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

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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 (Ngns) 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
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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.

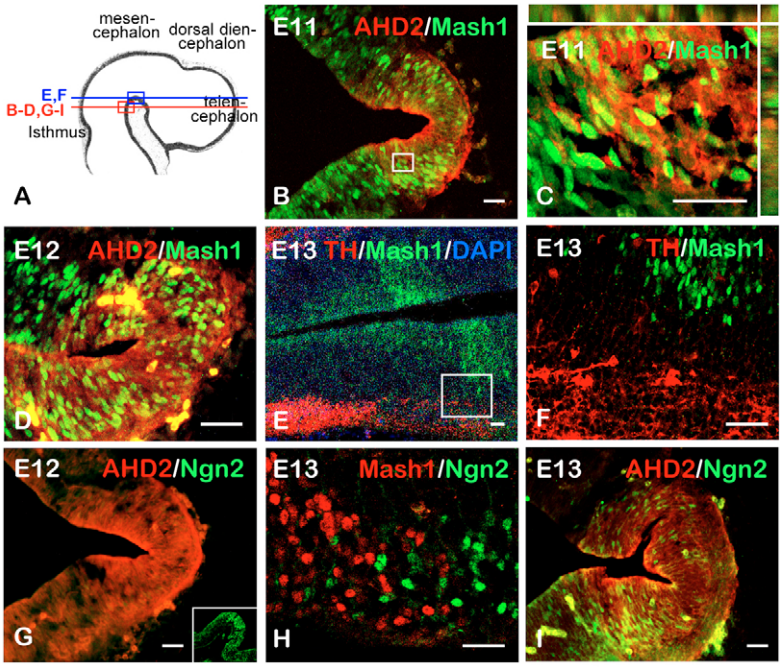

Fig. 1. Expression of Mash1 and Ngn2 in the developing ventral midbrain. (A) Embryonic brains were cryosectioned along the red (B-D,G-H) and blue (E,F) lines, and the immunohistochemical images were taken from the areas in red (B-D,G-H) and blue (E,F) boxes with confocal microscopy. (B-D) Localization of Mash1 expression in AHD2-positive DA progenitor cells in the ventral midbrains at E11 (B,C) and E12 (D). The boxed area of B is magnified in C. Panel C shows micrographs of the *z*-stacked confocal picture along the y-axis (right) and the x-axis (upper). (E,F) Expression patterns of Mash1 and TH in the E13 ventral midbrain. Note that TH expression is confined to the mantle region in the ventral midbrain at E13, whereas Mash1 is expressed in the proliferating ventricular region. Panel F is a higher magnification image of the white boxed area in E. The expression pattern of Ngn2 in the embryonic ventral midbrain is shown in panels G-I. Ngn2 expression is not detected in the ventral midbrain at E12 (G). Inset, Ngn2 expression in the diencephalic area at E12 (positive control). Ngn2-expressing cells are detected in the ventral midbrain at E13 (H,I) and a subpopulation of AHD2-positive cells co-express Ngn2 (I). Bars, 10 μm.

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

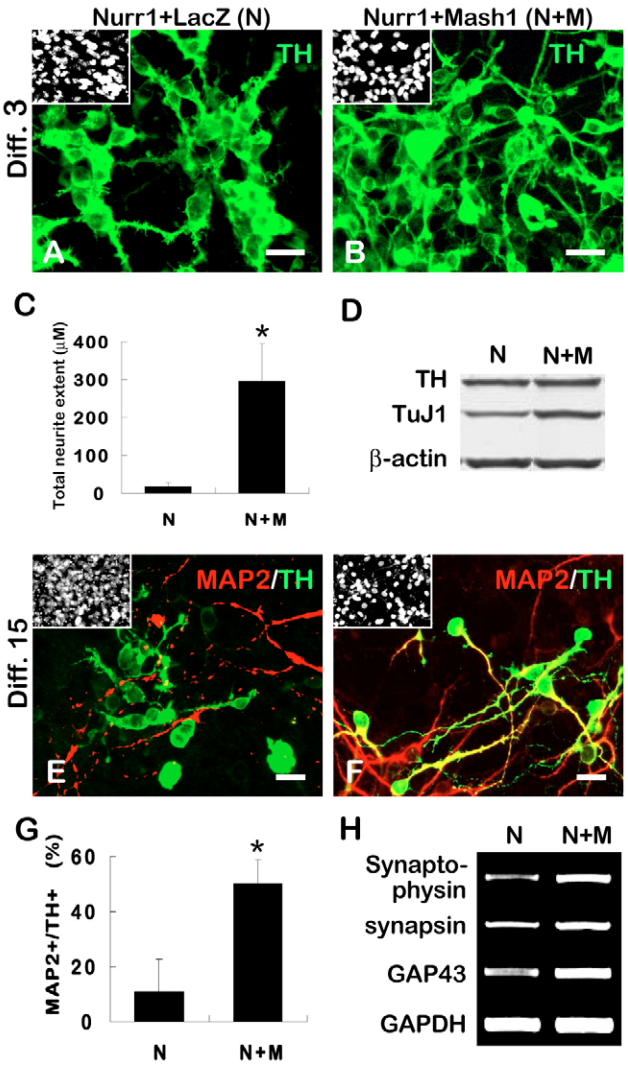

Fig. 2. Mash1-induced neuronal differentiation of Nurr1-TH cells. Mash1 or LacZ (control) was co-expressed with Nurr1 in the precursor cells isolated from rat embryonic cortices using a retroviral vector system. Differentiation of the precursors was induced and immunocytochemical, immunoblot and RT-PCR 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 C 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

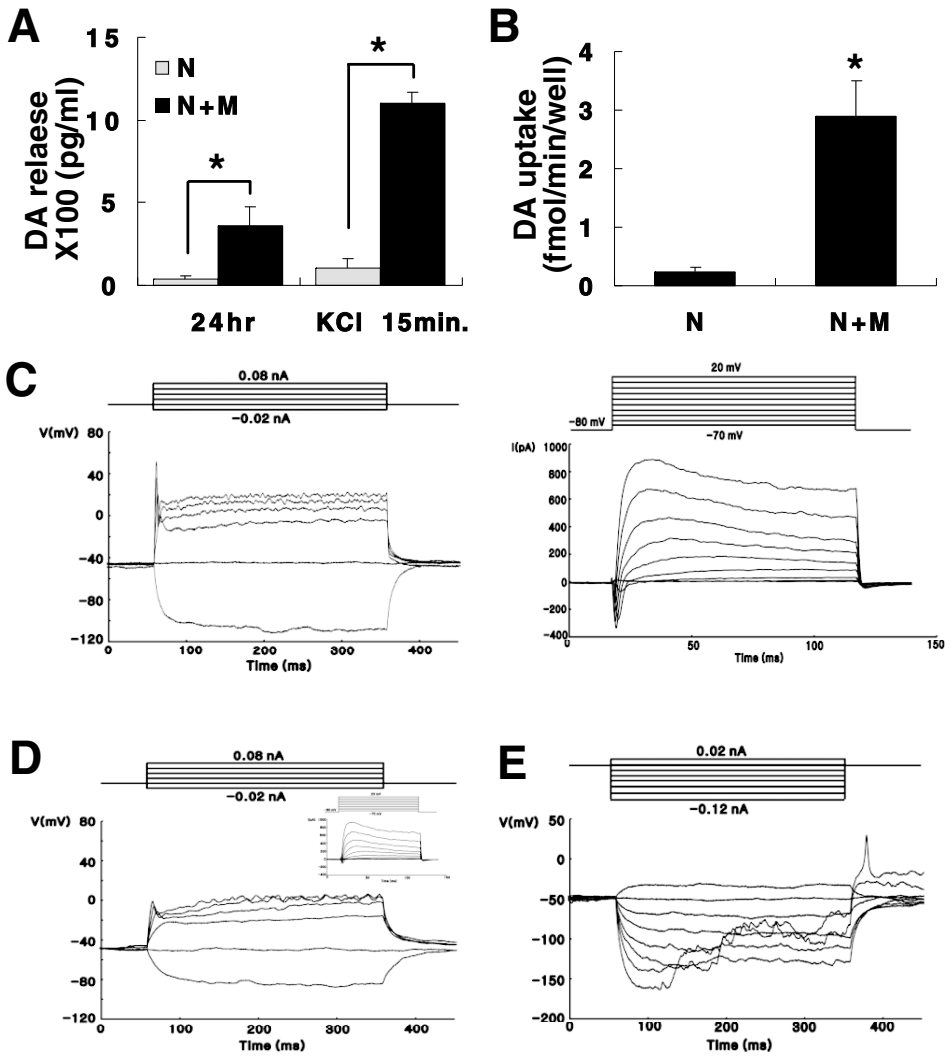

Fig. 3. Acquisition of presynaptic neuronal function by Nurr1-induced DA cells with Mash1. Precursor cells from E14 cortices were transduced with Nurr1 and Mash1 (N+M) or Nurr1 and LacZ (N, control), and the functional analyses were performed after 10 days of in vitro differentiation. (A) HPLC quantification 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 Nurr1 alone (or Nurr1 and LacZ), although the other electrophysiological properties described above could be detected in the Nurr1-transduced cells with well-differentiated neuronal shape.

Inhibitory role of atonal related bHLHs in Nurr1-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 Nurr1 dramatically repressed Nurr1-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 Nurr1-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).

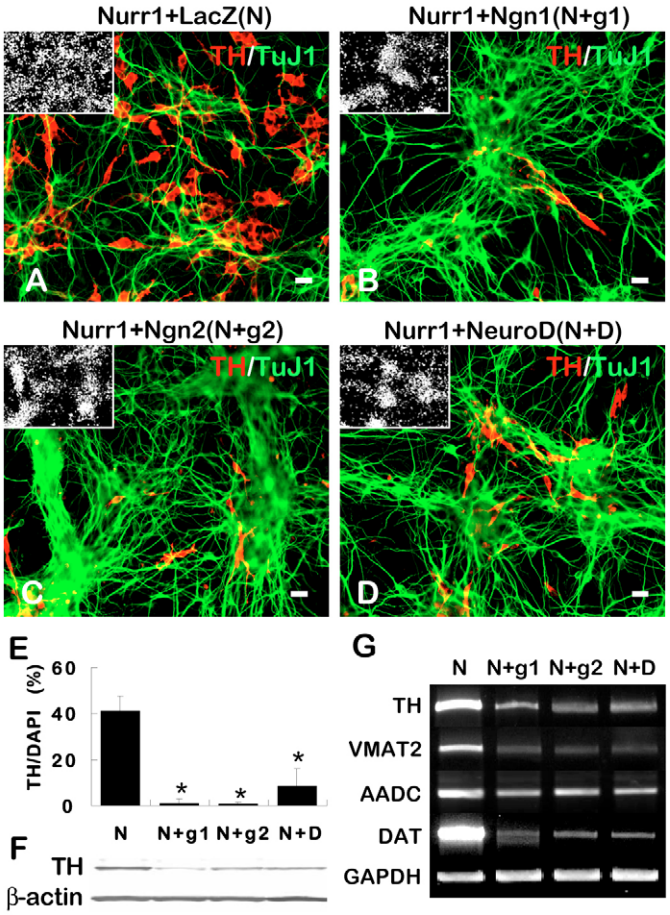

Fig. 4. Inhibitory activity of the atonal-related bHLH proteins on Nurr1-induced expression of DA neuronal phenotype. The cortical precursor cells, at day 3 of in vitro expansion, were transduced with Nurr1 plus Ngn1, Ngn2, NeuroD or LacZ (control) using retroviral infection. After 3 days of differentiation, the cultures were subjected to immunocytochemical (A-E), immunoblot analyses (F) for TH and RT-PCR analyses (G) for TH, AADC (DA synthesis), VMAT2 (DA storage) and DAT (DA uptake). (A-D) Representative images of TH/TuJ1 immunocytochemistry on the cells transduced with LacZ (A), Ngn1 (B), Ngn2 (C), and NeuroD (D) along with Nurr1. Note that TH^+ cell numbers were reduced by the bHLH co-expression, with increases of $TuJ1^+$ neurons. Insets, DAPI nuclear staining of the same fields. The graph in E represents percentage of TH^+ cells out of total ($DAPI^+$) cells. *Significantly different from the control at $P<0.001$. Bars, 10 μm.

Helix-Loop-Helix domains are responsible for the specificity of the bHLH effects in Nurr1-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 Nurr1-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 Nurr1-infected cultures did not alter the proportion of TH^+ cells, compared with the control cultures transduced with Nurr1 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

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

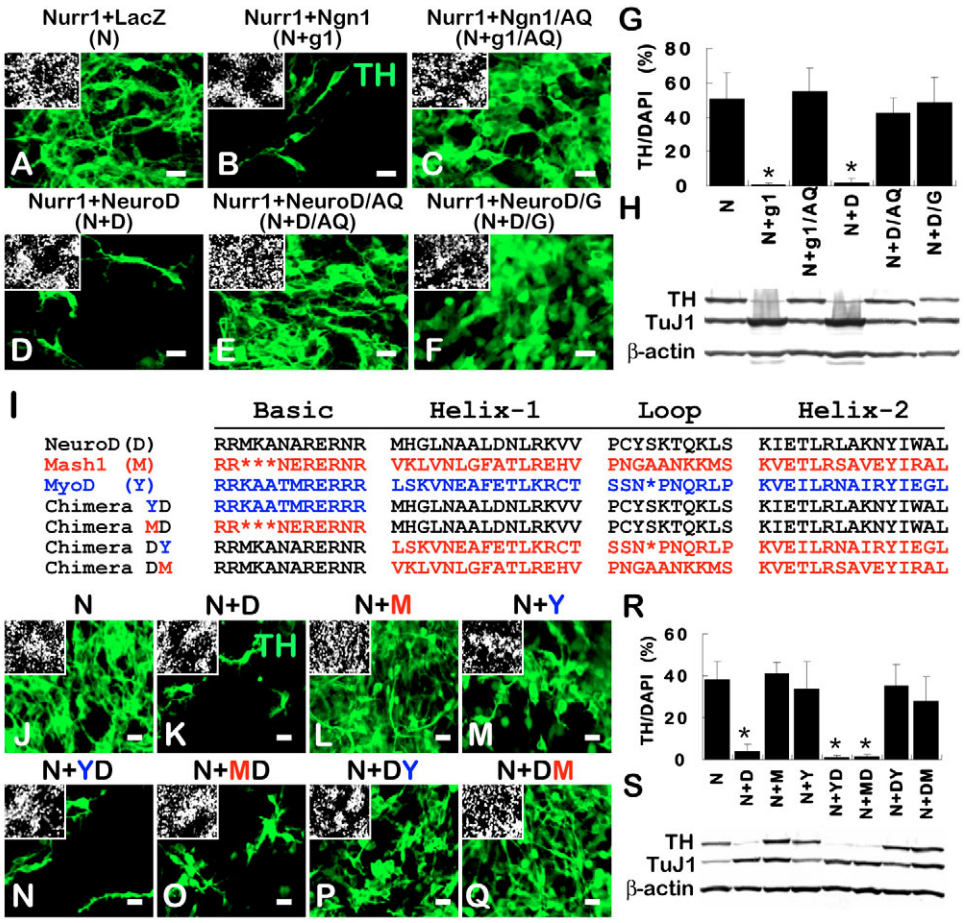

Fig. 5. Essential structural components of Ngn1 and NeuroD required for the inhibitory actions on Nurr1-induced TH expression. (A-H) Effect of the abolishment of DNA-binding capacity of Ngn1 and NeuroD. Each of the wild types and the DNA-binding mutants of Ngn1 and NeuroD was introduced into Nurr1-transduced 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 immunochemical (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 AG 490 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.

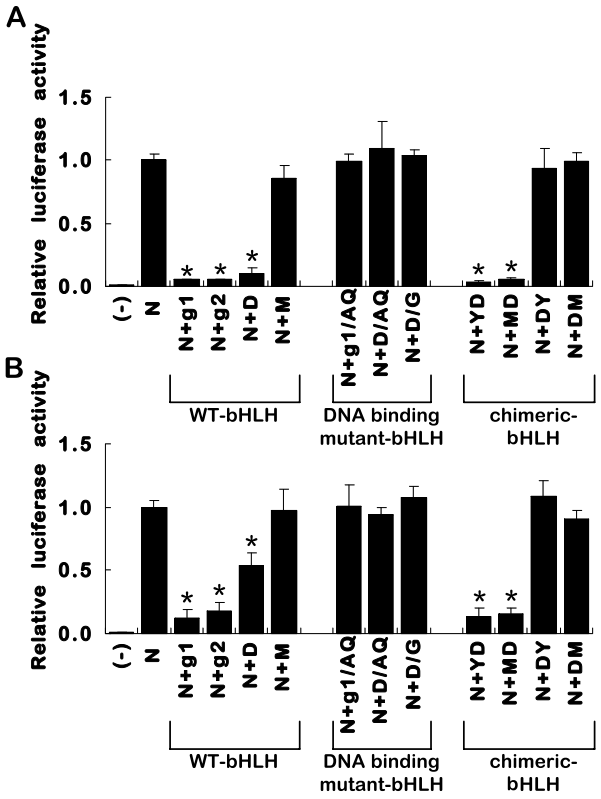

Fig. 6. Atonal-related bHLHs repress Nurr1-induced transactivation of TH promoter activity. Promoter assays using the reporter vectors driven by TH6.0 (A) and NL3 (B) were performed as described in Materials and Methods. The data represent mean percentages (± s.e.m.) of luciferase activities relative to that of the control (sample transfected with pEF1α-Nurr1 plus pEF1α-EGFP). Titles of the treatments including the bHLH mutants and chimeras were abbreviated as shown in Fig. 5. Luciferase activity was determined at day 2 of post-transfection and normalized to the activity of the β-galactosidase. *Significantly different from the control at P<0.001 in the duplicates of three independent experiments.

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 and achaete-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 viscero-motor neurons (Dubreuil et al., 2002; Pattyn et al., 2004) and serotonergic 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 Ngn2 expression seen in the telencephalon and spinal cord is not observed in the embryonic midbrain (Fig. 1H). Rather, Ngn2 expression shows a lag and could not be detected in the ventral midbrain during the early embryonic ages (E11 and E12). By E13, when Ngn2 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 Ngn2 have recently been demonstrated in the generation of dorsal spinal cord interneurons: Mash1 is required for the formation of dorsal interneuron 3 (dI3) and dI5 neurons, whereas Ngn2 acts to limit the number of these neuronal populations (Helms et al., 2005). Similarly, the late appearance and inhibitory action of Ngn2 in the ventral midbrain may imply that the role of Ngn2 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 Nurr1-induced DA neuronal differentiation. Mash1 was expressed in midbrain DA neuronal progenitors and its forced expression caused immature Nurr1-induced DA cells to differentiate into mature and functional DA neurons with presynaptic DA neuronal markers. By contrast, atonal-related bHLHs (Ngn1, Ngn2 and NeuroD) repressed Nurr1-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 Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (CMF-HBSS; Invitrogen, Carlsbad, CA). Cells were plated at 8000 cells/cm^2 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) (Johe 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% CO_2, 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 Nurr1 were constructed by inserting Nurr1 cDNA into the monocistronic retroviral vector pCL or into the bicistronic vector pIRES-LacZ-CL or pIRES-eGFP upstream to IRES element as described previously (pNurr1-IRES-LacZ or pNurr1-IRES-eGFP) (Kim, J. Y. et al., 2003). The rat Mash1, mouse Ngn1, mouse Ngn2 and hamster NeuroD cDNAs were amplified using high fidelity *Taq* polymerase® (Invitrogen) and cloned into pGEM T-Easy® (Promega, Madison, WI). pCL retroviral vectors harboring these genes were generated by inserting *Nde*I and *Sal*I fragments derived from each pGEM T-Easy intermediate. The vectors pNurr1-IRES-Mash1, pNurr1-IRES-Ngn1, pNurr1-IRES-Ngn2 and pNurr1-IRES-NeuroD, designed for co-expressing each of the bHLH genes with Nurr1, were constructed by replacing LacZ of pNurr1-IRES-LacZ with each of the bHLH cDNAs. The two DNA-binding mutants, Ngn1/AQ (Sun 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 acid R of the basic region was substituted with G in a similar fashion to the previously described mutant for Mash1/G (Ellenberger et al., 1994; Ma et al., 1994; Nakada et al., 2004). The basic region of NeuroD was fused to the HLH domains of Mash1 (M) and MyoD (Y), to generate the chimeric proteins DM and DY, respectively. Similarly, the chimeras MD 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 HLH 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 of the bHLH regions used for the chimeric proteins and mutants are shown in Fig. 5I. The retroviral vectors were introduced into the retrovirus packaging cell line 293gpg (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 Nurr1, each of the viruses of the monocistronic pCL constructs [(pCL-Mash1, pCL-Ngn1, pCL-Ngn2, pCL-NeuroD, bHLH mutant constructs, and pCL-LacZ (control)] was mixed with that of pCL-Nurr1 1:1 (v:v) and applied to cells for infection. In certain experiments, viruses of the bicistronic vectors pNurr1-IRES-Mash1, pNurr1-IRES-Ngn1, pNurr1-IRES-Ngn2, pNurr1-IRES-NeuroD and pNurr1-IRES-LacZ (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% paraformaldeyde solution overnight and cryopreserved by sinking 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 at the concentrations given: anti rabbit-aldehyde dehydrogenase 2 (AHD2, a kind gift from G. Duester, Burnham Institute for Medical Research, La Jolla, CA; 1:300), TH (Pel-Freez, Rogers, AR, 1:250), neuron-specific class III β-tubulin (TuJ1, Covance, Richmond, CA, 1:2000), Nurr1 (Chemicon, 1:100), anti-mouse-Mash1 and Ngn2 (a kind gift from D. J. Anderson, Howard Hughes Medical Institute, Pasadena, CA; 1:1000 and 1:5, respectively), TH (Sigma, 1:10,000), Bromodeoxyuridine (BrdU, Accurate Chemical, Westbury, NY, 1:400), microtubule-associated protein 2 (MAP2, Sigma, 1:500) and GFP (Roche, Mannheim, Germany, 1:400). Appropriate fluorescence-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 a 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 $MgCl_2$ 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′-GGCATTGCTCTCAATGACAA-3′ and 5′-AGGG-CCTCTCTCTTGCTCTC-3′, 25 cycles, 60°C, 165 bp); synapsin I (5′-CCACCCC-CACAAGGCCAGCAACA-3′ and 5′-GGTCCCCCGGCAGCAGCAATGATG-3′, 29 cycles, 58°C, 512 bp); synaptophysin (5′-TGGTATCCTACCGCATTC-3′ and 5′-ACTCACCTCATAGCTCC-3′, 26 cycles, 58°C, 379 bp), GAP43 (5′-AGAAA-GCAGCCAAGCTGAGGAGG-3′ and 5′-CAGGAGAGACAGGGTTCAGGTGG-3′, 24 cycles, 58°C, 167 bp), TH (5′-TGTCAGAGGAGCCCGAGGTC 3′ and 5′-CCAAGAGCAGCCCATCAAAG-3′, 35 cycles, 60°C, 412 bp); DAT (5′-GGA-CCAATGTTCTTCAGTGGTGGC-3′ and 5′-GGATCCATGGGAGGTCCATGG-3′, 30 cycles, 58°C, 289 bp); VMAT2 (5′-CTTTGGAGTTGGTTTTGC-3′ and 5′-GCAGTTGTGGTCCATGAG-3′, 33 cycles, 55°C, 301 bp); AADC (5′-CCT-ACTGGCTGCTCGGACTAA-3′ and 5′-GCGTACCAGTGACTCAAACTC-3′, 30 cycles, 58°C, 163 bp). Mash1 (5′-GGCTCAACTTCAGTGGCTTC-3′ and 5′-TGG-AGTAGTTGGGGGAGATG-3′, 28 cycles, 55°C, 291 bp); Ngn1 (5′-CCAGGAC-GAAGAGCAGGAA-3′ and 5′-GGTGAAGTCTTCTGAAGCCGA-3′, 35 cycles, 60°C, 468 bp); Ngn2 (5′-TTCGTCAAATCTGAGACTCTGG-3′ and 5′-TGCG-CGCGACGGGCGACGCT-3′, 35 cycles, 59°C, 273 bp); NeuroD (5′-CTCAG-

TTCTCAGGACGAGGA-3′ and 5′-TAGTTCTTGGCCAAGCGCAG-3′, 30 cycles, 60°C, 368 bp). RT-PCR products were analyzed with agarose gel electrophoresis.

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 PMSF, 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 (1:5,000), 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 (Amersham 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 Axiovert phase-contrast microscope equipped with an Axiocam 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 in 500 μl N2 plus AA medium or in the same medium supplemented with 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 that of Anton and Sayre (Anton and Sayre, 1962). Samples (40 μl) were injected with a Rheodyne injector and separated with a reverse-phase μ-Bondapak C18 column (150×3.0 mm, Eicom, Japan) maintained at 32°C with a column heater (Waters, Cotland, NY). The mobile phase consisted of 0.05 M citric acid, 0.05 M disodium phosphate (pH 3.1), 3.2 mM 1-octanesulfonic acid (sodium salt), 0.3 mM EDTA and 12% methanol which was pumped at a flow rate of 0.5 ml/minute using a Waters solvent delivery system. Electroactive compounds were analyzed at +750 mV using an analytical cell and an amperometric detector (Eicom, Model ECD-300, Japan). DA levels were calculated using external DA standard injected immediately before and after each experiment.

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 [^{3}H]DA in PBS (5 μCi/mmol, Amersham, Buckinghamshire, UK) with or without 10 μM nomifensine (RBI, Natick, MA), a dopamine transporter (DAT) blocker, to determine the level of nonspecific uptake. After incubation for 10 minutes at 37°C, the uptake reactions were terminated by aspiration of the reaction solution and washing twice with ice-cold PBS. Cells were lysed in 0.5 M NaOH and the radioactivity was measured by liquid scintillation counting (MicroBeta® TriLux ver. 4.4, Wallac, Turku, Finland). The specific DA-uptake level was calculated by subtracting nonspecific uptake (with nomifensine) from uptake value without nomifensine.

Electrophysiology

Electrophysiological properties of Nurr1-expressing cells were examined using the whole-cell recording configuration obtained by the conventional 'dialysed' whole-cell patch-clamp technique. Precursor cells isolated from E14 rat cortices were cultured on 12 mm diameter coverslips and infected with the retroviral constructs of pNurr1-IRES-eGFP plus pCL-Mash1 (or pCL-LacZ for a control) as described above. After 10 days of in vitro differentiation, individual Nurr1-expressing cells, identified by GFP expression under a GFP-filtered microscope, were selected for the electrophysiological analysis. Patch electrodes were fabricated from a borosilicate glass capillary (Sutter Instrument, San Rafael, CA) by using a vertical micropipette puller (Narishige, Tokyo, Japan). The patch electrodes were fire-polished on a microforge (Narighige) and had resistances of 2-4 MΩ when filled with the internal solution described below. The cell membrane capacitance and series resistance were compensated (typically >80%) electronically using a patch-clamp amplifier (Axopatch-200A; Axon Instruments, Foster City, CA). Current protocol generation and data acquisition were performed using pClamp 8.2 software on an IBM computer equipped with an analog-to-digital converter (Digidata 1322A; Axon Instruments). Voltage traces were filtered at 5 KHz by using the four-pole Bessel filter in the clamp amplifier and stored on the computer hard drive for subsequent analyses. All experiments were performed at room temperature (21-24°C). For recording of membrane potential in current clamp mode, the patch pipette solution contained 134 μM KCl, 1.2 μM $MgCl_2$, 1 μM MgATP, 0.1 μM Na_2GTP, 10 μM EGTA, 14 μM glucose and 10.5 μM HEPES (pH adjusted to 7.2 with KOH). The bath solution contained 134 μM NaCl, 5 μM KCl, 2.5 μM $CaCl_2$, 1.2 μM $MgCl_2$, 14 μM glucose and 10.5 μM HEPES (pH adjusted to 7.4 with NaOH).

BrdU-incorporation assay

The effect of Ngn1 on cell proliferation was determined using BrdU assay and compared with the effect of the bFGF-signaling blockers SU5402 (20 μM, Calbiochem) and PD98059 (25 μM, Calbiochem). Cells were treated with 10 μM BrdU (Roche, Basel, Switzerland) and then examined by immunocytochemistry with anti-BrdU antibody.

Promoter Assay

The luciferase reporter vectors pTH6.0-GL3 and p(NL3)4-GL3 have been constructed by engineering the 6.0 kb upstream sequences of the rat TH gene and four copies of NL3 sequence respectively into pGL3® (Promega) bacterial luciferase plasmid (Kim, K. S. et al., 2003). PCR-amplified coding fragments of Nurr1, Mash1, Ngn1, Ngn2, NeuroD, the bHLH mutants and the chimeric-bHLHs were cloned into the multiple cloning sites of pEF6/V5-HisA® (Invitrogen) to generate the pEF1α-based expression constructs in which the cloned gene is designed to be expressed under the control of EF1α promoter. Plasmids for transfection were prepared using Qiagen columns (Qiagen, Santa Clarita, CA). The proliferating precursor cells cultured in 24-well plates as described above were transfected using Lipofectamine 2000® (Invitrogen) with a reporter construct (0.05 pmole), pEF1α-βgal (0.05 pmole, to correct the difference of transfection efficiencies), pEF1α-Nurr1 (0.1 pmole) and pEF1α-bHLH (or pEF1α-eGFP as a negative control for the bHLH effect, 0.1 pmole). 48 hours later, transfected cells were examined with a luciferase assay kit (BD Bioscience Pharmingen, Franklin Lakes, NJ) and measured with a luminometer (Orion I microplate luminometer, Berthold detection system, Oak Ridge, TN). To correct for differences in transfection efficiencies among different DNA precipitates, luciferase activity was normalized to that of β-galactosidase activity estimated by ELISA reader (model 550, BioRad).

Cell counting and statistic 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 with Tukey post-hoc analysis (SPSS 11.0; SPSS, Chicago, IL) when more than two groups were involved.

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References

Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995). PD 98059 is a specific inhibitor of the activation of mitogen activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.* **270**, 27489-27494.

Anton, A. H. and Sayre, D. F. (1962). A study of the factors affecting the aluminum oxide-trihydroxyindole procedure for the analysis of catecholamines. *J. Pharmacol. Exp. Ther.* **138**, 360-375.

Berke, J. D. and Hyman, S. E. (2000). Addiction, dopamine, and the molecular mechanisms of memory. *Neuron* **25**, 515-532.

Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517-530.

Castillo, S. O., Baffi, J. S., Palkovits, M., Goldstein, D. S., Kopin, I. J., Witta, J., Magnuson, M. A. and Nikodem, V. M. (1998). Dopamine biosynthesis is selectively abolished in substantia nigra/ventral tegmental area but not in hypothalamic neurons in mice with targeted disruption of the Nurr1 gene. *Mol. Cell. Neurosci.* **11**, 36-46.

Chung, S., Sonntag, K. C., Andersson, T., Bjorklund, L. M., Park, J. J., Kim, D. W., Kang, U. J., Isacson, O. and Kim, K. S. (2002). Genetic engineering of mouse embryonic stem cells by Nurr1 enhances differentiation and maturation into dopaminergic neurons. *Eur. J. Neurosci.* **16**, 1829-1838.

Dubreuil, V., Hirsch, M. R., Jouve, C., Brunet, J. F. and Goridis, C. (2002). The role of Phox2b in synchronizing pan-neuronal and type-specific aspects of neurogenesis. *Development* **129**, 5241-5253.

Ellenberger, T., Fass, D., Arnaud, M. and Harrison, S. (1994). Crystal structure of transcription factor E47: E-box recognition by a basic region helix-loop-helix dimmer. *Genes Dev.* **8**, 970-980.

Fode, C., Ma, Q., Casarosa, S., Ang, S. L., Anderson, D. J. and Guillemot, F. (2000). A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. *Genes Dev.* **14**, 67-80.

Fukada, T., Hibi, M., Yamanaka, Y., Takahashi-Tezuka, M., Fujitani, Y., Yamaguchi,

T., Nakajima, K. and Hirano, T. (1996). Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. *Immunity* **5**, 449-460.
Gazit, A., Osherov, N., Posner, I., Yaish, P., Poradosu, E., Gilon, C. and Levitzki, A. (1991). Tyrphostins. 2. Heterocyclic and alpha-substituted benzylidenemalononitrile tyrphostins as potent inhibitors of EGF receptor and ErbB2/neu tyrosine kinases. *J. Med. Chem.* **34**, 1896-1907.
Goridis, C. and Brunet, J. F. (1999). Transcriptional control of neurotransmitter phenotype. *Curr. Opin. Neurobiol*. **9**, 47-53.
Goridis, C. and Rohrer, H. (2002). Specification of catecholaminergic and serotonergic neurons. *Nat. Rev. Neurosci*. **3**, 531-541.
Graybiel, A. M., Aosaki, T., Flaherty, A. W. and Kimura, M. (1994). The basal ganglia and adaptive motor control. *Science* **265**, 1826-1831.
Hatakeyama, J., Tomita, K., Inoue, T. and Kageyama, R. (2001). Roles of homeobox and bHLH genes in specification of a retinal cell type. *Development* **128**, 1313-1322.
Helms, A. W., Battistte, J., Henke, R. M., Nakada, Y., Simplicio, N., Guillemot, F. and Johnson, J. E. (2005). Sequential roles for Mash1 and Ngn2 in the generation of dorsal spinal cord interneurons. *Development* **132**, 2709-2719.
Hirsch, M. R., Tiveron, M. C., Guillemot, F., Brunet, J. F. and Goridis, C. (1998). Control of noradrenergic differentiation and Phox2a expression by MASH1 in the central and peripheral nervous system. *Development* **125**, 599-608.
Johe, K. K., Hazel, T. G., Muller, T., Dugich-Djordjevic, M. M. and McKay, R. D. (1996). Single factors direct the differentiation of stem cells from the fetal and adult nervous system. *Genes Dev*. **10**, 3129-3140.
Kageyama, R. and Nakanishi, S. (1997). Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. *Curr. Opin. Genet. Dev.* **7**, 659-665.
Kim, J. H., Auerbach, J. M., Rodriguez-Gomez, J. A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S. H., Nguyen, J., Sanchez-Pernaute, R., Bankiewicz, K. et al. (2002). Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* **418**, 50-56.
Kim, J. Y., Koh, H. C., Lee, J. Y., Chang, M. Y., Kim, Y. C., Chung, H. Y., Son, H., Lee, Y. S., Studer, L., McKay, R. et al. (2003). Dopaminergic neuronal differentiation from rat embryonic neural precursors by Nurr1 overexpression. *J. Neurochem*. **85**, 1443-1454.
Kim, K. S., Kim, C. H., Hwang, D. Y., Seo, H., Chung, S., Hong, S. J., Lim, J. K., Anderson, T. and Isacson, O. (2003). Orphan nuclear receptor Nurr1 directly transactivates the promoter activity of the tyrosine hydroxylase gene in a cell-specific manner. *J. Neurochem*. **85**, 622-634.
Lee, J. E. (1997). Basic helix-loop-helix genes in neural development. *Curr. Opin. Neurobiol.* **7**, 13-20.
Lee, S. H., Chang, M. Y., Lee, K. H., Park, B. S., Lee, Y. S., Chin, H. R. and Lee, Y. S. (2000). Importance of valine at position 152 for the substrate transport and 2beta-carbomethoxy-3beta-(4-fluorophenyl)tropane binding of dopamine transporter. *Mol. Pharmacol.* **57**, 883-889.
Lee, S. K. and Pfaff, S. L. (2003). Synchronization of neurogenesis and motor neuron specification by direct coupling of bHLH and homeodomain transcription factors. *Neuron* **38**, 731-745.
Lo, L., Tiveron, M. C. and Anderson, D. J. (1998). MASH1 activates expression of the paired homeodomain transcription factor Phox2a, and couples pan-neuronal and subtype-specific components of autonomic neuronal identity. *Development* **125**, 609-620.
Ma, P., Rould, M., Weintraub, H. and Pabo, C. (1994). Crystal structure of MyoD bHLH domain-DNA complex: perspectives on DNA recognition and implications for transcriptional activation. *Cell* **77**, 451-459.
Marin, F., Herrero, M. T., Vyas, S. and Puelles, L. (2005). Ontogeny of tyrosine hydroxylase mRNA expression in mid- and forebrain: neuromeric pattern and novel positive regions. *Dev. Dyn.* **234**, 709-717.
Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K., Hubbard, S. R. and Schlessinger, J. (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276**, 955-960.
Nakada, Y., Hunsaker, L., Henke, R. M. and Johnson, J. E. (2004). Distinct domains within Mash1 and Math1 are required for fuction in neuronal differentiation versus neuronal cell-type specification. *Development* **131**, 1319-1330.
Nieoullon, A. (2002). Dopamine and the regulation of cognition and attention. *Prog. Neurobiol.* **67**, 53-83.
Ory, D. S., Neugeboren, B. A. and Mulligan, R. C. (1996). A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. *Proc. Natl. Acad. Sci. USA* **93**, 11400-11406.
Parras, C. M., Schuurmans, C., Scardigli, R., Kim, J., Anderson, D. J. and Guillemot, F. (2002). Divergent functions of the proneural genes Mash1 and Ngn2 in the specification of neuronal subtype identity. *Genes Dev*. **16**, 324-338.
Pattyn, A., Morin, X., Cremer, H., Goridis, C. and Brunet, J. F. (1999). The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. *Nature* **399**, 366-370.
Pattyn, A., Goridis, C. and Brunet, J. F. (2000). Specification of central noradrenergic phenotype by the homeobox gene Phox2b. *Mol. Cell. Neurosci*. **15**, 235-243.
Pattyn, A., Simplicio, N., van Doorninck, J. H., Goridis, C., Guillemot, F. and Brunet, J. F. (2004). Ascl1/Mash1 is required for the development of central serotonergic neurons. *Nat. Neurosci*. **7**, 589-595.
Perez, S. E., Rebelo, S. and Anderson, D. J. (1999). Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo. *Development* **126**, 1715-1728.
Rayport, S., Sulzer, D., Shi, W. X., Sawasdikosol, S., Monaco, J., Batson, D. and Rajendran, G. (1992). Identified postnatal mesolimbic dopamine neurons in culture: morphology and electrophysiology. *J. Neurosci*. **12**, 4264-4280.
Rohrbacher, J., Ichinohe, N. and Kitai, S. T. (2000). Electrophysiological characteristics of substantia nigra neurons in organotypic cultures: spontaneous and evoked activities. *Neuroscience* **97**, 703-714.
Ross, S. E., Greenberg, M. E. and Stiles, C. D. (2003). Basic helix-loop-helix factors in cortical development. *Neuron* **39**, 13-25.
Sakurada, K., Ohshima-Sakurada, M., Palmer, T. D. and Gage, F. H. (1999). Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. *Development* **126**, 4017-4026.
Saucedo-Cardenas, O., Quintana-Hau, J. D., Le, W. D., Smidt, M. P., Cox, J. J., De Mayo, F., Burbach, J. P. and Conneely, O. M. (1998). Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. *Proc. Natl. Acad. Sci. USA* **95**, 4013-4018.
Shirasaki, R. and Pfaff, S. L. (2002). Transcriptional codes and the control of neuronal identity. *Annu. Rev. Neurosci*. **25**, 251-281.
Sonntag, K. C., Simantov, R., Kim, K. S. and Isacson, O. (2004). Temporally induced Nurr1 can induce a non-neuronal dopaminergic cell type in embryonic stem cell differentiation. *Eur. J. Neurosci*. **19**, 1141-1152.
Sun, Y., Nadal-Vicens, M., Misono, S., Lin, M. Z., Zubiaga, A., Hua, X., Fan, G. and Greenberg, M. E. (2001). Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. *Cell* **104**, 365-376.
Wagner, J., Akerud, P., Castro, D. S., Holm, P. C., Canals, J. M., Snyder, E. Y., Perlmann, T. and Arenas, E. (1999). Induction of a midbrain dopaminergic phenotype in Nurr1-overexpressing neural stem cells by type 1 astrocytes. *Nat. Biotechnol*. **17**, 653-659.
Wallen, A., Zetterstrom, R. H., Solomin, L., Arvidsson, M., Olson, L. and Perlmann, T. (1999). Fate of mesencephalic AHD2-expressing dopamine progenitor cells in NURR1 mutant mice. *Exp. Cell Res*. **253**, 737-746.
Zetterstrom, R. H., Williams, R., Perlmann, T. and Olson, L. (1996). Cellular expression of the immediate early transcription factors Nurr1 and NGFI-B suggests a gene regulatory role in several brain regions including the nigrostriatal dopamine system. *Mol. Brain Res*. **41**, 111-120.
Zetterstrom, R. H., Solomin, L., Jansson, L., Hoffer, B. J., Olson, L. and Perlmann, T. (1997). Dopamine neuron agenesis in Nurr1-deficient mice. *Science* **276**, 248-250.