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Development of the mesencephalic dopaminergic neuron system is compromised in the absence of neurogenin 2

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Neurogenin 2 (*Ngn2*) is a proneural gene involved in neuronal differentiation and subtype specification in various regions of the nervous system. In the ventral midbrain, Ngn2 is expressed in a spatiotemporal pattern that correlates with the generation of mesencephalic dopaminergic (mesDA) neurons. We show here that lack of Ngn2 impairs the development of mesDA neurons, such that less than half of the normal mesDA neuron number remain in *Ngn2* mutant mice at postnatal stages. Analysis of *Ngn2* mutant mice during mesDA neurogenesis show that medially located precursors are formed but are arrested in their differentiation at a stage when they have not yet acquired the characteristics of mesDA neuron precursors. Loss of Ngn2 function appears to specifically affect the generation of DA neurons, as the development of other types of neurons within the ventral midbrain is unaltered. *Ngn2* is the first example of a gene expressed in progenitors in the ventricular zone of the mesDA neuron domain that is essential for proper mesDA neuron differentiation, and whose loss of function causes impaired mesDA neurogenesis without other major abnormalities in the ventral midbrain.

KEY WORDS: Parkinson's disease, Proneural genes, Differentiation, Midbrain, Tyrosine hydroxylase, Mouse

INTRODUCTION

The mesencephalic dopaminergic (mesDA) neurons have been studied extensively owing to their involvement in Parkinson's disease, as well as in psychiatric and affective disorders. The mesDA neurons are located in the substantia nigra (SN, cell group A9), the ventral tegmental area (VTA, cell group A10) and the retro-rubral area (RRA, cell group A8). DA neurons in the SN project to the dorsolateral striatum, and it is the loss of this innervation that is the leading cause of the debilitating motor symptoms in Parkinson's disease. The more medially located VTA neurons project to the limbic areas and parts of the cerebral cortex, and are implicated in motivational and cognitive behavior. The secreted factors Shh and Fgf8 are known to be critical for the induction of ventral midbrain (VM) identity at early stages of neural development (Hynes et al., 1995; Ye et al., 1998). In addition, loss-of-function studies have shown that genes such as Nurr1 (Nr4a2 – Mouse Genome Informatics), Pitx3, Lmx1b, and Engrailed (En) 1 and 2 are important for the later differentiation of the mesDA neurons (Simon et al., 2001; Smidt et al., 2000; van den Munckhof et al., 2003; Zetterstrom et al., 1997). However, much is still unknown regarding the genes and factors involved in generation of mesDA neurons in the developing VM.

The proneural gene neurogenin 2 (*Ngn2*; *Neurog2* – Mouse Genome Informatics) is member of a family of bHLH transcription factors (Gradwohl et al., 1996; Sommer et al., 1996), and is important not only for neuronal differentiation (Fode et al., 1998), but also for neuronal subtype-specification in various regions of the nervous system (Ma et al., 1999; Scardigli et al., 2001). Within the VM, Ngn2 expression is restricted to the ventricular zone (VZ), and its expression correlates both spatially and temporally with the generation of mesDA neurons, suggesting that Ngn2 is involved in DA neuron development (Thompson et al., 2006). To investigate the importance of Ngn2 in mesDA neuron development, we studied the VM phenotype of a *Ngn2*-knock-out mutant mouse. Our analysis shows an initial loss of DA neurons in the developing VM followed by a partial recovery later during embryogenesis, such that the number of DA neurons in the *Ngn2* mutant amounts to less than half of the number in the wild type at postnatal stages. Other populations of neurons in the VM are not affected, suggesting that, in the VM, Ngn2 is involved in differentiation of the mesDA neurons specifically.

MATERIALS AND METHODS

Mouse line and genotyping

Mice used in these studies were maintained and handled according to the guidelines set by the Ethical Committee for the use of animals at Lund University. Heterozygous *Ngn2-GFP* mice were maintained on a CD1/129 background and crossed to produce homozygous embryos or mice. Genotyping of the *Ngn2* wild-type allele has been described previously by Fode et al. (Fode et al., 1998). For genotyping of the mutant allele the following primers were used: Ngn2KI5 (5′-GGA CAT TCC CGG ACA CAC AC-3′) and Ngn2KImutant3 (5′-GCA TCA CCT TCA CCC TCT CC-3′; giving a 440 bp fragment). PCR conditions for the primers were: 30 cycles of 94°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute. For staging of embryos, the morning of the vaginal plug was considered as embryonic day (E) 0.5.

BrdU injections

Timed pregnant females were injected intraperitoneally (i.p.) with 50 μg BrdU/g body weight, once at E13.5 (45-minute incorporation), or twice with a three-hour interval at E15.5 and E16.5.

Immunohistochemistry

For histological analysis, heads or dissected brains (E11.5-P0) were immersion fixed overnight with 4% paraformaldehyde (PFA) in PBS at 8°C. P18 mice received lethal doses of pentobarbitone and were transcardially perfused with 0.9% saline followed by 4% PFA. The brains were post-fixed for 2 hours. Following fixation, the tissue was cryoprotected overnight in 30% (E11.5-E13.5) or 25% (E15.5-P18) sucrose. Sections were blocked in 2% normal serum/0.25% Triton X-100/PBS prior to

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incubation with primary and secondary antibodies. Primary antibodies used following this procedure were: rabbit anti-AADC (1:1000; Chemicon), rat anti-BrdU (1:100; Oxford Biotechnology; DNA was denaturated in 1 M HCl at 65°C for 30 minutes prior to preincubation, incubation with antibody at 8°C), rabbit anti-En1/2 (1:1000; gift from A. L. Joyner, New York, NY), rabbit anti-Isl1 (1:100; Abcam), rabbit anti-Isl1/2 (1:500; gift from H. Edlund and T. Edlund, Umeå, Sweden), chicken anti-GFP (1:5000; Chemicon), mouse anti-nestin (1:1000; BD), goat anti-Neurod1 (1:400; Santa Cruz), rabbit anti-Nurr1 (1:1000; Santa Cruz), mouse anti-PSANCAM (1:200; Chemicon), mouse anti-Th (1:4000; Chemicon), and rabbit anti-Th (1:1000; Pelfreeze). The first blocking step was replaced by 20 minutes in boiling 10 mM citrate buffer (pH 6) for primary antibodies against: rabbit anti-calbindin D28k (1:1000; Chemicon), rabbit anti-Girk2 (1:80; Alomone Labs), mouse anti-Isl1 (1:100; Hybridoma Bank), rat anti-Ki67 (1:50; DAKO), mouse anti-Ngn2 (1:20; gift from D. J. Anderson, Pasadena, CA), rabbit anti-Pitx3 (1:400; gift from J. P. Burbach, Utrecht, The Netherlands), and rabbit anti-VMAT (1:1000; Chemicon). The staining protocol for mouse anti-Brn3a (1:50; Santa Cruz) included boiling in citrate buffer in addition to a blocking step prior to both primary and secondary antibodies (blocking solution: 1% milk/10% normal serum/1 mg/ml BSA/PBS). Cell nuclei were visualized by staining with DAPI (1:1000; Sigma). Apoptotic cells were detected by TUNEL staining using the ApopTag Red Kit (Chemicon).

Measurement of DA and DOPAC in forebrain tissue

Tissue samples were homogenized in 0.4 M perchloric acid, diluted in milliQ water and filtered. The samples were injected into an ESA Coulochem III with electrochemical detection by a cooled autosampler (Midas). The potential of the second electrode was set at +320 mV. The mobile phase (sodium acetate 5 g/l, Na_2-EDTA 30 mg/l, octane-sulfonic acid 100 mg/l, methanol 12%, pH 4.2) was delivered at a flow rate of 500 μl/minute to a reverse-phase C18 column (4.6 mm diameter, 150 mm length, CHROMPACK). The peaks were processed by Azur chromatographic software.

In vitro differentiation

VM and dorsal midbrain (DM) were dissected from E11.5 wild-type embryos and mechanically dissociated into single cell suspensions. Cells were plated on Matrigel (BD Biosciences) at a density of 100,000 cells/0.8 cm^2 in DMEM/F12 medium supplemented with B27, 20 ng/ml EGF, 10 ng/ml bFGF, and 40 U heparin. The cultures were transduced with VSV-G-pseudotyped retroviruses containing a *Ngn2-IRES-GFP* vector (Falk et al., 2002) or a *GFP* control vector, 15 hours after plating. The medium was replaced with differentiation medium (DMEM/F12 with 1% FCS, 10 ng/ml GDNF, and 100 μM ascorbic acid) after an additional 10 hours of incubation. The cultures were fixed in 4% ice-cold PFA for 15 minutes after 5 days under differentiation conditions.

Cell counts

Tyrosine hydroxylase (Th)-positive cells were quantified in P0 and P18 *Ngn2* mutants and littermates. Every tenth section (16 μm) was collected from P0 pups and every fifth section (30 μm) was collected from P18 pups. Three sections were used for quantification using the procedure of Sauer et al. (Sauer et al., 1995), one containing the interpeduncular nucleus at the level of oculomotor nerve exit, the one previous and the one following (indicated with a star in Fig. 2J,K). The average cell number per section was calculated bilaterally for each animal. An estimation of the reduction in total Th-positive cell number in the P18 pups was obtained by counting Th-positive cells in all sections containing SN and VTA in the series (every fifth section stained). The average total numbers of Th-positive cells counted were calculated. All Isl^+ and $Brn3a^+$ cells within the oculomotor and red nuclei, respectively, were counted bilaterally, and the mean total number was compared between wild-type and *Ngn2* mutant mice. At E13.5, all DAPI-stained nuclei (within the MZ) and BrdU-positive cells within the mesDA neuron domain were counted in a series of every tenth section. The number of GFP/GLAST double-positive cells within a distance of 2 μm from the ventricular surface at one confocal plan per animal was counted. All numbers were corrected for split cell counts using the Abercrombie formula (Abercrombie, 1946).

Statistical analysis

Cell counts were compared using an unpaired Student's *t*-test. The statistical significance level was set at $P<0.05$. Data is expressed as the group mean ±1 s.e.m.

RESULTS

Lack of Ngn2 causes a reduction in the number of DA neurons in the developing VM

In mice, the mesDA neurons develop over a period of 3-4 days with the first postmitotic Th-expressing cells seen at around E11, and Ngn2 is expressed during this time in the VZ cells giving rise to mesDA neurons (Fig. 1A). At E11.5, Ngn2 expression is confined to the cells within the proliferative VZ, with the exception of a few single cells that express Nurr1, which marks postmitotic mesDA neuron precursors (Fig. 1B,C). In order to follow the development of the mesDA neurons in $Ngn2^{-/-}$ mice, heterozygous *Ngn2-GFP* ($Ngn2^{+/-}$) mice were crossed and the litters taken at different timepoints, starting at E11.5. The mice were then analysed for the expression of mesDA neuron markers.

In wild-type embryos at E11.5, some scattered Th-positive cells are seen at the ventral rim of the VM, and, at E13.5, Th-positive cells fill the mantle zone (MZ) forming the beginnings of the mesDA nuclei (Fig. 1D,F). In E11.5 *Ngn2* mutants, only single Th-positive cells were seen (Fig. 1E), and some of the embryos analyzed (3/7) did not display any Th-positive cells at all. At E13.5, the number of Th-positive neurons in *Ngn2* mutants was approximately one-tenth of that seen in wild-type mice. The Th-positive cells present in the mutants were primarily located in thin stripes at the lateral edges of the expected DA neuron domain (Fig. 1G). Later, at E15.5, the number of Th-positive cells in *Ngn2* mutants was still dramatically reduced compared with in wild-type mice and the pattern of the existing Th-positive neurons was similar to that seen at E13.5 (Fig. 1H,I). At E17.5, however, more Th-positive neurons had been generated in the *Ngn2* mutants and the discrepancy between wild-type and *Ngn2* mutant mice, with respect to both number and distribution of the Th-positive cells, was less pronounced (Fig. 1N,O). *Ngn2-GFP* heterozygotes did not differ from wild-type mice at any stage of development. In further analyses, we therefore used the heterozygous animals interchangeably with wild-type mice.

The expression of Aadc (Ddc – Mouse Genome Informatics), which is expressed in both DA neuron precursors and mature DA neurons, and Vmat2 (Slc18a2 – Mouse Genome Informatics), a marker for more mature DA neurons, showed the same pattern of expression as Th did in the *Ngn2* mutant (Fig. 1J-M). Therefore, the reduction of Th-positive neurons in the *Ngn2* mutant does not appear to be caused by a specific downregulation of the *Th* gene but represents a reduction in the number of mesDA neurons.

Compromised mesDA neuron system in postnatal *Ngn2* mutants

The *Ngn2* mutants were born with the expected frequency (15/80), but most of them died within the first days of birth. Because the *Ngn2* mutants were among the smaller pups it was possible to reduce the size of the litter, which was beneficial for the survival of the mutants. Reducing the litters to ⩽5 pups allowed for one mutant for every two litters to survive (8 mutants in 15 litters). The mutants had a limited weight gain when compared with wild-type and heterozygous pups, and weighed on average one-third of what their littermates did at P18 (Fig. 2C). They had an emaciated appearance but showed no obvious abnormal behavior. At approximately 3 weeks of age their condition began to deteriorate, and no mutants survived beyond P25 (see also Fode et al., 1998). To assess the fate

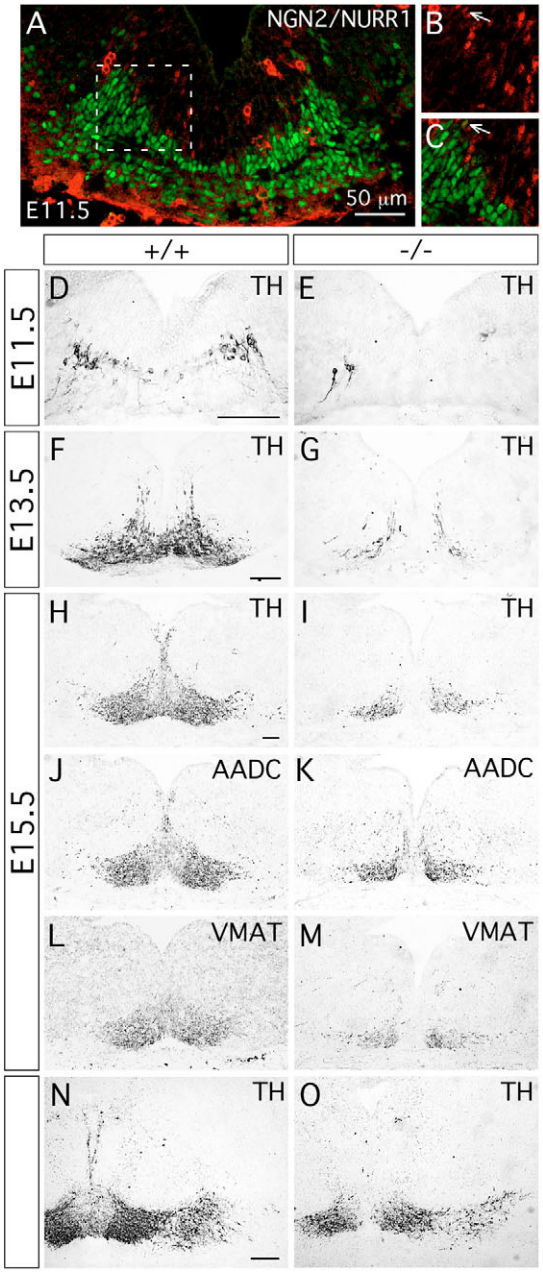

Fig. 1. Impaired development of Th-positive mesDA neurons in Ngn2 mutant mice. (**A-C**) In the VM Ngn2 (red) was expressed in part of the VZ lying immediately above the Nurr1-expressing mesDA neuron precursors (green, A). A few single Ngn2-positive cells were seen to co-express Nurr1 (arrow, B,C). (**D-I**) At E11.5-E15.5, the number of Th-positive cells in the VM was dramatically reduced, by approx 90%, in the homozygous (–/–) *Ngn2* mutants (E,G,I), compared with the wild-type (WT, +/+) mice (D,F,H). The remaining Th-positive cells were located at the lateral edges of the mesDA neuron domain, whereas the medial portion was almost completely devoid of Th-positive cells. (**J-M**) Parallel sections stained for Aadc and Vmat2 showed the same degree of cell loss, with positive cells remaining in the same lateral population. (**N**,**O**) At later embryonic stages (E17.5), more Th-positive neurons had been generated in the *Ngn2* mutant and the difference between wild type and *Ngn2* mutants with respect to both the number and distribution of Th-positive neurons was less pronounced. Scale bars: 50 μm in A; 100 μm in D-O.

of the mesDA system postnatally, the expression of Th was analyzed in neonatal (P0) and P18 *Ngn2* mutants and littermates. At P0 there was no obvious difference in brain size between mutant and wild-type pups. However, at P18 the size of *Ngn2* mutant brain was considerably reduced and weighed approximately 80% of that of wild-type littermates. Notably, the number of sections retrieved from the midbrain through the mesDA domain at P18 was roughly 20% fewer in the mutants, as shown in Fig. 2J,K. In all mutants analyzed at this stage (n=5), the ventricular system, including the midbrain aqueduct, was enlarged. At P0, the distribution of Th-positive cells was similar in wild-type and mutant mice; however the number of Th-positive cells was markedly reduced both in VTA, SN (Fig. 2A,B) and RRA (not shown). This reduction was also apparent in P18 mutants when comparing sections of similar rostrocaudal levels (Fig. 2D,E,J,K). For quantification, Th-positive cells were counted bilaterally in two to three sections per animal and the average number of cells/section for the VTA and SN was compared in mutants and littermates at P0 and P18. At P0, the number of Th-positive cells in the *Ngn2* mutants was reduced by about 50%, and at P18 by about 60%, in both VTA and SN (Fig. 2N,O; P<0.01 for all comparisons). Heterozygous littermates were also analyzed at P18. There was a highly significant difference in Th-positive cell number between homozygotes and heterozygotes (P<0.001 for both SN and VTA), but no difference between heterozygotes and wild-type mice (P=0.47; Fig. 2O). Because the SN-VTA region was reduced in size in the *Ngn2* mutants at P18 it is likely that the above figure underestimates the reduction of the mesDA cell number. In order to estimate the magnitude of the total cell loss at P18, we counted Th-positive cells in all sections that included SN or VTA (7 sections in the wild type and heterozygotes, and 5 sections in the mutants). The average number of counted cells in wild type, heterozygotes and mutants were 815, 964 and 262 in SN, and 816, 954 and 254 in VTA, respectively, representing an average mesDA neuron loss of about 70% in the *Ngn2* mutant at P18.

As observed at embryonic stages, the staining pattern of Vmat2 was identical to that of Th in consecutive sections at P18 (Fig. 2H,I). Because this was the case also for other mesDA neuron markers (see below), this again indicates an actual loss of DA neurons rather than a selective downregulation of the *Th* gene itself. None of the mesDA nuclei appeared to be preferentially affected or missing in either the P0 or P18 mutants, with the exception of a population of Th-positive cells surrounding the aqueduct, which was almost completely lost in the mutant mice (Fig. 2F,G).

In Th immunostained sections from the forebrain, the distribution of Th-positive fibers in the striatum and the adjacent limbic and cortical forebrain areas in the *Ngn2* mutants did not differ from that seen in wild-type littermates. No single forebrain area was deprived of Th-positive innervation, suggesting that the DA neurons remaining in the mutant SN and VTA were projecting to their appropriate target areas (Fig. 2L,M).

Neurochemical analysis at P18 showed reduced total levels of both DA (38% of wild type) and DOPAC (48% of wild type) in forebrain of *Ngn2* mutants (Table 1). Even when correcting for the reduced weight of the brain, the tissue concentration of both DA and DOPAC in the mutant forebrain was less than 65% of that measured in wild-type mice. The turnover of DA, as assessed by the DOPAC/DA ratio, did not differ between wild-type and *Ngn2* mutant mice.

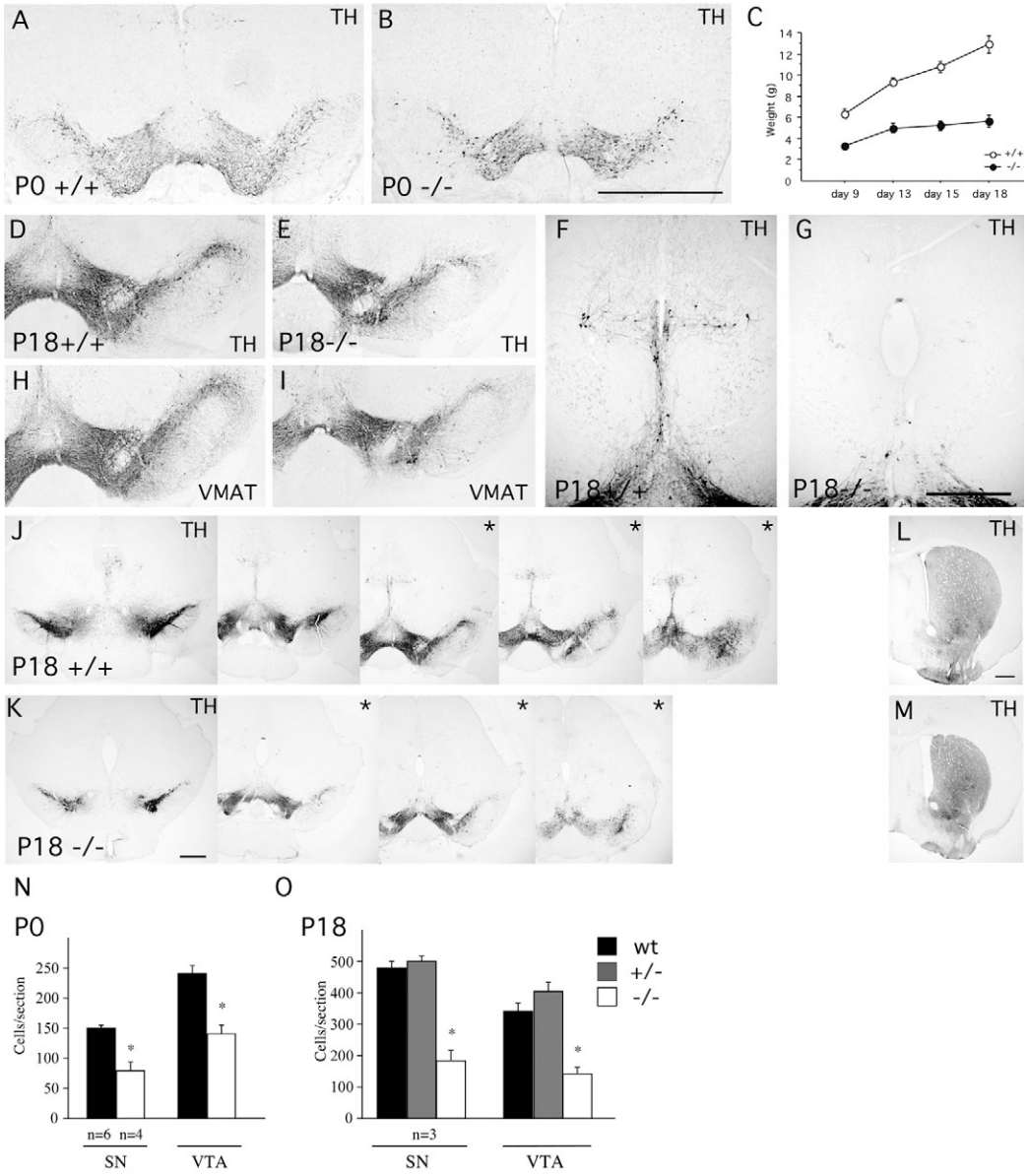

Fig. 2. Loss of mesDA neurons in postnatal Ngn2 mutant mice. (**A-M**) In postnatal *Ngn2* mutants, the number of Th-positive neurons was markedly reduced in both the SN and the VTA (A,B,D,E), and a population of Th-positive cells located close to the midbrain aqueduct was completely missing (F,G). Parallel sections stained for Vmat2 showed that no other cells present in the SN-VTA area displayed a DA phenotype (H,I). The Th-positive innervation of striatal and limbic forebrain areas was normal, although the target areas were reduced in size (L,M). The *Ngn2* mutants were similar in size to wild-type mice at birth but showed a reduced weight gain postnatally (C), and the size of their brain was reduced by about 20% (J,K). Stars indicate the rostrocaudal levels used for cell counting. (**N**,**O**) Cell counts at P0 and P18 revealed a more than 50% loss of Th-positive neurons in the SN and VTA of the *Ngn2* mutants. *$P \leqslant 0.01$ relative to wild type (N), and in wild type adn heterozygous (O) (Student´s *t*-test). Scale bars: 500 μm.

Appropriate specification of remaining mesDA neurons in *Ngn2* mutants

The mesDA system contains two major subtypes of DA neurons, the SN and VTA DA neurons. The two best-described markers that distinguish these subtypes are Girk2 and calbindin, which are preferentially expressed by SN and VTA neurons, respectively (Liang et al., 1996; Schein et al., 1998). We compared the distribution of Girk2 and calbindin in postnatal *Ngn2* mutant and wild-type mice in order to investigate whether the reduced number of mesDA neurons resulted from a selective loss of one of the two DA neuron subtypes. In both wild type and *Ngn2* mutants, Th-positive neurons in the VTA co-expressed calbindin, whereas the SN DA neurons were negative for this marker (P0, Fig. 3A-F). The typical expression profile of Girk2 is first seen at later postnatal stages, we thus studied the expression of Girk2 at P18. At this age, in both wild type and *Ngn2* mutants, Girk2 was preferentially expressed in the SN, and to a lesser extent in DA neurons located in the zone between VTA and SN (Fig. 3G-L).

In the ventral spinal cord, Ngn2 is required for the correct expression of a number of homeodomain (HD) transcription factors involved in neuronal subtype differentiation (Scardigli et al., 2001). To study whether Ngn2 in a similar fashion is important for the correct expression of HD transcription factors within the VM, we analysed the expression of Pitx3, a HD transcription factor

Table 1. Tissue wet-weight, DA and DOPAC content in the forebrain of *Ngn2* mutant mice

	Wild type (*n*=6)	*Ngn2*$^{-/-}$ (*n*=4)
Weight (mg)	305±4	231±5 (75%)*
DA total (pmol)	1207±33	458±48 (38%)*
DOPAC total (pmol)	185±13	88±8 (48%)*
DOPAC/DA ratio	0.15	0.19
DA concentration (pmol/mg)	3.96±0.11	1.99±0.14 (50%)*
DOPAC concentration (pmol/mg)	0.61±0.03	0.38±0.02 (62%)*

*Statistical significance at $P<0.01$ (unpaired Student's *t*-test).
The percentage of wild-type value is shown in parentheses.

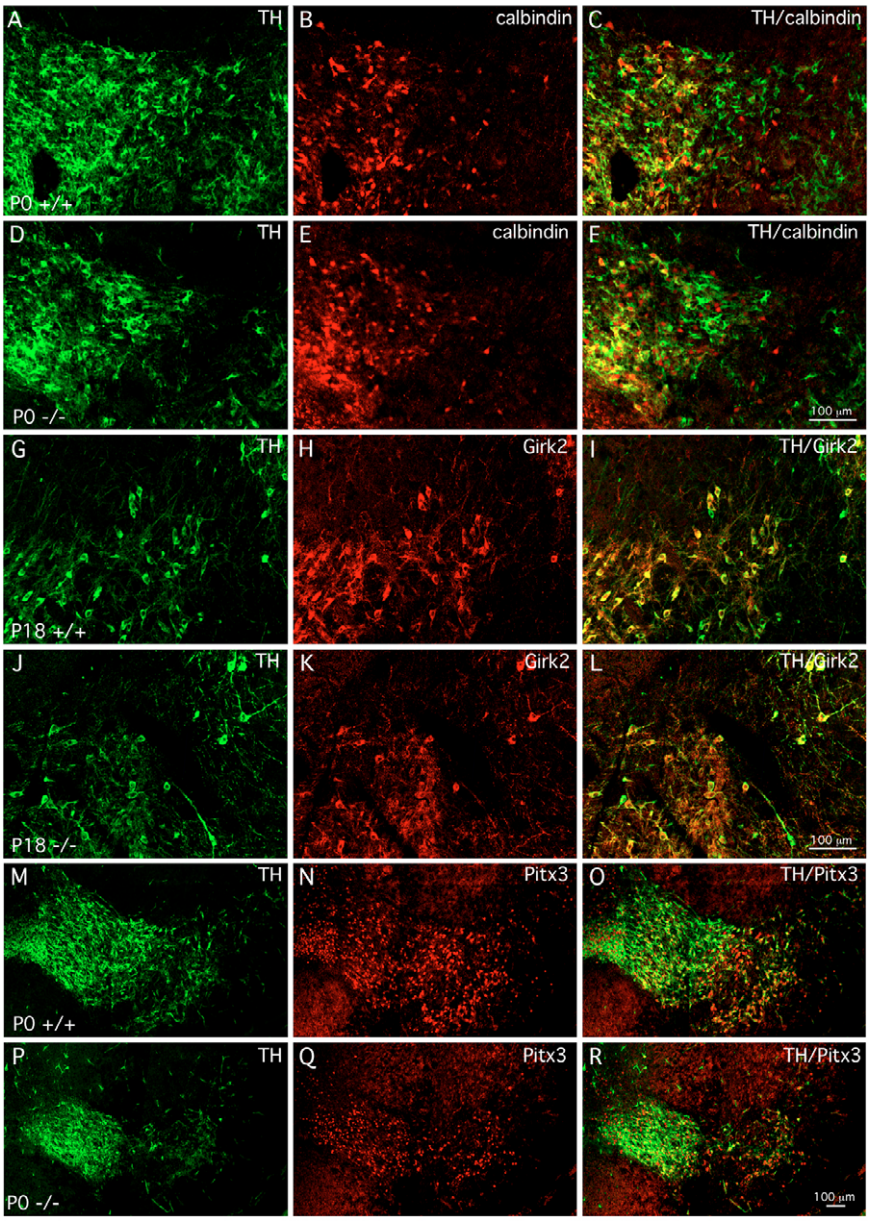

Fig. 3. Correct specification of mesDA neurons in Ngn2 mutants. In wild-type mice, the two major mesDA neuron subtypes, the SN and VTA neurons, are characterized by their expression of Girk2 (in SN DA neurons) and calbindin (in VTA DA neurons). (**A**-**L**) In postnatal *Ngn2* mutant mice (D,E), double-stained sections showed that the expression of calbindin (red, **E**,**F**) in the Th-positive VTA neurons (green) was similar to that seen in wild-type mice (B,C), and Girk2 (red, **K**,**L**) was expressed in Th-positive SN neurons (green) in a pattern indistinguishable from that of wild type (H,I). (**M-R**) The expression of Pitx3 (red) was maintained in all Th-positive neurons (green) in *Ngn2* mutants (R). Scale bars: 100 μm.

specifically expressed in mesDA neurons (Smidt et al., 1997). As previously reported (Smidt et al., 2004; Zhao et al., 2004), Pitx3 was expressed in virtually all Th-positive neurons in the SN and VTA in wild-type mice. Similarly, all remaining Th-positive DA neurons in the SN and VTA in the postnatal *Ngn2* mutant expressed Pitx3. Two other HD transcription factors, En1 and En2, are also expressed in developing and mature mesDA neurons, but are not as specific to this cell population as Pitx3 (Davis and Joyner, 1988; Simon et al., 2001). We found that En1/2 was co-expressed with Th in *Ngn2* mutants to the same extent as in wild-type mice (data not shown).

These data indicate that the mesDA neurons formed in *Ngn2* mutant mice are correctly specified. Notably, all six mesDA neuron markers analyzed (in addition to Th) showed the same expression pattern and DA neuron cell loss in the *Ngn2* mutant mice, which points to a reduction of DA neuron number, rather than a misregulation of genes important for the DA neurotransmitter phenotype.

Loss of medially located mesDA neuron precursors in the *Ngn2* mutant

The analysis of the development of mesDA neurons in the *Ngn2* mutants showed a substantial early loss of Th-positive neurons. To clarify if the mesDA neuron precursors are generated normally in the mutant, but fail to mature to more differentiated neurons, we studied the expression of the orphan nuclear receptor Nurr1. MesDA neuron precursors express Nurr1 prior to Th, at the time of migration through the postmitotic intermediate zone (IZ, defined here as the zone between the proliferative VZ and the MZ) (Zetterstrom et al., 1997). In the *Ngn2-GFP* heterozygotes, at E11.5, the entire IZ is filled with Nurr1-positive cells (Fig. 4A), and at E13.5 the further differentiated mesDA neurons in the MZ also express Nurr1 (Fig. 4G). In the *Ngn2* mutants, the region around the midline was completely void of Nurr1-positive mesDA neuron precursors, at both E11.5 and E13.5 (Fig. 4D,J). Two streams of Nurr1-positive cells were located at the lateral edges of the presumptive mesDA precursor domain, similar to the Th

expression pattern previously seen at these stages (Fig. 4D,J). More Nurr1-positive cells were detected at E15.5, but their spatial distribution remained the same (Fig. 4P). At E13.5, Pitx3 expression, which has an onset that is slightly earlier than Th (Zhao et al., 2004), showed the same loss of medially located mesDA neuron precursors in the *Ngn2* mutant mice as did Nurr1 (data not shown).

The *Ngn2-GFP* mouse used in this study, expresses GFP from the *Ngn2* locus. In these mice, the GFP protein is detectable not only in the Ngn2-expressing cells in the VZ, but also in their immediate progeny in the IZ (Fig. 4H). This may be explained either by the longer half-life of the GFP protein, or by differences in the post-transcriptional regulation of Ngn2 and GFP. Because the progeny of Ngn2-expressing precursors continue to stain for GFP for a limited time, short-term lineage tracing of these cells is possible. Analysis of GFP expression in the *Ngn2* mutants showed the presence of GFP-positive cells in the medial sector, as well as in more lateral positions (Fig. 4E,K,Q). This demonstrates that the VM cells lacking Ngn2 survive, at least initially. However, although the cells at the lateral edges of the mesDA neuron domain express Nurr1 as they migrate ventrally (Fig. 4L,R), the GFP-positive cells within the medial sector do not show any Nurr1 or Pitx3 expression. These results show that the absence of Th-positive neurons in the *Ngn2* mutant mice is matched by a lack of Nurr1- and Pitx3-expressing mesDA neuron precursors.

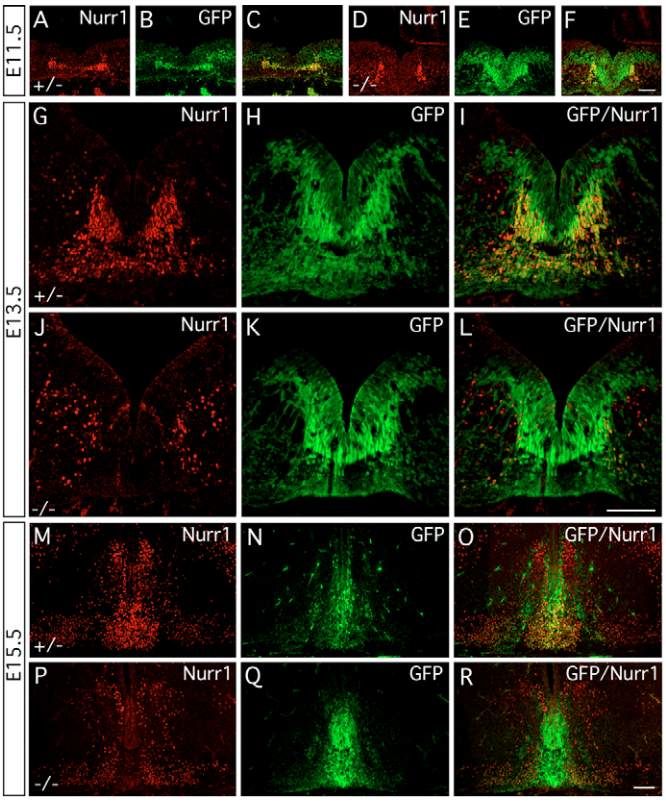

Fig. 4. Loss of medially located mesDA neuron precursors in the Ngn2 mutant. (**A**,**D**,**G**,**J**,**M**,**P**) At all stages of mesDA neurogenesis (E11.5-E15.5), Nurr1 staining showed a lack of Nurr1-positive mesDA neuron precursors in the medial region of the VM in *Ngn2* mutants. (**E**,**K**,**Q**) Interestingly, GFP expressed from the *Ngn2* locus revealed the presence of cells in the medial region of the *Ngn2* mutants. (**B**,**E**,**H**,**K**,**O**,**S**) GFP staining; (**C**,**F**,**I**,**L**,**O**,**R**) merged images. Scale bars: 100 μm.

Delayed differentiation and migration of the mesDA neurons to the MZ in *Ngn2* mutant mice

Immunostaining for PSA-NCAM (data not shown) and β-III-tubulin in E13.5 *Ngn2* mutants showed the presence of neurons in the medial section of the MZ lacking Th and Nurr1 expression (Fig. 5B-D,G-I). However, the number of cells within the MZ was reduced to 26% of wild-type levels as detected by the nuclear stain DAPI (heterozygotes, 1400±75; mutants, 369±19; n=4, P<0.001; Fig. 5E,J). The reduction in cell number was most prominent at caudal levels of the mesDA neuron domain where the MZ was completely devoid of cells (data not shown). Additionally, the DAPI stain revealed retention of cells in the medial sector such that a densely packed VZ/IZ was formed in the *Ngn2* mutant mice (Fig. 5E,J). On closer examination of the VZ/IZ in the *Ngn2* mutants, we noticed a displacement of expressing GFP-positive cells towards the ventricle and a tendency of these cells to maintain contact with the ventricular surface (Fig. 6I). This is in contrast to the heterozygotes, where the highest expression of GFP was observed in the postmitotic cells in the IZ (Fig. 6A). We stained for GLAST to determine if the cells that remained in contact with the ventricular surface were radial glia and thus likely to act as neural progenitors (Anthony et al., 2004). The results showed a threefold increase in the percentage of GFP/GLAST-positive cells within a 2 μm distance from the ventricular surface in the *Ngn2* mutants compared with in heterozygotes (heterozygotes, 8.8±2.7%; mutants, 27.3±6.9%; n=3, P<0.05), and a clear increase in the area of the ventricular surface occupied by GFP/GLAST-positive endfeets (arrows in Fig. 6C,K). Thus, the absence of Ngn2 leads to an increase in the number of (GFP/GLAST-positive) neural progenitor cells.

Next, we wanted to investigate the phenotype of the remaining cell accumulation in the *Ngn2* mutants in more detail. The proliferative marker Ki67 (data not shown) and BrdU labeling at E13.5 showed that proliferation was limited to a three to five cell-diameter-thick zone along the ventricular surface, in a pattern indistinguishable from the distribution in heterozygous mice, and that there was no increase in cell division within the cell accumulation compared with heterozyotes (BrdU: heterozygotes, 299±87; mutants, 263±40; Fig. 6D,E). Thus, the border between the VZ and the IZ persists in the *Ngn2* mutants, and the absence of Ki67-positive and BrdU-labeled cells in the mutant IZ indicates that the Nurr1-negative cells that accumulate in this zone are postmitotic. The neuronal determination factor Neurod1 (Lee et al., 1995) was expressed in the IZ of heterozygous mice at E13.5; however, similar to the expression pattern of Nurr1, Neurod1-positive cells were only seen at lateral positions in the mutant IZ (Fig. 6L,M,O,P). The lack of Neurod1 expression in the *Ngn2* mutants was specific for the mesDA neuron domain, as Neurod1 expression was unaffected in other parts of the midbrain (data not shown). At the same time, none of the cells in the mutant IZ expressed the neuronal markers PSA-NCAM (data not shown) and β-III-tubulin (Fig. 5B,G), which does not support the presence of immature or mature neurons among the arrested cells within the IZ. Finally, no expression of GFAP could be detected in this region of the *Ngn2* mutants (data not shown).

To determine the fate of the arrested cells in the mutant IZ, we performed a TUNEL assay. Single labeled cells were detected at E13.5 in the VM of wild-type mice, and up to six labeled cells in one section in *Ngn2* mutant mice (n=6; data not shown). This suggests that some cells will go through apoptosis when lacking the differentiation cues provided by Ngn2; however most of them will remain. It seems likely, therefore, that cells appearing in the MZ from E15.5 (Fig. 6F,N) and onwards are derived from accumulated cells that are released from the differentiation arrest. The release of

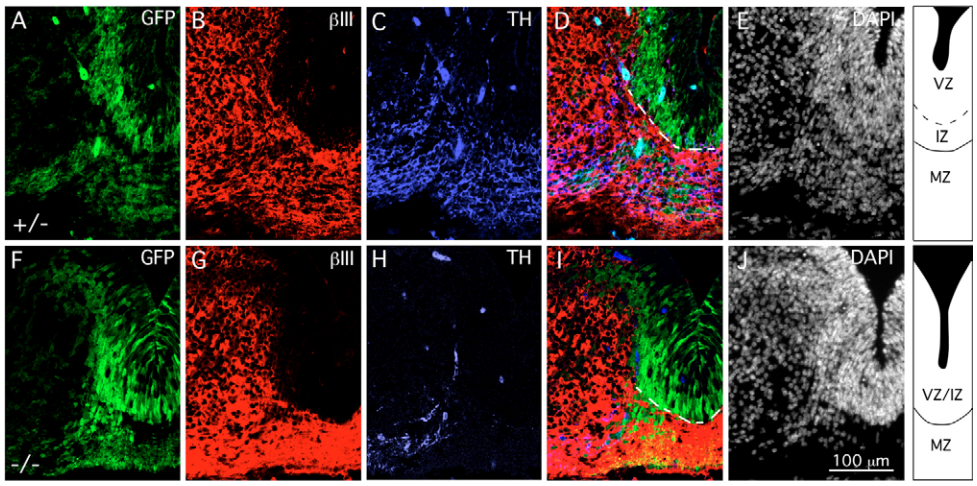

Fig. 5. Absence of Ngn2 causes a reduction of the MZ and retention of cells within the VZ/IZ. (**A-D**,**F-I**) β-III-tubulin staining at E13.5 revealed the presence of neurons within the MZ of the mesDA domain despite the almost complete lack of Th-positive neurons. (**E**,**J**) However, DAPI staining showed a reduction in total cell number in the MZ and an accumulation of retained cells in the VZ/IZ of the *Ngn2* mutants in comparison to heterozygous mice. Diagrams on the right show the positioning of the VZ, IZ and MZ in the heterozygous and mutant mice.

cells to the MZ also corresponds well with the catch-up of the mesDA neurons seen between E15.5 and E17.5. Alternatively, proliferating VZ cells could generate the late-appearing mesDA neurons. To investigate this possibility, we exposed E15.5 and E16.5 embryos to BrdU, i.e. at time-points when mesDA neurogenesis is complete in wild-type mice. Incorporation of BrdU in mesDA neurons was examined at P0. No BrdU/Th double-labeled mesDA neurons were observed in either wild-type or *Ngn2* mutant mice, despite a high number of BrdU-labeled cells in this area (data not shown). Therefore, the increase in mesDA neurons between E15.5 and E17.5 is unlikely to be explained by a prolonged neurogenesis.

Together, these results suggest that, in the *Ngn2* mutant mice, most precursors within the mesDA domain are initially arrested in their differentiation, either at the level of neural radial glia, or at a postmitotic stage when they have not acquired the characteristics of neuronal or mesDA neuron precursors. Some of the precursors appear to be eventually released from this arrest.

Differentiation of non-DA neurons in the VM is unaffected in the absence of Ngn2

To ensure that the reduced and delayed genesis of DA neurons is not a consequence of a general impairment, or delay in neurogenesis, we examined the development of other types of neurons within the VM. The possibility that other cell populations may expand at the expense of DA neurons in the *Ngn2* mutants was also investigated. In this analysis, the transcription factors islet 1 (Isl1) and Brn3a (Pou4f1 – Mouse Genome Informatics) were used as markers of precursors of the neurons within the oculomotor and red nucleus, respectively. These nuclei develop within the part of the VM that is lateral to the mesDA neuron domain (Agarwala et al., 2001; Wallen et al., 1999). At E11.5, the number of Isl1-positive neurons in *Ngn2* mutants was indistinguishable from that of wild-type mice (Fig. 7A,D). When quantified at P0, the number of Isl1-positive oculomotor neurons (Fig. 7H,K) was 259.7±23.3 in the *Ngn2* mutants compared with 255.9±14.2 in wild-type mice (n=3, P=0.90). Loss of Ngn2 thus did not cause either a decrease or an increase of oculomotor neurons.

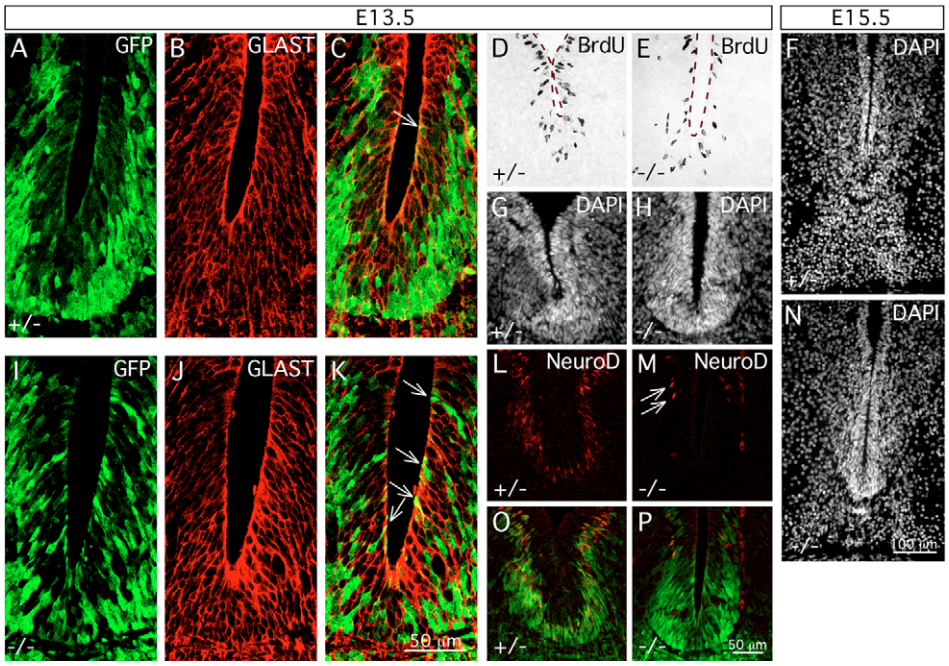

Fig. 6. Build-up of unspecified postmitotic precursor cells in the IZ of the Ngn2 mutants. (**A-C**,**I-K**) Lack of *Ngn2* function caused an increase in the number of GFP/GLAST-positive neural progenitors with maintained contact to the ventricular surface compared with wild type (arrows). (**D**,**E**) BrdU labeling, however, did not show an increase in the number of proliferating cells or an expansion of the VZ in the *Ngn2* mutants. (**L**,**M**,**O**,**P**) Staining for the neuronal determination factor Neurod1 (double staining with GFP) showed that these IZ cells, in addition to being Nurr1-negative, were Neurod1-negative, with the exception of a few laterally positioned precursors. (**G**,**H**) Nuclear DAPI stain of the Neurod1/GFP double-stained sections shows the distribution of cells in the IZ. (**F**,**N**) At E15.5, the accumulation of cells, as detected by DAPI, in the VZ/IZ has diminished and more cells are present in the MZ compared with at E13.5 (Fig. 3E,J).

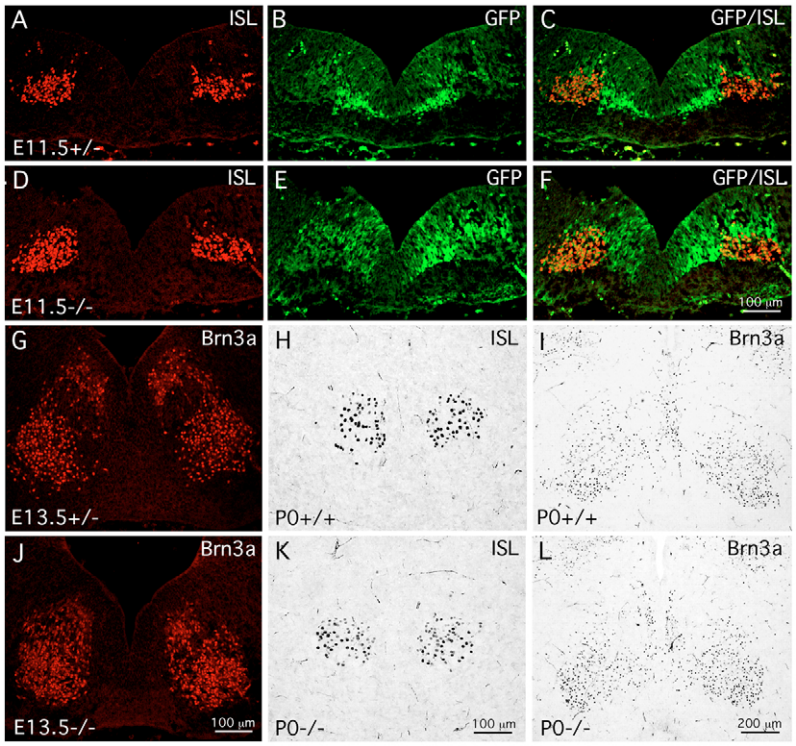

Fig. 7. The development of non-DA neurons in the VM is unaffected in the Ngn2 mutant. In the heterozygotes, the Isl1-positive oculomotor neurons show weak GFP-expression at E11.5, and are thus presumably derived from the Ngn2-expressing VZ progenitors cells. Nevertheless, the number of Isl1-positive cells was unaffected in the *Ngn2* mutants, both at E11.5 (**A**,**D**) and at P0 (**H**,**K**). Similarly, the Brn3a-positive neurons of the red nucleus were not affected in the *Ngn2* mutant, neither at E13.5 (**G**,**J**), nor at P0 (**I**,**L**). (**B**,**E**) GFP staining at E11.5; (**C**,**F**) Merged image of A,B and D,E, respectively.

Because the neurogenesis of oculomotor neurons is ongoing at E11.5, these results also indicate that genesis of these neurons is not delayed in the *Ngn2* mutants, as is the case for mesDA neurons. Interestingly, Isl1-positive cells show weak GFP expression at E11.5 (Fig. 7C,F). Hence, Ngn2 is likely to be expressed in oculomotor neuron precursors without being fundamental for their development. Similarly, no difference was detected in the number of Brn3a-positive neurons at E13.5 (Fig. 7G,J), and quantitative analysis of Brn3a-positive neurons in the red nucleus at P0 did not reveal any difference between wild-type and *Ngn2* mutant mice (total cell number: wild type, 1194±39; mutant, 1113±37; n=4, P=0.19; Fig. 7I,L). Hence, within the VM, loss of Ngn2 function specifically affects the differentiation of DA neurons, whereas the development of other neuronal subtypes is unaltered.

Ngn2 is not on its own sufficient to induce mesDA neuron fate

These results raise the question of whether Ngn2 by itself is sufficient to induce a mesDA neuron fate in neural precursors. We therefore ectopically expressed Ngn2 in primary cultures from E11.5 mouse dorsal midbrain (DM), by transduction with *Ngn2-IRES-GFP*; *IRES-GFP* retroviruses were used as a control. After 5 days of differentiation, the cultures were analyzed for the presence of β-III-tubulin- and Th-positive neurons. Ngn2 transduction increased the number of β-III-tubulin-positive neurons from 15.7±1.3% in the GFP-control transduced cultures to 32.6±1.0% (n=3, P=0.005). However, Ngn2 did not promote mesDA neuron differentiation in the DM cultures (Th-positive cells per well: control, 38.3±9.4; Ngn2, 37.4±13.3; transduction efficiency 66%) nor did it increase the number of Th-positive neurons in the similarly infected VM (Th-positive cells per well: control: 696.7±61.3; Ngn2, 681.3±124.4). This experiment shows that, even though lack of Ngn2 within the VM specifically affects the differentiation of mesDA neurons, Ngn2 is not sufficient to induce a mesDA neuron fate.

DISCUSSION

Ngn2, and other proneural genes, are known to be involved in the acquisition of neuronal fate, as well as in neuronal-subtype specification, exerting their action on proliferating neural progenitor cells (Bertrand et al., 2002). In the VM, Ngn2 is expressed in the proliferative VZ in a spatiotemporal pattern that correlates with mesDA neurogenesis, suggesting that Ngn2 is expressed in the precursors of mesDA neurons (Fig. 1A) (Thompson et al., 2006). In this study, we show that the generation of the mesDA neuron system is greatly impaired in *Ngn2* knockout mice, indicating that Ngn2 function is important for the normal development of the mesDA neuron system; other neuronal populations within the VM, notably the Isl1-expressing oculomotor neurons and the Brn3a-expressing neurons of the red nucleus, are unaffected. In the *Ngn2* mutant, the number of Th-positive neurons in the VM was initially reduced by about 90%, as seen at E11.5-E13.5. At later stages of development, i.e. between E15.5 and E17.5, there was a partial recovery to about 30-50% of normal levels, as seen postnatally. The remaining mesDA neurons, most of which were formed with several days delay, were correctly specified with respect to the expression of appropriate HD proteins and other transcription factors, and differentiation into VTA and SN neuron subtypes, as well as their axonal projections in the forebrain.

Specific loss of mesDA neurons in the absence of Ngn2

Ngn2 is known to be important for promoting neuronal differentiation in many areas of the CNS. Our data show that the VM phenotype of *Ngn2* mutants includes loss of mesDA neurons, a smaller midbrain and an enlarged aqueduct, indicating that some cell populations are indeed lost or never formed in the mutant VM. Within the VM, the reduction of neuron number appears to be specific for the mesDA neurons, as the number of Isl1- and Brn3a-positive neurons, i.e. the neurons forming the oculomotor and red nuclei, respectively, is not affected. At early stages of mesDA

neurogenesis (E11.5), Ngn2 is expressed more broadly within the VZ of the VM, in a band that extends lateral to the presumptive DA progenitor domain. In the heterozygous *Ngn2-GFP* mice, where GFP expression can be used as a short-term lineage tracer, Isl1-positive cells show weak GFP expression at this stage, suggesting that they are derived from this lateral Ngn2-expressing progenitor population. In the developing spinal cord, proneural genes, such as Ngn2, interact with HD proteins to couple neurogenesis and subtype specification (Lee and Pfaff, 2003), and in the *Ngn2* mutant embryos, the expression of the HD proteins Lim1/2, En1 and Chx10, which are markers for subtypes of ventral spinal cord interneurons, is almost completely missing. In addition, the number of neurons expressing the HD protein Isl1, a marker of the cholinergic motoneurons, is reduced by about 50% (Scardigli et al., 2001). Our data show, that in contrast to the motoneurons in the ventral spinal cord, the expression of Isl1 in oculomotor neurons in VM does not depend on Ngn2 expression. Interestingly, the mesDA neurons that are formed in the *Ngn2* mutants show the expected expression of Pitx3 and En1/2, indicating that the expression of these characteristic HD proteins is maintained also in the absence of Ngn2.

Although Ngn2 function is necessary for proper development of the mesDA neuron system, our gain-of-function experiment showed that Ngn2 is not by itself sufficient to ectopically promote mesDA neuron differentiation in primary DM cultures. This is consistent with the view that Ngn2 has primarily a permissive, rather than an instructive, role in neuronal subtype specification, and that its function therefore, to a large extent, is dependent on the cellular context (Bertrand et al., 2002; Parras et al., 2002).

Partial rescue of DA neurons in *Ngn2* mutant mice

The initial loss of Th-positive neurons in the VM, seen at E11.5-E13.5, was compensated for during later stages of development. A similar phenotype has been reported for the large diameter sensory neurons of the dorsal root ganglion (DRG) in *Ngn2* mutants (Ma et al., 1999). However, in contrast to the DRG where the normal number of neurons is seen within a few days after cessation of neurogenesis, the recovery of the mesDA neuron system was only partial. It seems possible that the incomplete recovery could be explained by an increase in apoptosis, as seen at E13.5 in the *Ngn2* mutants. Alternatively, some of the accumulated precursors may have been re-specified into other types of neurons. From our analysis, it is clear that the numbers of neurons in the oculomotor and red nuclei are unchanged in the absence of Ngn2. However, other types of neurons, such as GABAergic neurons are present in the VM. In the absence of data relevant to the development of these neuronal populations, we cannot exclude that they may be generated in excess numbers in the mutant mice.

The mesDA neurons that were formed in the *Ngn2* mutants at the expected time of mesDA neurogenesis (E11.5-E13.5) were located in a distinct lateral population, which suggested that they could represent a specific subtype of mesDA neuron. In the PNS, Ngn2 is expressed in progenitors that later form subclasses of sensory neurons, and in the *Ngn2* mutants only these neurons are affected (Fode et al., 1998; Ma et al., 1999). There are no markers to identify distinct subtypes of mesDA neurons during neurogenesis; however, the number of neurons in the SN and VTA, i.e. the two major subtypes that are discernible in the adult, were equally affected. In addition, the remaining SN and VTA neurons were correctly specified with respect to the expression of the distinguishing markers Girk2 and calbindin, and the distribution of Th-positive fibers in the striatum and the adjacent limbic and cortical areas indicate that the SN and VTA populations projected to their expected targets in the forebrain.

Delayed differentiation of mesDA neuron precursors in the absence of Ngn2

In the *Ngn2* mutants, we noticed a retention of cells in the medial part of the mesDA neuron domain, which was most pronounced at E12-E13.5. This was observed as an accumulation of cells in the mutant VZ/IZ, matched by a reduction of cells in the MZ, as evidenced by DAPI staining. Closer examination of the mutant VZ/IZ indicated that Ngn2 is important at two steps of the differentiation process. First, lack of Ngn2 function caused an increase in GFP-positive radial glia cells, suggesting that a fraction of cells remain as early neural progenitors in the absence of Ngn2. Importantly, BrdU labeling showed that Ngn2 is not essential for cell cycle exit, as the VZ was not increased in size in the *Ngn2* mutants. Second, the Nurr1-negative cells within the mutant IZ also lacked Neurod1 expression. Neurod1 is a downstream target of Ngn2 and a known neuronal determination factor (Lee et al., 1995; Ma et al., 1996; Mattar et al., 2004). Lack of Neurod1 in the mutant IZ therefore implies that Ngn2 function is important for the progenitor cells within the IZ to acquire a neuronal fate, which can explain the lack of mesDA neuronal markers. It is worth noticing that this requirement of Ngn2 for Neurod1 expression was specific for the mesDA neuron domain, as Neurod1 expression was unaffected in other parts of the midbrain, and that, despite an almost complete lack of mesDA neurons, a substantial number of β-III-tubulin-positive neurons were present at E13.5 in the part of the MZ where the mesDA neurons are normally located.

It is likely that, due to the lack of specification, cells within the mutant VZ/IZ cannot respond to the migratory cues present in the surrounding environment and thus stay close to the ventricle. This is reminiscent of the phenotype seen in distal cranial ganglia of *Ngn2* mutants, where delamination and migration of sensory neuron precursors from the epibranchial placodes is blocked, and the expression of neuronal markers is missing (Fode et al., 1998). In the cranial sensory neurons, the block in neurogenesis is later compensated for and the geniculate ganglia are present at birth in *Ngn2* mutants, although diminished in size. Although the recovery of geniculate ganglion neurons is most likely due to neural crest-derived precursors populating the structure, we investigated alternative explanations for the compensation in the mesDA system. BrdU labeling at E15.5 and E16.5 showed that the increase of Th-positive neurons between E15.5 and E17.5 is not due to a prolonged neurogenesis of mesDA neurons in the *Ngn2* mutants within this period. Because the accumulation of cells in the VZ/IZ decreased as development progressed, there is a possibility that some of these cells later differentiate into mesDA neurons. In support of this idea, Kele et al. show that another proneural gene, *Mash1*, is expressed within the VZ of the mesDA neuron domain, and that the recovery of Th-positive neurons, seen at E17.5 in the *Ngn2* mutant, is abolished in mice lacking both *Ngn2* and *Mash1* genes (Kele et al., 2006). Moreover, Kele et al. have observed that *Mash1* when knocked into the *Ngn2* locus can partially restore the number of Th-positive neurons that develop at this stage (Kele et al., 2006).

Ngn2 functions upstream of Pitx3, Nurr1 and En1/2 in mesDA neuron differentiation

A number of developmental genes, such as En1/2, Lmx1b, Pitx3 and Nurr1, are expressed in mesDA neuron precursors in the VM, and have been shown to be of major importance for the development of the mesDA neuron system (Simon et al., 2001; Smidt et al., 2000; van den Munckhof et al., 2003; Zetterstrom et al., 1997). For the most part, however, these genes are expressed at postmitotic stages, and are important for the terminal

differentiation, maintenance or survival of the mesDA neurons. The only mutant where defects in the early differentiation into mesDA neurons have been suggested is the *Lmx1b* mutant. In the absence of Lmx1b, Pitx3 is never expressed, and Nurr1 and Th expression is lost after E16.5. However, the defects are not limited to the mesDA neuron system, as the VM is reported to show other major malformations (Smidt et al., 2000). Hence, *Ngn2* is the first example of a gene, expressed in the VM VZ cells, that is essential for proper mesDA neuron differentiation, and where loss of function causes impaired mesDA neurogenesis without other major abnormalities in the VM.

The proneural genes are known to be expressed in proliferating neural progenitor cells in diverse regions of the nervous system, where they promote neuronal differentiation and suppress glial phenotypes (Bylund et al., 2003; Sun et al., 2001). The spatiotemporal pattern of Ngn2 expression in the developing VM suggests that Ngn2 plays a role in the differentiation of VZ progenitors within the mesDA neuron domain, and that it acts upstream of Nurr1, Pitx3 and En1/2 in mesDA neuron differentiation. *Ngn2* is thus the first gene shown to be involved in the regulation of mesDA neuron differentiation at the level of the early progenitor cell.

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