to ten microscopic fields were randomly chosen in each well and three to six culture wells were analyzed in each experiment. Data are expressed as mean ± s.e.m. Statistical comparisons were made by ANOVA followed by Tukey post-hoc analysis (SSPS 11.0; SPSS, Chicago, IL) when more than two groups were involved.

We would like to thank Dr. Duester for his kind gift of AHD2 antibody and David Anderson for his kind gift of Mash1 and Ngn2 antibodies. This work was supported by SC2150, SC2130 (Stem Cell Research Center of the 21st Century Frontier Research Program) and NLR Grant MI-0318-00-0290 funded by the Ministry of Science and Technology, Republic of Korea, and the NIH grant MH88866.

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