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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; Guillemot, 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 and Bally-Cuif, 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

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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 (Würst et al., 1994; Hanks et al., 1995; Danielian et al., 1996), and *Pax2* and *Pax5* (Urbanek 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 Aldh1 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 Munckhof 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 to 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), $Ngn2^{KIGFP}$ (Seibt et al., 2003), $Mash1;Ngn2^{KIGFP}$, $Mash1^{KINgn2}$ (Parras et al., 2002), $Ngn2^{KIMash1}$ (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 pulses 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-Ychloro-3-indolyl phosphate (BCIP) (purple) substrates. The following mouse antisense RNA probes were used: *Th* (Perlmann and Jansson, 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).

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 (1:400, 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% H_2O_2 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.

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

DEVELOPMENT

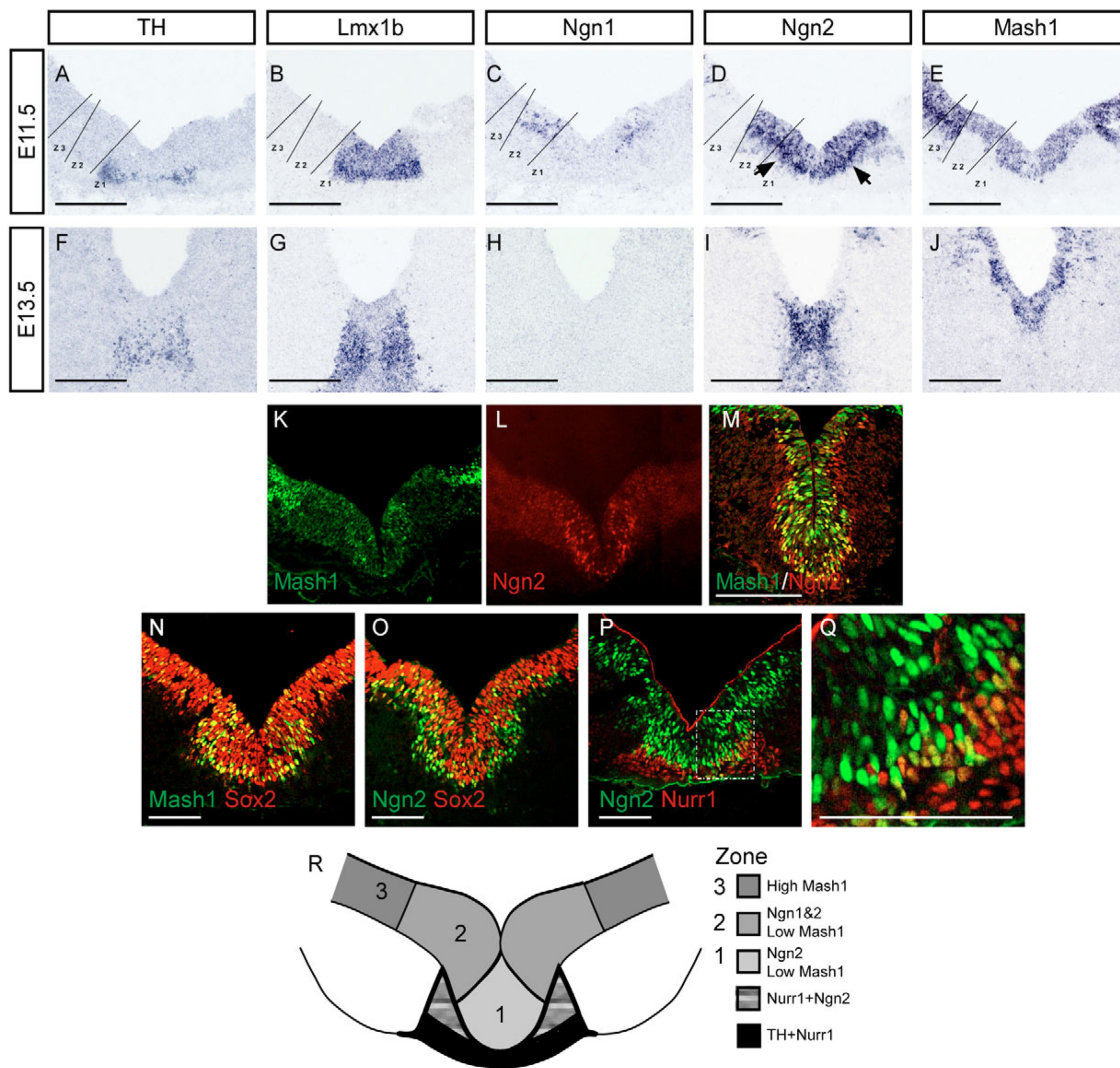

Fig. 1. Expression of proneural bHLH genes in the developing ventral midbrain in relation to tyrosine hydroxylase (*Th*), *Lmx1b* and *Nurr1* expression. (**A**-**Q**) Coronal sections of the ventral midbrain. ISH at E11.5 and E13.5 revealed *Th* mRNA in DA neurons (A,F) and *Lmx1b* mRNA expression in 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 seen also 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$^{KIGFP/+}$ embryos at E11.5 (P,Q). A summary of all the expression data at E11.5 is shown in **R**. Scale bar: 100 μm.

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. 4C). To determine whether Mash1 and Ngn2 are expressed in proliferating progenitors, we therefore colocalised the expression of these proteins with Sox2 in the VM in wild-type embryos at E11.5 by double immunohistochemistry. All Mash1$^+$ cells expressed Sox2, indicating that Mash1 is only expressed in proliferating VZ progenitors (Fig. 1N). By contrast, Ngn2 was co-expressed with Sox2$^+$ proliferating progenitors, as well as in Sox2$^-$ postmitotic cells outside the VZ (Fig. 1O). Consistent with this result, Ngn2 was also co-expressed with some postmitotic DA precursors in the intermediate zone (IZ) of the ventral midbrain (Fig. 1P,Q) that express the orphan nuclear receptor Nurr1 (Wallen et al., 1999). The IZ is defined here as the zone between the Sox2$^+$ VZ and the marginal zone (MZ) containing Th$^+$ neurons. By contrast, Ngn2 was not expressed in Nurr1$^+$Th$^+$ cells in the marginal zone (data not shown). Our data therefore indicate that Mash1 and Ngn2 are expressed in some Sox2$^+$ VZ progenitors, and Ngn2 is also expressed in postmitotic Nurr1$^+$ DA

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+

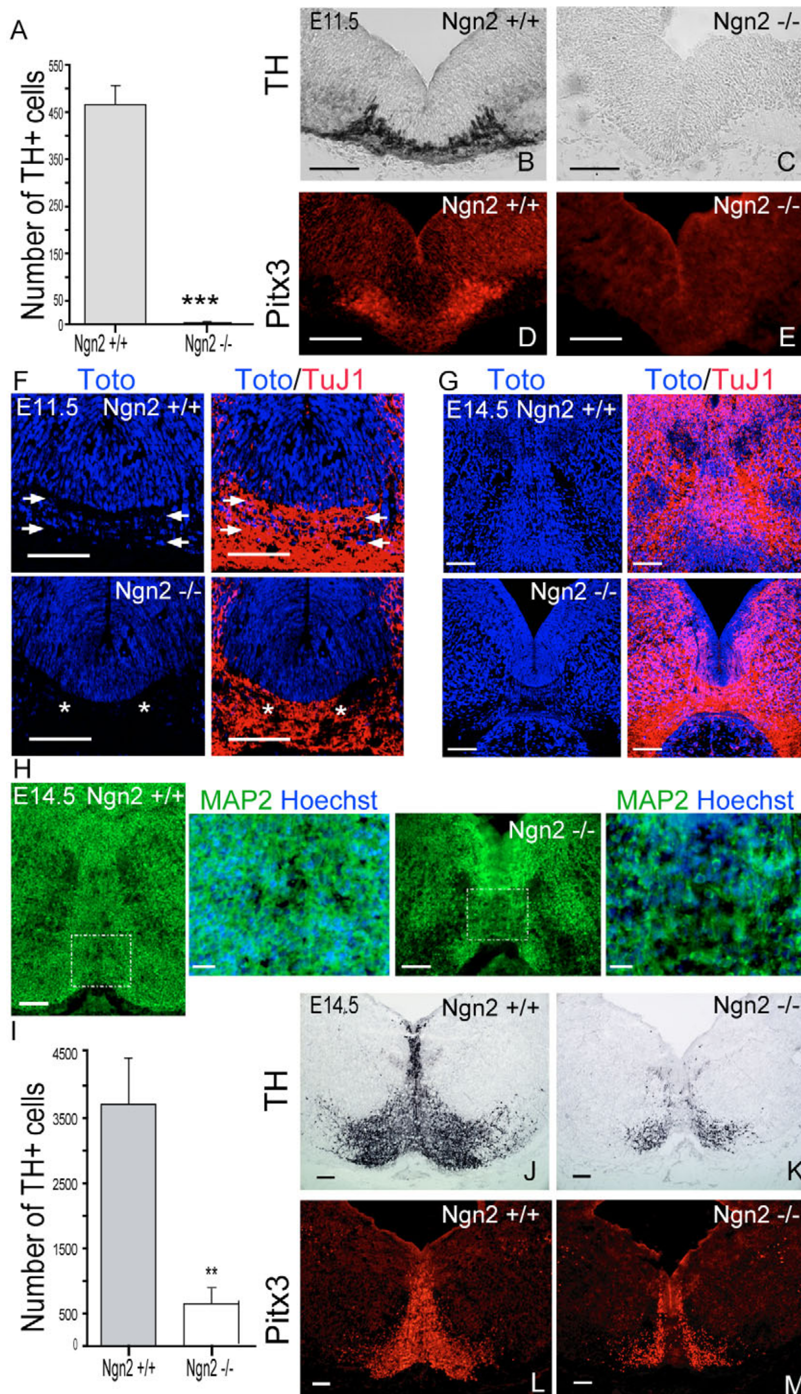

Fig. 2. Loss of DA neurons in *Ngn2* null mutant mice. An almost complete ablation of DA neurons was detected at E11.5 as assessed by Th (**A-C**) and Pitx3 (**D**,**E**) immunostaining. The expression of the pan-neuronal marker, βIII tubulin, together with Toto nuclear labeling, revealed a complete loss of neuronal cell bodies in the midline region of *Ngn2* mutant mice at E11.5 (**F**). Arrows point to Toto-labeled nuclei and asterisks denote the absence of cell bodies, with only βIII tubulin+ processes present. At E14.5, Toto/Tuj1 and MAP2/Hoechst expression revealed a reduction of the size of the marginal zone in mutant embryos, indicating a severely reduced number of neurons (**G**,**H**). A severe reduction in number of Th+ cells (**I-K**) and in Pitx3 staining (**L**,**M**) was also detected. Note that Th+ cells in mutant embryos are localized lateral to the midline. ***P<0.001 and **P<0.01, as assessed by Student´s *t*-test with Welch´s correction (n=4-5 in A,H). Scale bar: 100 μm in B-E,G,H,J-M; 10 μm in F.

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, $GAD^{65/67}$, serotonin, islet 1 and Brn3a did not show any

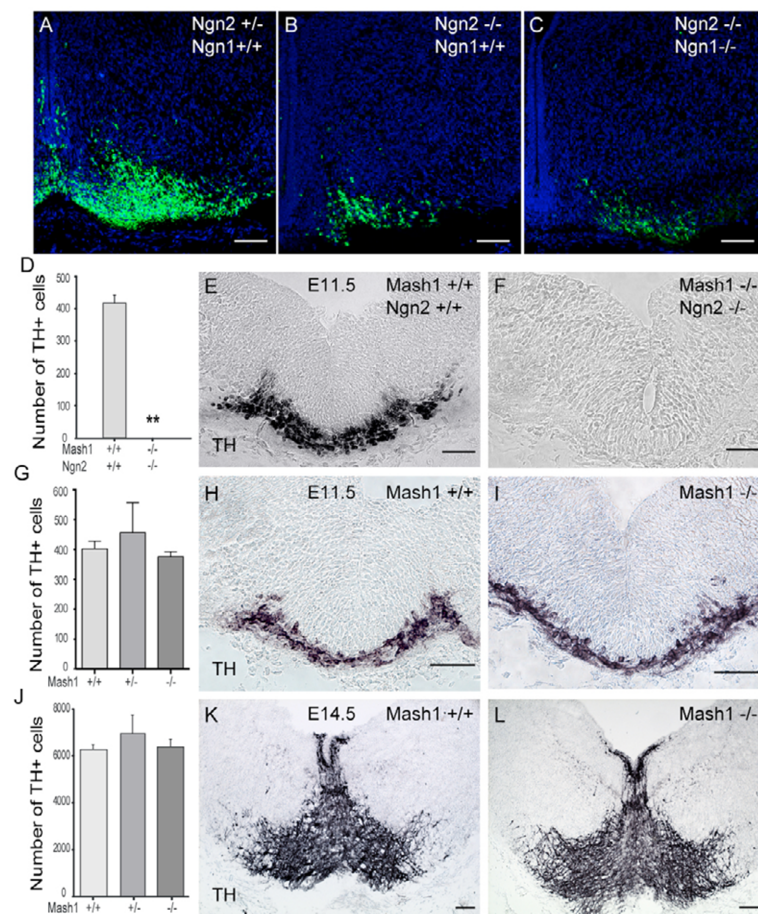

Fig. 3. Mash1 and Ngn1 do not contribute significantly to the loss of Th^+ DA neurons in Ngn2 mutant mice. Mice double mutant for *Ngn2* and *Ngn1* do not show any further loss of Th^+ cells at E14.5, compared with *Ngn2* single mutants (**A-C**), indicating that only *Ngn2* is required for the development 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.

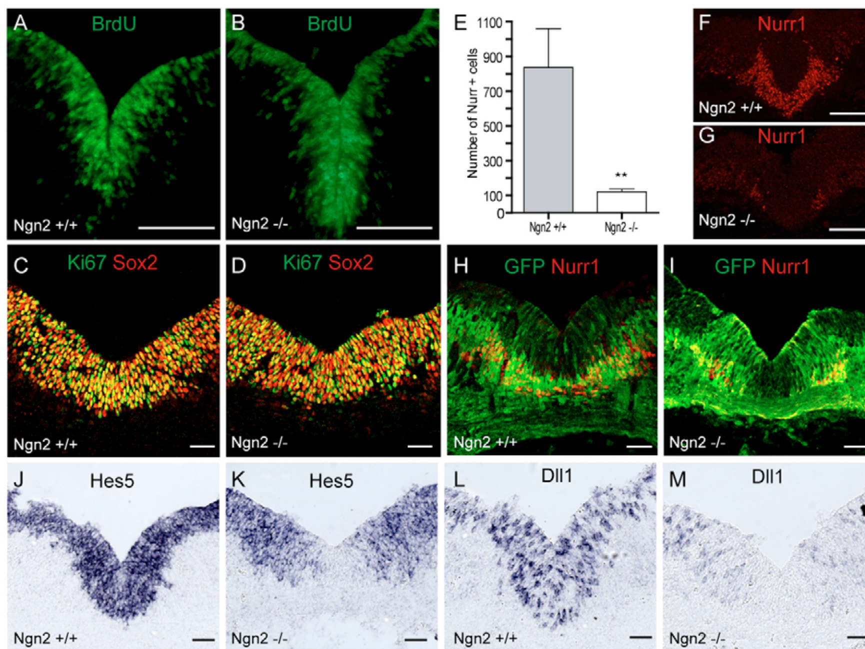

Fig. 4. Loss of Ngn2 results in a decrease in number of Nurr1$^+$ cells at E11.5. (**A-D**) No change in BrdU incorporation (A,B) or in Sox2$^+$/Ki67$^+$ double labeled cells (C,D) was found in *Ngn2*$^{-/-}$ mice compared with wild types at E11.5. (**E-G**) A simultaneous decrease in number of Nurr1$^+$ cells was detected in the intermediate and marginal zones (E, *n*=5; ***P*<0.01, Student´s *t*-test with Welch´s correction). (**H**,**I**) GFP$^+$/Nurr1$^+$ cells were also missing directly under the ventral midline in *Ngn2*$^{Gfp/Gfp}$ embryos. (**J-M**) The expression of *Hes5* (J,K) and *Dll1* (L,M), two markers of proneural activity, was also reduced in the ventricular zone. Scale bar: 100 μm.

increase, or decrease, in cell numbers (not shown). Similarly, premature differentiation of ventricular zone precursors into astrocytes was not detected (data not shown). Thus, the loss of DA neurons in *Ngn2* mutants is not due to the misspecification of DA progenitors into another VM cell type. However, VZ progenitors in *Ngn2* mutants present a defect in the expression of two markers of proneural activity, *Hes5*, an effector of Notch signaling, and *Dll1*, a Notch ligand. In wild-type mice, *Hes5* and *Dll1* were expressed in the VZ of the VM in a 'salt-and-pepper' pattern. The expression of both *Hes5* and *Dll1* was specifically reduced in zone 1 in *Ngn2* mutant mice (Fig. 4J-M), indicating that some aspect of neurogenesis was impaired.

We next examined the status of postmitotic DA precursors expressing Nurr1 in *Ngn2* mutants at E11.5. Dramatically, the overall number of Nurr1$^+$ cells detected at E11.5 was reduced to 14% of normal levels (Fig. 4E-G), and these cells were located in a lateral position. The Ngn2 mutant line contained GFP inserted into the Ngn2 locus (*Ngn2*$^{KI\text{GFP}}$) (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 *Ngn2*$^{KIGFP/+}$ and *Ngn2*$^{KIGFP/KIGFP}$ 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 (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 *Ngn1* 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) part of 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 (*Ngn2*$^{KIMash1/KIMash1}$) (Fode et al., 2000), or by deleting both genes in double mutant mice

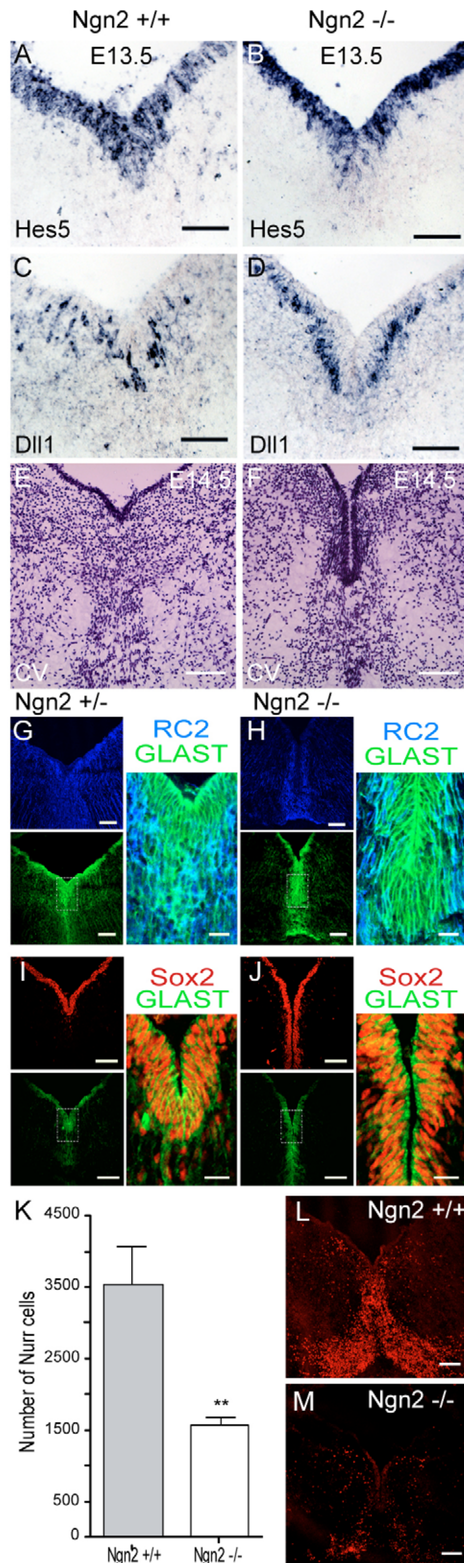

Fig. 5. Radial glia cells continue to accumulate in *Ngn2* mutant mice, but the number of Nurr1^{+} cells partially recovers at E14.5. (**A-D**) By E13.5, Hes5 (A,B) and Dll1 (C,D) expression are significantly recovered in zone 1 VZ. (**E**,**F**) Cresyl violet staining highlights the enlarged ventricular zone in *Ngn2* null mice at E14.5. (**G-J**) This cyto-architectural change is mirrored by the accumulation of radial glia cells, marked by RC2 /GLAST (G,H) or Sox2/GLAST (I,J) double staining. (**K-M**) At E14.5, the reduction in the number of Nurr1^{+} cells in *Ngn2* mutants was less pronounced than at E11.5 (see Fig. 4G), indicating a partial rescue of the block in differentiation of DA neuron precursors (K, n=3; ***P*<0.01, Student's *t*-test with Welch's correction). Scale bar: 100 μm.

(*Ngn2*$^{-/-}$;*Mash1*$^{-/-}$). 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*$^{KIMash1/KIMash1}$ 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*$^{KIMash1/KIMash1}$ 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*$^{KIMash1/KIMash1}$ 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$^+$/GFP$^+$ 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 *Hes5* 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.

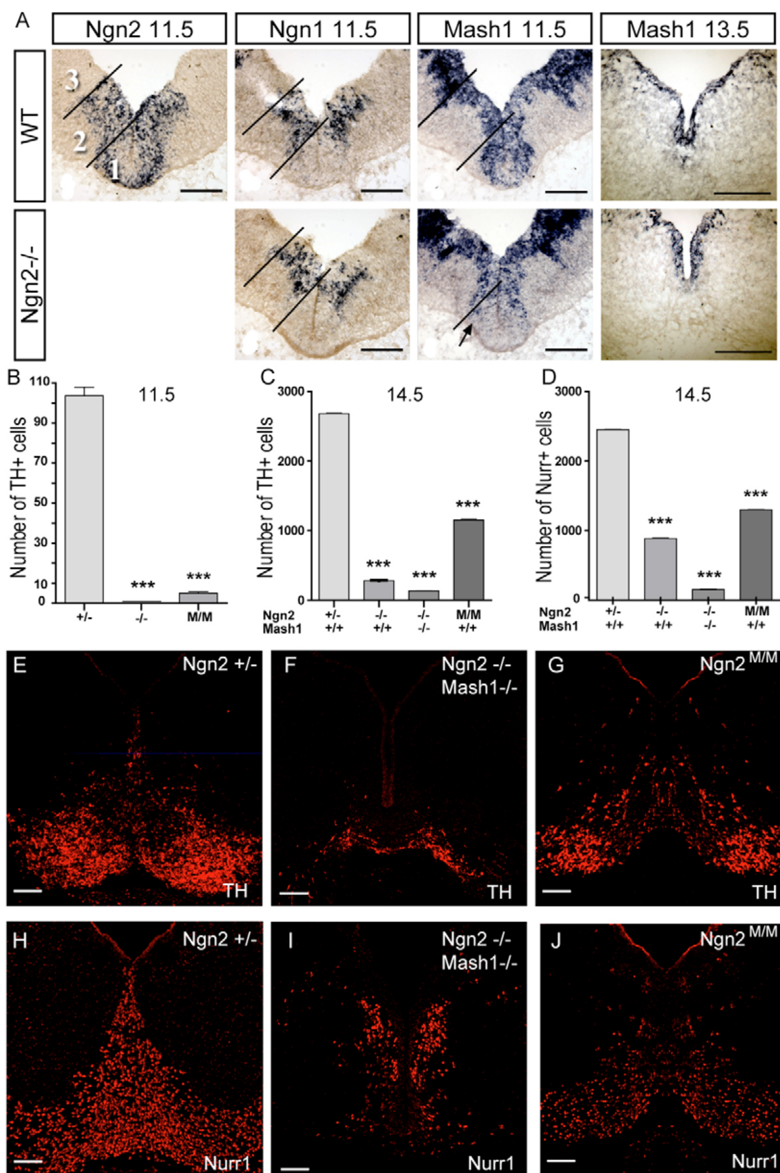

Fig. 6. Partial rescue of the loss of Nurr1$^+$ and Th$^+$ cells in *Ngn2* mutant mice by endogenous or overexpressed Mash1. (**A**) At E11.5, *Mash1* is transiently downregulated in zone 1 of *Ngn2*$^{-/-}$ mice (arrow), but has partially recovered by E13.5. (**B**) Substitution of *Ngn2* expression by *Mash1* (*Ngn2*$^{KIMash1/KIMash1}$, abbreviated to *Ngn2*$^{M/M}$) did not rescue the reduction in number of Th$^+$ cells observed in *Ngn2* mutants at E11.5. (**C**,**E-G**) However, *Mash1* overexpression partially rescues the loss of Th$^+$ DA neurons at E14.5. (**D**,**H-J**) The greater loss of Nurr1$^+$ cells in *Ngn2*;*Mash1* double mutants than in Ngn2 single mutants indicates that endogenous *Mash1* partially rescues the loss of Nurr1$^+$ precursors at E14.5 (B-D, *n*=3-6; ****P*<0.001, one-way ANOVA, with Bonferroni's correction for multiple comparisons). Scale bar: 100 μm in A; 20 μm in E-J.

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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 candiate 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 posibility 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 $Ngn2^{KIMash1/KIMash1}$ 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 $Ngn2^{KIMash1/KIMash1}$ 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).

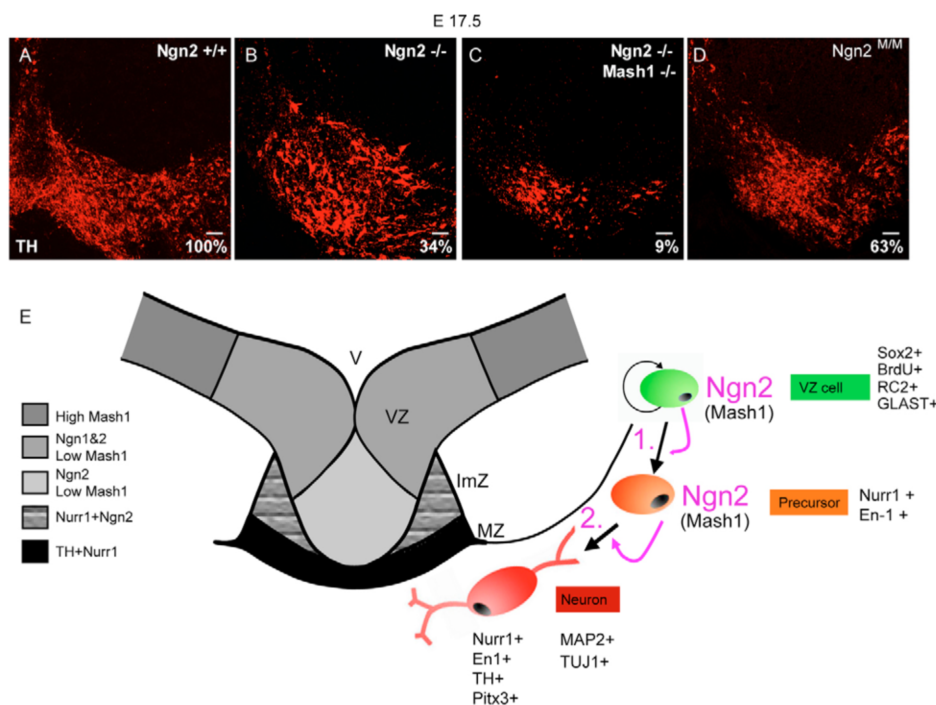

Fig. 7. Further but incomplete rescue of Th expression at E17.5, and a schematic model of the expression and function of proneural bHLH in the development of midbrain DA neurons. (**A**-**D**) The number of Th$^+$ neurons is further increased in *Ngn2* and $Ngn2^{KIMash1/KIMash1}$ mutants, but remains 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 ($Ngn2^{KIMash1/KIMash1}$). 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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