Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons

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Proneural genes are crucial regulators of neurogenesis and subtype specification in many areas of the nervous system; however, their function in dopaminergic neuron development is unknown. We report that proneural genes have an intricate pattern of expression in the ventricular zone of the ventral midbrain, where mesencephalic dopaminergic neurons are generated. Neurogenin 2 (Ngn2) and Mash1 are expressed in the ventral midline, while Ngn1, Ngn2 and Mash1 are co-localized more laterally in the ventricular zone. Ngn2 is also expressed in an intermediate zone immediately adjacent to the ventricular zone at the ventral midline. To examine the function of these genes, we analyzed mutant mice in which one or two of these genes were deleted (Ngn1, Ngn2 and Mash1) or substituted (Mash1 in the Ngn2 locus). Our results demonstrate that Ngn2 is required for the differentiation of Sox2+ ventricular zone progenitors into Nurr1+ postmitotic dopaminergic neuron precursors in the intermediate zone, and that it is also likely to be required for their subsequent differentiation into tyrosine hydroxylase-positive dopaminergic neurons in the marginal zone. Although Mash1 normally has no detectable function in dopaminergic neuron development, it could partially rescue the generation of dopaminergic neuron precursors in the absence of Ngn2. These results demonstrate that Ngn2 is uniquely required for the development of midbrain dopaminergic neurons.

KEY WORDS: Proneural genes, Cell fate specification, Differentiation, Sox2, Nurr1, Stem cells, Parkinson’s disease

INTRODUCTION

During the initial phases of nervous system development, progenitor cells in the neural tube proliferate and divide symmetrically to give rise to identical multipotent neuroepithelial cells. These progenitors subsequently divide asymmetrically to generate cells that are fated to differentiate into a neuron, sometimes following additional cycles of cell division. This process is regulated by the activity of transcription factors with basic helix-loop-helix (bHLH) motifs, including the neurogenin and Mash1 proneural factors involved in initiating neurogenesis, and other bHLH factors, such as Neurod, that are involved in terminal neuronal differentiation.

The proneural genes of the bHLH class were first identified in *Drosophila* as key regulators of neural lineage development (Brunet and Ghysen, 1999; Guillaumot, 1999). The three most extensively studied genes in rodents are the mouse *achaete-scute* homologue (*Mash1*) and the members of the *atonal*-related family of genes, neurogenins (*Ngn*) 1 and 2 (*Neurog1 and 2 – Mouse Genome Informatics*). Mash1 and NgnS are sufficient for the initiation of a generic neurogenic program in a variety of progenitor cells, both in vitro (Lo et al., 1998; Farah et al., 2000; Sun et al., 2001) and in vivo (Ma et al., 1996; Blader et al., 1997; Mizugushi et al., 2001). At a mechanistic level, the proneural activity of NgnS involves the promotion of neurogenesis and the concomitant repression of the alternative glial fate (Tomita et al., 2000; Nieto et al., 2001; Sun et al., 2001). Interestingly, proneural bHLH genes also contribute to the specification of diverse neurotransmitter identities/neuronal subtypes (Bertrand et al., 2002). In the mammalian peripheral nervous system, for example, NgnS, but not Mash1, promote a sensory neuron identity (Perez et al., 1999; Lo et al., 2002). In the central nervous system, Mash1 influences neuronal fate decisions in noradrenergic neurons (Hirsch et al., 1998; Lo et al., 1998), ventral and dorsal telencephalic GABAergic neurons (Fode et al., 2000; Casarosa et al., 1999), spinal cord interneurons (Parras et al., 2002; Helms et al., 2005) and serotonergic neurons (Pattyn et al., 2004), whereas NgnS are involved in the differentiation of dorsal telencephalic glutamatergic neurons (Fode et al., 2000; Schuurmans et al., 2004) and the specification of motoneurons in the ventral spinal cord (Mizuguchi et al., 2001; Novitch et al., 2001). These studies indicate that proneural bHLH genes contribute to a unique transcriptional code for generating neuronal diversity, and coordinate generic and cell-type-specific neurogenesis in a region-specific manner.

In recent years, stem cells have raised important expectations and have been considered as attractive candidates in cell replacement therapies for neurodegenerative disorders (Lindvall et al., 2004). The promise of stem cell therapies in diseases such as Parkinson’s disease has renewed the interest in gaining a deeper understanding of the signals and mechanisms that regulate the differentiation of stem/progenitor cells into specific neuronal populations, such as dopaminergic (DA) neurons. It is known that DA neurons in the ventral midbrain (VM) require for their development sonic hedgehog (Shh), as a ventralizing signal (Hynes et al., 1995), and signals derived from the isthmic organizer for anteroposterior specification (Wurst et al., 2001; Rhinn and Brand, 2001). The isthmic organizer is induced at the midbrain-hindbrain border and its position is controlled by two homeodomain transcription factors, Otx2 in the midbrain and Gbx2 in the hindbrain. Organizer-derived signals, such as Fgf8 (Ye...
et al., 1998) and Wnt1 (McMahon and Bradley, 1990; Thomas and Cappechi, 1990), maintain the expression pattern of a number of genes, including engrailed 1 and 2 (Würg et al., 1994; Hanks et al., 1995; Danielian et al., 1996), and Pax2 and Pax5 (Urbaneck et al., 1997), that contribute to the development of most neuronal cell types in the mid- and hindbrain region. DA neurons are first detected in the mantle zone of the mouse VM midline at embryonic day (E) 10.5 and DA neurogenesis continues until E13 (DiPorzio et al., 1990). These cells are generated from proliferating precursor cells in the ventricular zone (VZ) of the VM. Proliferating precursors express Aldhl1 and give rise to postmitotic DA precursors that express Nurr1, an orphan nuclear receptor required for the differentiation of DA precursors into tyrosine hydroxylase (Th) DA neurons (Zetterstrom et al., 1997; Saucedo-Cárdenas et al., 1998; Castillo et al., 1998). Additional genes required for DA neuron development include the LIM homeodomain Lmx1b (Smidt et al., 2000) and the Pitx3 homeodomain genes, essential for DA neuron survival in the substantia nigra pars compacta (SNc) (Nunes et al., 2003; Van den Munchof et al., 2003; Hwang et al., 2003; Smidt et al., 2004). Surprisingly, no study has yet investigated the role of proneural bHLH genes in DA neurogenesis, in the specification of the VM DA neuronal identity or in the differentiation of DA precursors into neurons. We hereby report that Ngn2, but not Ngn1 or Mash1, is required for the generation of DA neurons. Our findings indicate about 86% and 66% of Th+ DA neurons fail to develop in Ngn2 mutants at the end of the neurogenic period (E14.5) and at E17.5, respectively. The partial rescue of Th+ DA neurons between E14.5 and E17.5 requires Mash1 activity, as this rescue does not occur in Ngn2;Mash1 double mutants. Importantly, endogenous Mash1 can partially compensate, after a delay, for the loss of Th+ neurons in Ngn2 mutants. However, even ectopic expression of Mash1 from the Ngn2 promoter could not completely rescue the generation of DA neurons, indicating that Ngn2 has a unique role in DA neurogenesis. Thus, our results indicate that Ngn2 is required for the development of midbrain DA neurons.

MATERIALS AND METHODS

Animals and tissue preparation

Male and female wild-type CD-1 mice (25–35 g, Charles River, Uppsala) were housed, bred and treated according to the guidelines of the European Community (86/609/EEC), the Society for Neuroscience (January 1985), and all experiments were approved by the local ethical committee. Timed-pregnant females were obtained by overnight mating. The day of detection of the vaginal plug was considered as E0.5. Mash1 (Guillemot et al., 1993), Ngn2KIGFP (Seibt et al., 2003), Mash1;Ngn2KIGFP, Mash1;Pitx3/null (Parras et al., 2002), Ngn2KIGMash1 (Parras et al., 2002) and Ngn1;Ngn2 (Fode et al., 2000) were genotyped and processed as previously described. For analysis of BrdU incorporation, 6-hour pulsures were performed with 100 µg BrdU /gram of animal.

In situ hybridization and immunohistochemistry

For in situ hybridization (ISH), embryos were fixed (4% paraformaldehyde in phosphate-buffered saline; PBS at 4°C) for 20 minutes (E10.5), 30 minutes (E11.5), 90 minutes or overnight (E14.5) before being cryopreserved in 20% sucrose, frozen in OCT and coronally sectioned (12–14 µm) onto slides (SuperFrost®Plus). ISH was performed as described (Conlon and Herrmann, 1993). ISH was performed on fresh frozen or fixed tissue with digoxigenin-labelled single-stranded RNA probes at 55°C or at 70°C, followed by incubation with nitroblue tetrazolium (NBT) plus 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (purple) substrates. The following mouse antisense RNA probes were used: Th (Perlmann and Janson, 1995), Lmx1b (Chen et al., 1998), Mash1 (Guillemot and Joyner, 1993), Ngn1 (Fode et al., 1998), Ngn2 (Fode et al., 1998), Dll1 (Bettenhausen et al., 1995) and Hes5 (Akazawa et al., 1992).

RESULTS

Expression domains of proneural bHLH genes in the VM

In order to characterize the temporal and spatial pattern of proneural bHLH gene expression in the VM during the DA neurogenic period, we performed in situ hybridization (ISH) studies of mouse embryos at E11.5 and E13.5, and correlated their expression domains with those of other markers of DA neurons, such as Th (Fig. 1A,F) and Lmx1b (Fig. 1B,G) (Smidt et al., 2000). At E11.5 and E13.5, Ngn1, Ngn2 and Mash1 showed distinct profiles of expression in the VM; however, Ngn1 expression was extinguished by the latter stage (Fig. 1C-E, H-J). The combined expression patterns of these genes revealed the existence of three distinct zones differing in their expression patterns of proneural genes at E11.5, referred to in the rest of the manuscript as zones 1, 2 and 3. Ngn2 and Mash1 were expressed in zone 1 (Fig. 1D,E,R), which also expressed Lmx1b (Fig. 1B). Ngn1, Ngn2 and Mash1 were all expressed in zone 2 (Fig. 1C,E-R), while Mash1 was the only proneural gene expressed in zone 3 (Fig. 1E,R). The dorsal boundary of zone 3 corresponds to the alar-basal boundary. As both Ngn2 and Mash1 mRNAs were detected in the VZ of zone 1, we examined whether both proteins were expressed in the same or distinct precursor pools. Double immunohistochemistry at E11.5 showed that Mash1 and Ngn2 proteins had a similar distribution to the corresponding transcripts. In particular, Mash1 was expressed for immunohistochemistry, coronal sections (12-14 µm thick) were pre-incubated for 1 hour in blocking solution [PBS, 0.1% bovine serum albumin (BSA), 0.1-0.3% Triton-X 100 and 5-10% normal serum] followed by incubation at 4°C overnight with one or more of the following primary antibodies diluted in blocking solution: mouse monoclonal anti-Mash1 (1:1, gift from D. J. Anderson); mouse anti-Ngn2 (1:20, gift from D. J. Anderson), rabbit anti-GFP (1:1000, Molecular Probes), rat anti-bromodeoxyuridine (BrdU; 1:20, Immunological Direct), rat anti-BrdU (1:150, Abcam), rabbit anti-Cleaved Caspase-3 (1:100, Cell Signaling), guinea pig anti-glutamate transporter GLAST (1:200-1:2000, Chemicon), mouse anti-MAP2 (1:750, Sigma), rat anti-Ki67 (1:80, Dako), rabbit anti-Nurr1 (1:100, gift from T. Perlmann, Karolinska Institute, Stockholm), rabbit anti-Pitx3 (1:200, gift from P. Burbach, Rudolf Magnus Institute of Neuroscience, Utrecht); mouse anti-RC2, (1:200, Developmental Studies Hybridoma Bank); rabbit anti-Sox2 (1:500, Chemicon; 1:25, R&D Systems; 1:3000, gift from T. Edlund, Umea University, Umea); rabbit anti-Th (1:250, PelFreeze), sheep and rabbit anti-Th (1:1000, Chemicon), mouse anti-βIII-tubulin (1:1000, Sigma) followed by nuclear staining with Toto-3 iodide (1:1000, Molecular Probes). Pre-treatment with 2N HCl for 15 minutes prior to pre-incubation with primary antibody was needed for the detection of BrdU. After washing, slides were incubated for 1-2 hours at room temperature with the appropriate secondary antibodies: biotinylated (1:100, Jackson Laboratories), fluorophore conjugated (Cy2-, Cy3- and Cy5-, 1:300, Jackson Laboratories), or secondary antibodies conjugated with a fluorochrome (Molecular Probes). Hoechst nuclear stain (5 mg/ml, 1:5000, Sigma) was performed for visualization of all cells. Biotinylated secondary antibodies were visualized with the Vector Laboratories ABC immunoperoxidase kit, using 3-3′ diaminobenzidine tetrahydrochloride (DAB 0.5 mg/ml/nickel chloride (1.6 mg/ml) substrate. Where appropriate, endogenous peroxidase activity was quenched for 20 minutes with 5% H2O2 prior to pre-incubation with secondary antibody. Sections were washed and mounted using glycerol or Aquapolymount mounting media (Poly-Labo). Cresyl violet staining solution was 0.25%. Quantitative immunocytochemical data represent mean ± standard deviation for cell counts in consecutive sections through the entire substantia nigra, every 70 µm, in three to eight animals per condition. Photos were acquired with a Zeiss Axioplan 100M microscope and collected with a Hamamatsu camera C4742-95 (with the Openlab™ 3.1.7 imaging software). Confocal pictures were taken with a LSM510 Zeiss microscope.

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at high levels in zone 3, and both Mash1 and Ngn2 proteins were expressed in zones 1 and 2, where they were co-expressed in some VZ cells (Fig. 1K-M).

The HMG-box transcription factor Sox2 is expressed by most progenitors of the developing CNS, and is generally downregulated by neural cells as they exit cell cycle and differentiate (Graham et al., 2003). Co-expression of Sox2 and Ki67, a marker of proliferating cells, indicates that all Sox2+ VZ progenitors correspond to proliferating progenitors in the VM at E11.5 (Fig. 1H). Ngn1 mRNA was expressed only in zone 2 (C), Ngn2 was expressed in zones 1 and 2 (D), and Mash1 was expressed in zones 1, 2 and 3 at different levels (E). At E11.5, Mash1 and Ngn2 protein were expressed in a ‘salt-and-pepper’ pattern in the VZ with some double-labelled cells (K-M). Mash1 (N) and Ngn2 (O) are co-expressed with some Sox2+ progenitors. In addition, Ngn2 is also expressed in Sox2- cells (O). Nurr1 and Ngn2 proteins are co-expressed in some cells in the IZ of Ngn2<sup>KIGFP/+</sup> embryos at E11.5 (P,Q). A summary of all the expression data at E11.5 is shown in R. Scale bar: 100 μm.

Fig. 1. Expression of proneural bHLH genes in the developing ventral midbrain in relation to tyrosine hydroxylase (Th), Lmx1b and Nurr1 expression. (A-Q) Coronal sections of the ventral midbrain. ISH at E11.5 and E13.5 revealed Th mRNA in DA neurons (A,F) and Lmx1b mRNA expression in both DA progenitors and neurons in zone 1 of the VM (B,G). The expression of the proneural bHLH genes was restricted to the ventricular zone, with the exception of Ngn2, which is also seen in the IZ (P,Q). Ngn1 mRNA was expressed only in zone 2 (C), Ngn2 was expressed in zones 1 and 2 (D), and Mash1 was expressed in zones 1, 2 and 3 at different levels (E). At E11.5, Mash1 and Ngn2 protein were expressed in a ‘salt-and-pepper’ pattern in the VZ with some double-labelled cells (K-M). Mash1 (N) and Ngn2 (O) are co-expressed with some Sox2+ progenitors. In addition, Ngn2 is also expressed in Sox2- cells (O). Nurr1 and Ngn2 proteins are co-expressed in some cells in the IZ of Ngn2<sup>KIGFP/+</sup> embryos at E11.5 (P,Q). A summary of all the expression data at E11.5 is shown in R. Scale bar: 100 μm.
precursors, but not in differentiated Th+ DA neurons (Fig. 1R), suggesting possible functions for these genes in VM DA neurogenesis.

**Ngn2, but not Ngn1 or Mash1, is required for the development of DA neurons**

To address the role of proneural bHLH genes in development of the VM, we examined whether any of the VM cell populations were affected in mice mutant for these genes. Given that Th+ cells are found in the MZ of the ventral midline domain, and that the adjacent VZ expressed both Mash1 and Ngn2, we first examined whether these genes were required for DA neurogenesis.

Analysis of Ngn2 null mutant mice revealed a near complete loss of DA neurons at E11.5, as assessed by the expression of Th (Fig. 2A-C) and Pitx3 (Fig. 2D-E) (Van der Munckhof et al., 2003). Examination of the expression of the pan-neuronal marker βIII tubulin, together with the nuclear marker Toto, revealed the absence of Tuj1+/Toto+ cells normally positioned in the MZ (Fig. 2F). This domain was acellular and only Tuj1+/Toto- fibers also present in wild-type embryos were found in mutant embryos. By contrast, Tuj1+/Toto+ neurons were observed in the Ngn2 null embryos at E14.5, but were reduced in number compared with in wild-type embryos (Fig. 2G). Similarly, the number of microtubule-associated protein 2 (MAP2+) (Mtap2+)/Hoechst+
cells decreased in the Ngn2 null embryos at E14.5 (Fig. 2H), indicating that only a few neurons are generated in the absence of Ngn2. Accordingly, a very substantial loss of DA neurons was detected, with only 17% of the Th+ neurons normally present in the developing VM remaining (Fig. 2I-K). These residual Th+ cells were found in a lateral position, suggesting that the loss of Ngn2 primarily affected midline, but, to a lesser extent, also lateral DA neurons. Previous studies have proposed that prospective substantia nigra (SN) DA neurons could be born in a more lateral position than ventral tegmental area DA neurons (Hanaway et al., 1971; Smidt et al., 2004). We thus examined whether the expression of Pitx3 was differentially affected. The expression pattern of Pitx3 at E14.5 (Fig. 2L,M) revealed no difference from Th staining, suggesting that the loss of DA neurons in the Ngn2 null mice move did not affect the Pitx3-expressing DA neurons to a greater extent.

As 17% of VM DA neurons are still present at E14.5 in the absence of Ngn2, we next examined whether Ngn1 or Mash1, which are also expressed in the VZ of the VM, could be responsible for the birth of a subset of DA neurons in the absence of Ngn2. Analysis of Ngn1;Ngn2 double mutant mice showed no additional loss of Th+ DA neurons (10%) when compared with Ngn2 single mutants (11%, Fig. 3A-C) at E14.5. Consistently, Ngn1 single mutants showed no change in the number of Th+ neurons (data not shown) at E14.5. Similarly, analysis of Mash1;Ngn2 double mutant embryos showed a complete loss of DA neurons (Fig. 3D-F), identical to that observed in Ngn2 single mutants at E11.5. Moreover, analysis of Th expression in Mash1 mice revealed no abnormality in the staining pattern or the number of Th-positive cells during DA neurogenesis at E11.5 (Fig. 3G-I) or E14.5 (Fig. 3J-L), indicating that Mash1 is dispensable for VM DA neurogenesis. Thus, our results demonstrate that Ngn2, but not Ngn1 or Mash1, is required for DA neurogenesis.

**Ngn2 promotes the generation of DA precursors and their differentiation into DA neurons**

We next examined whether the loss of Th+ cells in the Ngn2 null mice at E11.5 was due to a failure in precursor survival, proliferation, fate specification or differentiation. We first assessed the possibility that VM precursors would die in the absence of Ngn2. The number of pyknotic nuclei and of active caspase 3 immunoreactive cells did not differ between wild-type and Ngn2 mutant mice, when the loss of Th+ cells was first detected at E11.5 (data not shown). We also examined whether the reduction in the number of Th+ cells was due to a defect in the proliferation of VM precursors. The number of proliferating cells that incorporated BrdU during S phase in zones 1 and 2 of the VZ did not differ between wild-type (1378±218, n=5) and Ngn2 null mice (1130.75±128, n=5) at E11.5 (Fig. 4A,B). In agreement with this, Sox2 and Ki67 double labeling also showed a similar expression in the VZ of the Ngn2 mutant when compared with wild-type embryos at this stage (Fig. 4C,D). Thus cell death or defects in cell proliferation are not responsible for the reduction in the number of Th+ cells in Ngn2 mutants at E11.5.

We therefore examined whether the specification or differentiation of VZ and postmitotic IZ precursors was affected in these mutants. Expression of different neuronal markers, including GABA, GAD65/67, serotonin, islet 1 and Brn3a did not show any

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**Fig. 3. Mash1 and Ngn1 do not contribute significantly to the loss of Th+ DA neurons in Ngn2 mutant mice.** Mice double mutant for Ngn2 and Ngn1 do not show any further loss of Th+ cells at E14.5, compared with Ngn2 single mutants (A-C), indicating that only Ngn2 is required for the development of midbrain DA neurons. A severe reduction in the number of Th+ cells is detected at E11.5 in Mash1;Ngn2 double mutant mice (D-F), but the defect is not more severe than in Ngn2 single mutant mice (see Fig. 2). Consistently, no alteration in the number or distribution of Th+ cells is detected in Mash1 single mutant mice at E11.5 (G-I) or E14.5 (J-L). *P<0.01, as assessed by Student’s t-test with Welch’s correction (D) or one-way ANOVA, with Bonferroni’s correction for multiple comparisons (G,J). n=3-6. Scale bar: 100 μm.
This result is in agreement with the loss of TOTO+ cells underneath precursors and neurons in the medial region of the VM (Fig. 4I).

Ngn2KI 14% of normal levels (Fig. 4E-G), and these cells were located in a transiently follow Ngn2+ cells in the VM of Ngn2KIGFP embryos (Seibt et al., 2003) and GFP therefore mimicked the expression domain of Ngn2 in the midbrain (data not shown). We made use of the stability of the GFP protein to transiently follow Ngn2+ cells in the VM of Ngn2KIGFP embryos. Double immunohistochemistry with GFP and Nurr1 revealed that VM progenitors give rise to GFP+Nurr1+ cells that span the ventral midline in wild-type embryos at E11.5. (Fig. 4H). By contrast, only lateral GFP+Nurr1+ cells are observed in Ngn2 mutants at E11.5, indicating a failure to generate DA precursors and neurons in the medial region of the VM (Fig. 4I). This result is in agreement with the loss of TOTO+ cells underneath the ventral midline in Ngn2 mutants (Fig. 2F). Taken together, these data indicate that more medially located zone 1 VM progenitors fail to generate postmitotic Nurr1+ DA precursors and neurons at E11.5.

By E13.5, Hes5 and Dll1 expression in zone 1 VM recovered, suggesting a partial rescue of neurogenesis (Fig. 5A-D). Accordingly, Toto+/Tuj1+ cells were seen in mutant embryos albeit significantly reduced when compared with wild-type embryos at E14.5 (Fig. 2G). Despite this delayed recovery, a ventral expansion of the medial VZ was visualized in cresyl violet-stained histological sections of the Ngn2 mutant compared with wild-type embryos at E14.5 (Fig. 5E,F). In order to further characterize the cells that were accumulating in the Ngn2 mutant embryos, we performed double immunohistochemistry for radial glial progenitor markers, GLAST (Slc1a3 – Mouse Genome Informatics) and RC2 (Ifaprc2 – Mouse Genome Informatics) (Fig. 5G,H), and also for Sox2 and GLAST (Slc1a3) and RC2 (Ifaprc2) immunohistochemistry for radial glial progenitor markers, GLAST (Slc1a3) and RC2 (Ifaprc2) (Fig. 5I,J). A dramatic increase in the number of GLAST+/RC2+ and Sox2+/GLAST+ cells in Ngn2 mutant compared with wild-type embryos at E14.5 indicated an expansion of radial glial VZ progenitors in the mutant zone 1. The partial rescue of neurogenesis also leads to an increase in the number of Nurr1+ cells (38%) in Ngn2 mutants at E14.5 (Fig. 5K-M).

Despite this overall increase in the fraction of Nurr1+ cells occurring in mutant mice between E11.5 and E14.5, the number of Th+ cells did not increase to the same extent, and there were twice as many Nurr1+ cells than Th+ cells in Ngn2 mutants at E14.5 (Fig. 5K and Fig. 2I, respectively). Thus, our results indicate that the loss of Ngn2 not only affects the early differentiation step of Sox2+ precursors into Nurr1+ postmitotic cells, but also the later differentiation of Nurr1+/Th– cells into Nurr1+/Th+ DA neurons. This difference in numbers of Nurr1+ cells and Th+ cells at E14.5 may reflect a delayed and partial rescue of Nurr1+ DA precursors that have not yet differentiated into Th+ DA neurons.

**Mash1 partially compensates for the loss of Ngn2**

We next examined whether the defects in the generation of DA neurons were solely attributable to the loss of Ngn2 or also involved misregulation of other proneural genes. We found expression of Mash1 mRNA in zone 2 in both wild-type and Ngn2+– mice at E11.5 (Fig. 6A). However, Mash1 mRNA was clearly reduced in zone 1 in Ngn2 mutant mice at E11.5, whereas it had partially recovered by E13.5 (Fig. 6A). Thus, our results suggested that: (1) the loss of Nurr1+ and Th+ cells observed at E11.5 could be contributed by a reduction of Mash1 in VZ precursors; and (2) the partial rescue in Nurr1+ cells observed at E14.5 could be mediated by Mash1. In order to examine these possibilities, we enhanced or decreased Mash1 levels in Ngn2 mutant mice by substituting Ngn2 expression with that of Mash1 in knock-in mice (Ngn2KIMash1/KIMash1) (Fode et al., 2000), or by deleting both genes in double mutant mice
We then analyzed the differentiation of Sox2 VZ precursors into Nurr1+ postmitotic precursors and into Th+ DA neurons. The number of Th+ cells in Ngn2-/- mice at E11.5 was similar to that found in Ngn2+/+ mice (5% of wild-type numbers, Fig. 6B), but the number of Nurr1+ cells was 56% (data not shown) of that observed in wild-type embryos, instead of 9% (data not shown) in Ngn2-/- mice. By the end of the neurogenic period (E14.5), the number of Nurr1+ cells had reached 38% in Ngn2 mutant embryos (Fig. 6D), and this was slightly improved in Ngn2;Mash1/KIMash1 mice (52% Nurr1+ cells compared with wild-type littermates, Fig. 6D,J). However, the number of Th+ cells had increased by then, from 11% in Ngn2 null mutants, to 43% and 63% in Ngn2;Mash1/KIMash1 mutants (Fig. 6C,E,G) at E14.5 and E17.5, respectively (Fig. 7A-D).

The progressive rescue of Nurr1+ cells in Ngn2 null mutants from E11.5 to E14.5 is matched by a parallel increase of Mash1 expression in zone 1 of these mutants between these two stages (Fig. 6A). This correlation suggested that upregulation of Mash1 in Ngn2 null mutants between E11.5 and E14.5 may account for the delayed increase in Nurr1+ cells in these mutants at E14.5. This hypothesis was supported by the finding that Ngn2;Mash1 double mutant embryos showed a more dramatic reduction in the number of Nurr1+ cells at E14.5 (7% of wild-type levels, Fig. 6D,I), than did Ngn2 single mutants (38%, Fig. 6D). In agreement with this, the rescue of Th+ neurons in Ngn2 single mutants between E11.5 (11%) and E17.5 (34%) also did not occur in Ngn2;Mash1 double mutants. as only 5% and 9% of Th+ DA neurons are present in these mutants at E14.5 (Fig. 6D) and E17.5 (Fig. 7A-D), respectively.

Altogether, these findings demonstrate that Mash1 can partially compensate for the loss of Ngn2 in the generation of Nurr1+ precursors and Th+ DA neurons. Mash1 endogenous expression is dependent on Ngn2 function at E11.5, and the loss of both Ngn2 and Mash1 results in an almost complete loss of Nurr1+ and Th+ cells at this stage. When endogenous Mash1 expression increases in a Ngn2-independent manner, it results in a partial rescue of Nurr1+ cells at E14.5 and of Th+ neurons at E17.5. Moreover, when Mash1 is exogenously provided by expression from the Ngn2 locus as early as E11.5, it results in a partial rescue of Nurr1+ cells at E11.5, and a delayed and partial rescue of Th+ DA neurons at E14.5 and E17.5, which leaves a permanent deficit in Th+ cells (Andersson et al., 2006).

**DISCUSSION**

Our study focused on the function of proneural bHLH genes in DA neurogenesis in the VM. Expression data showed that the VZ of the midbrain can be divided into three distinct zones based on proneural gene expression. Only Ngn2 and Mash1 were expressed by Sox2+ progenitors in zone 1, making them likely candidates for regulating neurogenesis in this domain. Moreover, Ngn2 was also expressed by Nurr1+ precursors in the IZ, suggesting a possible function in the DA lineage. Our results from the analysis of mutant
mice containing single or double mutations in these genes demonstrate that, although Mash1 is dispensable, Ngn2 is absolutely required for the differentiation of DA neurons. Hence Ngn2 represents a novel gene required by VM progenitors for DA neuron development.

**Ngn2 is required for the differentiation of Sox2+ ventricular zone progenitors into Nurr1+ IZ precursors**

Our immunohistochemical studies and data from the literature indicate that zone 1 VM progenitors (Ngn2+/Sox2+/GLAST+/Ki67+) generate postmitotic DA precursors (Ngn2+, Nurr1+, En1+) in the IZ, which subsequently differentiate further into postmitotic DA neurons (Nurr1+/En1+/Pitx3+/Th+/Tuj1+/MAP2+, see Fig. 7E) in the MZ. Ngn2 is required for the generation of postmitotic DA precursors, as zone 1 progenitors accumulate as Sox2+/Ki67+/GLAST+/GFAP+ cells in the VZ, indicating that these progenitors fail to exit the cell cycle and migrate into the IZ in Ngn2 mutants at E11.5. This requirement is partially rescued by endogenous Mash1 or by Mash1 expressed under the control of the Ngn2 promoter, indicating that part of Ngn2 function in the DA neuron lineage is not unique to this gene and can be partly compensated by Mash1. In agreement with this finding, Mash1 also partially rescued the generation of postmitotic cells in the IZ of Ngn2 mutant embryos at E14.5. In addition, the recovery of Hex5 and Dll1 expression, which is likely to be due to the recovery of Mash1 expression in zone 1 of Ngn2 mutant embryos at E13.5, also support a role for Ngn2 in a general program of neurogenesis underlying the production of DA precursors and DA neurons. Altogether, these results demonstrate that Ngn2 is required by some VZ progenitors to generate postmitotic precursors in the IZ. We therefore examined whether Ngn2 is sufficient to ectopically induce DA precursors or neurons. Preliminary studies indicate that when Ngn2 is ectopically expressed in the dorsal midbrain of mouse embryos at E10.5 by electroporation, enhanced neurogenesis measured by the number of βIII tubulin+ cells is observed two days later, but these cells do not express DA neuron markers such as Pitx3 and Nurr1 (W. Lin and S.-L.A., unpublished). Altogether, our findings indicate that Ngn2 is required, but not sufficient, for the generation of the majority of DA precursors.

![Fig. 6. Partial rescue of the loss of Nurr1+ and Th+ cells in Ngn2 mutant mice by endogenous or overexpressed Mash1.](image)
**Ngn2 may be required for the differentiation of Nurr1^+ postmitotic precursors into Th^+ DA neurons**

Because Ngn2 is also expressed in Nurr1^+ postmitotic precursors, this raises the possibility that Ngn2 also has a later role in these precursors for their differentiation. Inactivation of Ngn2, but not of Mash1, reduced the number of Th^+ cells down to 11-17% of wild-type levels at E11.5. In addition, at the end of the normal neurogenic period (E14.5), more Nurr1^+ precursors had been produced (38-43% of control, Fig. 5K and Fig. 6D), but the number of Th^+ cells did not increase to the same extent (11-17% of control, Fig. 2I, Fig. 6C) in Ngn2 mutants. Normal onset of expression of Th in postmitotic Nurr1^+ precursors occurs within half a day. Hence, the delay in expression of Th in Ngn2 nulls suggests an additional role of Ngn2 in regulating this differentiation step. Consistent with the idea of a delay in acquisition of Th expression, a further proportionate increase in Th^+ cells was observed in Ngn2 mutants at E17.5, following the increase of Nurr1^+ cells at E14.5. Alternatively, the delay might be due to a downregulation of additional factors that might be required by Nurr1 precursors for differentiation into DA neurons by E14.5. Wnts are one such candidate of an additional factor, as their expression is developmentally regulated during VM neurogenesis and Wnt1 or Wnt5a promote the differentiation of Nurr1^+ precursors into Th^+ neurons (Castelo-Branco et al., 2003). This possibility is further suggested by the finding that β-catenin binds directly to the promoter and activates the expression of proneural bHLH genes in neural progenitors (Israsena et al., 2004).

**Mash1 and Ngn2 double mutants showed a similar reduction in the number of Th^+ neurons, when compared with Ngn2 single mutants, indicating that Mash1 was not required for the differentiation of Nurr1^+ precursors into Th^+ cells. This finding is consistent with the lack of expression of Mash1 in postmitotic precursors. However, in Ngn2^+/Mash1/KIMash1 embryos, expression of Mash1 in postmitotic DA precursors partially accelerated their differentiation into Th^+ neurons at E14.5 and E17.5, suggesting Mash1 is also able to substitute for this later role of Ngn2 when ectopically expressed in postmitotic precursors.**

**Is there a function of Mash1 in DA neuron development?**

Mash1 substitution into the Ngn2 locus was previously reported to misdirect cortical progenitors to become GABAergic instead of glutamatergic (Fode et al., 2000). Unlike Ngn2, Mash1 has been attributed an instructive role in other systems, because it can re-specify neuronal lineages when expressed in Ngn2^−/− precursors in the cortex and hindbrain (Parras et al., 2002; Bertrand et al., 2002). Mash1 has also been implicated in the specification of GABAergic neurons in the dorsal midbrain (Miyoshi et al., 2004). Our findings, however, indicate that Mash1 is not sufficient to re-specify VM progenitors in Ngn2KIMash1/KIMash1 embryos into GABA or serotonin neurons, two phenotypes known to be specified by Mash1 (Fode et al., 2000; Pattyn et al., 2004). Thus, our results indicate that the function of Mash1 in normal VM development is more permissive than instructive, unlike in other neural systems (Parras et al., 2002; Bertrand et al., 2002).

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**Fig. 7. Further but incomplete rescue of Th expression at E17.5, and a schematic model of the expression and function of proneural bHLH in the development of midbrain DA neurons.**

(A-D) The number of Th^+ neurons is further increased in Ngn2 and Ngn2^+/Mash1/KIMash1 mutants, but remains low at 9% in Ngn2;Mash1 double mutants. (E) We show that Ngn2 is expressed in VZ cells that express Sox2 and radial glia markers. Ngn2 is required for the differentiation of Sox2^+ ventricular zone cells into Nurr1^+ DA precursors, as deletion of Ngn2 results in a decrease in the number of Nurr1^+ cells (step 1). This function is partially rescued by endogenous Mash1 recovered at E13.5 or by Mash1 expressed under the control of the Ngn2 promoter. Ngn2 is expressed by Nurr1^+ cells in the IZ and a second possible function of Ngn2 is to promote the differentiation of Nurr1^+ precursors into DA neurons (step 2). This step could be partially rescued by a substitution of Ngn2 by Mash1 (Ngn2^+/Mash1/KIMash1). Thus, our results suggest that Mash1 can also partially rescue the number of DA neurons. Scale bar: 100 μm.
Alternative neurogenic mechanisms

The timing of activation of Ngn2, Ngn1 and Mash1 expression precedes, or occurs at the same time as, the expression of postmitotic neuronal markers (data not shown), consistent with the idea that these represent key regulators of neurogenesis in this region. Interestingly, even the most medial region expresses Mash1 and Ngn2, unlike floor-plate cells in other regions of the CNS. This finding raises the strong possibility that ventral midline progenitors in the VM also undergo neurogenesis, a hypothesis that should be tested by further lineage and fate-mapping experiments.

It should also be noted that DA neuronal generation was not completely blocked by the deletion of Ngn2, as about 10-15% of TH+ cells were observed in the Ngn2 single, Ngn2;Ngn1 double and Ngn2;Mash1 double mutants at E14.5. Thus additional factor(s) are likely to be involved in the specification of DA neurons in the absence of Ngn2. Alternative and complementary neurogenic pathways have recently been described for cortical neuron specification (Schuurmans et al., 2004). First, a Ngn1/2-dependent process, and, second, a Ngn-independent but Pax6- and Tlx-dependent process, result in the generation of early-born and late-born glutamatergic cortical neurons, respectively. Preliminary results indicate that Pax6 is upregulated in zone 1 of the Ngn2 null mice (data not shown). Thus, it remains to be determined whether Pax6 could contribute to DA neurogenesis by a mechanism similar to that previously described in the cerebral cortex.

Concluding remarks

Our findings show that Ngn2 is a key regulator of midbrain DA neuron development and suggest that overexpression of Ngn2 in DA progenitors or stem cells, together with other transcription factors, may contribute to enhancing the DA differentiation of stem/precursor cells. Such strategies could contribute to the future development of transplantation- or endogenous neurogenesis-based cell replacement strategies for the treatment of neurodegenerative diseases such as Parkinson’s disease.

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References


